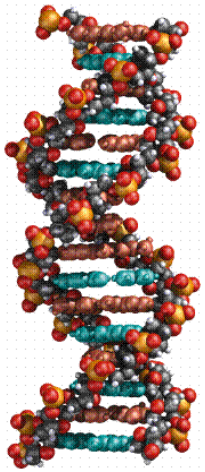
Principles of Toxicology



Course Notes

2013

for McGill University
Course OCCH-612

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http://www.invitroplus.mcgill.ca/Ftp/Toxicology Course Notes 2013.pdf

Determinants of Toxic Action

Modeling Principles

Original version: October 1993.
Revised versions: 1994, 1995, 1997, 1999, 2000, 2001, 2002, 2003, 2004, 2005, 2006, 2007, 2008, 2009, 2010, 2012, 2013.

Key to Assignment 1:_____ Solution:_____
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Key to Assignment 3:_____ Solution:_____

Contents

Scope of Toxicology

1 · Scope of Toxicology

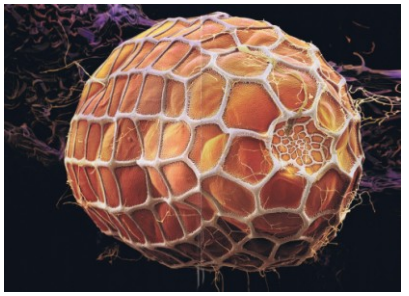
1.1. Toxicants

Toxicology studies the *injurious effects* of chemical and physical agents (including energy) on living organisms, observed as alterations in structure and function.
The variety of *injurious effects* becomes apparent if we examine the major causes of death (F1.1). Many of these diseases are caused or accelerated by exposure to toxic substances.
Toxicity data from various bio-medical sciences document the effects of exposure to natural♠ or artificial agents.
For more information on the history and scope of Toxicology, the student is referred to Casarett and Doull's *Toxicology. The Basic Science of Poisons.* 6th Edition, Curtis Klaassen, McGraw Hill, 2001, pp. 3-10.

♠ to dispel the idea that *natural is by definition healthy*, consider the following.

- many medicinal plants used by nearly 100 cultures on different continents are related, testifying to their efficacy. In excess, such natural medicines can involve risk.
- The hemlock plant (at right) was the state method of execution in ancient Greece, Socrates was one of its victims.
- Honey bees gather pollen from a variety of natural flowers which may contain toxins. From rhododendrons, one can get grayanotoxins, which bind reversibly to cell membranes, allowing the passage of sodium and leading to weakness, slow heartbeat, perspiration and nausea (the symptoms of a heart attack).
- Broccoli, considered a health food, contains methyl bromide, a commercial fumigant and pesticide. When crushed, its tissue generates isothiocyanates (toxic).
- The zebra longwing butterflys gather toxicants ingested from plants they eat, and produce eggs laced with cyanide and other toxins (the egg is illustrated below).

Hemlock (*Conium maculatum*)



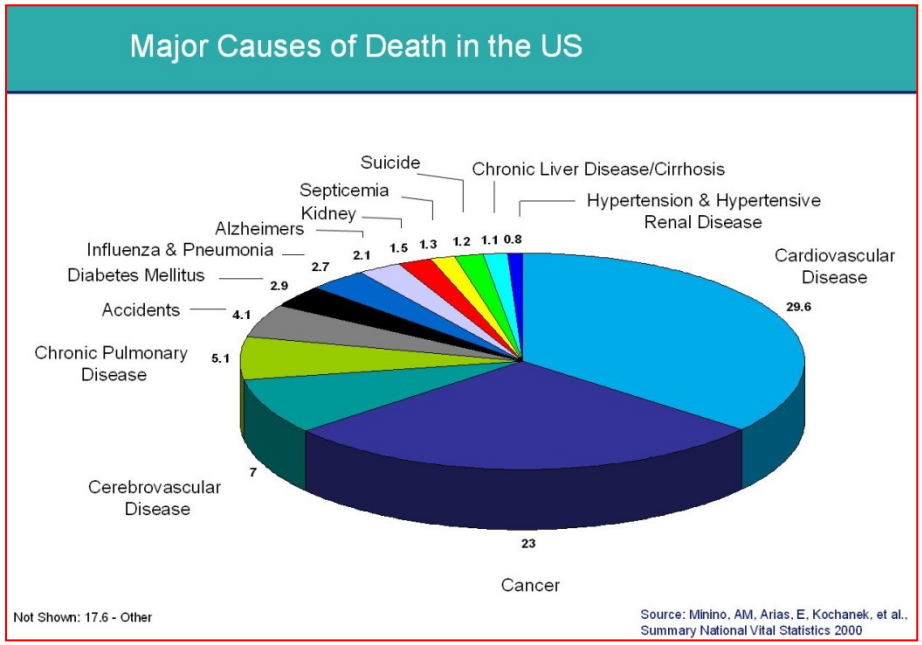
Egg of the zebra longwing butterfly

Toxicant Control

For pharmaceuticals and pesticides, producers have the burden of demonstrating safety to government. By contrast, for industrial chemicals, government must prove that a chemical poses a risk before it can restrict its production or use.
But companies have little incentive to develop information, since this increases the likelihood that evidence of harm will be uncovered. Changes are on the way, however:

- Canada recently completed the Domestic Substances List (DSL), mandated by law in 1999, which for the first time examined information available on the roughly 23,000 previously unassessed chemicals that have been in commerce in Canada over the last two decades, and identified more than 4,300 warranting further scrutiny.
- In the U.S., the voluntary High Production Volume (HPV) Chemical Challenge compiles basic hazard information on some 2,000 of the highest-volume chemicals in use.
- In Europe, a system of Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) was adopted in December 2006. It requires producers and users of 30,000 chemicals in commerce in Europe to register them and to provide information on their production, use, hazard and exposure potential. Chemicals of very high concern will be allowed only by authorization.

Dealing with natural toxicants, such as plant extracts, is different from dealing with a purified chemical. When preparing potions based on plant sources, one has to take into account the fact that the initial material may contain more or less of the active agent, depending on season, growth conditions or even time of day. This is why the pharmaceutical industry generally looks for natural remedies to provide *leads*



F1.1. Causes of Death involve many diverse diseases and body systems.

to useful molecules, as opposed to using the grown material itself. Scientists in laboratories are equally concerned with conducting studies using well defined agents. The supply of uniform products is better served by synthesis laboratories than agriculture. This has the consequence that most prepared drugs are based on a few purified molecules, as opposed to complex mixtures. This purity may have undesirable consequences, in particular by challenging metabolic pathways in very specific and forceful ways.

Almost any substance can be a toxicant. A single atomic species, such as metal exposure in industry (welding fumes), can have toxic effects. A protein, such as the *Clostridium Botulinum* toxin (F1.2), although much more complex, also has very strong toxic properties.

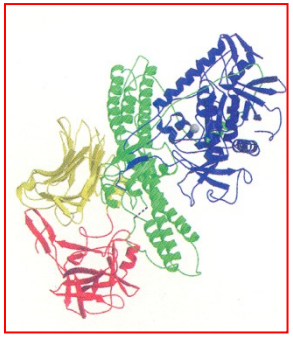
Subtle Toxicity

- In the liquid state, the O-H distance is 3 % shorter in deuterium than in normal water, and heavy water freezes at 4°C, rather than 0°C. Although these differences appear small, they are enough to underlie large toxicity differences. Heavy water interferes with the mitotic apparatus of higher organisms, including mammals, who soon become ill and die at the point where about half their body water has been replaced. Bacteria are only able to grow slowly in pure heavy water.
- Ionic pores are highly specific to metallic ions, but the selectivity of the pores is insufficient to avoid the substitution of lead for calcium, because both have similar atomic size (180 pm, see F7.4). This is also true for strontium, which has a slightly different size (200 pm), but a very similar electronic structure. Small differences in the properties of atoms have huge consequences for toxicity.
- Sometimes, the same molecule can have both a toxic and a benign character. Hemoglobin started its career in worms as a binding agent to detoxify oxygen. It was ultimately adopted by mammals in a slightly altered form for oxygen transport. Even within mammals, it transports life-giving oxygen to tissues, but will destroy the kidney's glomerulus, if liberated unchecked into the blood.

It seems, from these examples, as if toxicity occurs *by accident*.

Not-so-Subtle Toxicity

By contrast, some toxic molecules seem entirely conceived for nefarious goals. In the Botulinum toxin molecule of F1.2, *Blue* is the toxic fragment which paralyzes the muscles, *Green* is the pore opening the muscle's membrane, *Red-Yellow* binds to receptors on nerve cells, and if you look closely, a green loop is designed to mask the toxic part (*Blue*) from the immune system.



F1.2. Molecular Structure of the *Botulinum* toxin. Made of 1285 amino acids, 100 billionth of a gram is lethal through interference with acetylcholine.

As well, some substances are extremely hazardous when released accidentally as a result of chemical spills, industrial explosions, fires, or accidents involving railroad cars and trucks, or as a result of improper storage. These chemicals have been studied specifically to design emergency responses following releases.

Treating Acute Toxicity

Airway-breathing and circulation are always the most urgent considerations in emergency situations, to correct hypoxia and acidosis, and to maintain adequate circulation.
Documenting the intoxication is paramount.
Identification of electrolyte imbalances (renal function) and liver impairment should be assessed using basic laboratory studies (metabolic profile). Unless a specific antidote is available, management is supportive (treating symptoms) in many cases. Mental or hemodynamic status may deteriorate rapidly. Children are particularly susceptible.
Single-dose activated charcoal is the preferred gastrointestinal tract decontamination, up to 1 hour after ingestion.
The severity of symptoms and toxicokinetics of the ingested substance are considered in the disposition of an intoxicated person. Observation time is based on toxicity, half-life and amount ingested, particularly if signs or symptoms of toxicity do not reverse during the observation period.

Making Toxicity

The human body itself generates toxins[∂], and may transform molecules into more dangerous species by the process of bio-activation (see Chapter 8). Further, molecules in the body can become toxins as a result of changes in folding conformation, without any covalent chemical change. Such changes lead protein to form aggregates, or to be misrouted within cells. Examples of aggregation problems are found in diseases such as Alzheimer's, Creutzfeldt-Jakob and adult-onset diabetes for protein[1], and in Rett syndrome for chromatin[20]. Examples of misrouting are cystic fibrosis, systemic amyloidosis, nephrogenic diabetes insipidus, cancer, and so on.
Pairs of molecules that are mirror images of each other can have lethal limits that are 17-38 times that of the other (pyrethroid insecticides).

Fighting Toxicity

Our fight against toxicants is on on-going story. Analysis of samples collected in 2001 and 2002 by the CDC in the US shows that within 2 years, hygiene effectively reduced body burdens of lead (in children, a decline from 2.2 to 1.7 µg/dl),

[∂] A *toxin* is a metabolically produced toxicant. A *toxoid* is an altered form of a toxin, possessing little or no toxic power, but capable of inducing antibodies.

second-hand smoke (blood cotinine declined by 70 %) and mercury (an 18 % decline to 0.83 µg/dl for women of childbearing age), while other agents such as benzo(a)pyrene (found in a quarter of urine samples) and diisobutyl phthalate (majority of people) are found to be more widespread than expected[26].

1.1.1. Identifying Intoxication

Many intoxications cause common diseases and diffuse, non-specific symptoms. Exposure history is vital for correct diagnosis, but primary care physicians include mention of the patient's occupation in only 24 % of cases, and only 2 % include information about (1) toxic exposure, (2) work history and (3) environmental history.

1.1.1.1. Toxic Exposure

Current and past exposures to chemicals (air pollution, pesticide exposure, hazardous wastes/spill exposure, metals), dust and fibers, fumes, biological hazards, radiation, noise, and/or vibration should be documented. “Are family members experiencing the same, or unusual symptoms?”

1.1.1.2. Time-Course of Intoxication/Symptoms

The time-course of symptoms is a major clue to uncovering intoxication. Malaise can be periodic if the exposure is rhythmic, as occurring during a work shift.
“Do your symptoms get either worse or better at work?” and “Do your symptoms get either worse or better on weekends?”
In chronic intoxications, loss of appetite and weight are common symptoms.
The muscle inhibition of curare is more obvious than the nervous system hyperactivity connected with glutamate.
Generally, the slower the intoxication, the more difficult it is to relate to a specific situation.

In liver cirrhosis, alcohol can cause infiltrations in liver tissue over long periods of time. Nephrotoxic agents can induce very slow loss of nephrons in the kidney.

1.1.1.3. Work History

- Description of all previous jobs including short-term, seasonal, and part-time employment and military service.
- Description of typical workday (job tasks, location, materials, and agents used) and changes in routines or processes.
- Jobs of household members.

“Are other employees or family members similarly affected?”

1.1.1.4. Environment History

- Present and previous home locations, recent renovation/remodeling.
- Home ventilation/moisture control, insulation, heating and cooling systems.
- Home cleaning agents.
- Water supply.
- Hobbies (e.g., painting, photography, sculpting, welding, woodworking, piloting, restoring automobiles, shooting firearms, creating stained glass, creating ceramics, and gardening).

1.1.1.5. Forensics

To “home in” on a toxicant in the human body, blood can be used to indicate body status at a given time, but biopsies from heart, liver, kidney, lungs, brain, spleen, hair and nails are very useful to gauge chronic intoxication.
Unless you know what to look for, a large number of tests may be needed to finally detect a poison. But in acute cyanide poisoning, the odor is usually quite apparent at autopsy. You may perceive the bitter almond taste of cyanide if you crush 2 or 3 apple seeds between your teeth.

1.1.2. Identifying Toxicants

It is often difficult to distinguish cause from effect in biology, and this is particularly true in chronic toxicology. Symptoms can be connected to organs, which can be connected to histological changes, which may be connected to specific agents.
An important problem is that the reaction of the body to a toxicant may increase as a function of time because of bio-accumulation, or may attenuate because of physiological adaptation (toxicodynamics).

1.1.2.1. Structure-Activity Relationships

The ability to predict from molecular structure alone which molecules are toxic in the human body would be extremely precious. Unfortunately, our knowledge is not so advanced. Generally, real hints on toxicity purely from chemical structure are slim. In the absence of test data, Structure-Activity Relationship (SAR) analysis, which tries to predict a chemical's toxicity based on its chemical structure, is of limited usefulness. Accuracy of SAR analysis varies considerably depending on the particular chemical and on the predicted characteristic. Unless some toxicity information is already available on a specific molecule or on a closely related molecular structure, we depend on experiments of various kinds.

1.1.2.2. Toxicants and Evolution

In evolutionary development, tremendous efforts have been made to help animals identify toxicants. The largest gene superfamily in vertebrates is that of the olfactory receptor genes. In the mouse, 1,296 olfactory genes in 27 clusters have already been identified[13].

1.1.2.3. Reaching the Target

In the case of drugs, identification has traditionally been largely a matter of trial and error. A similar problem exists in the process of drug discovery, where a number of laboratories race to find molecules with specific medicinal actions.
The *Lipinsky*[11] *rules* predicting which molecules make good drugs (to be taken by mouth) relate essentially to *absorption* of the chemical, as opposed to its physiological action: molecules that cannot reach their targets cannot be effective drugs.

T1.3. LIPINSKY RULES FOR DRUGS
1. Molecular weight less than 500.
2. Log of the Partition Coefficient less than 5.
3. Number of hydrogen bonds donors less than 5 (expressed as the sum of OHs and NHs).
4. Number of hydrogen bond acceptors less than 10 (expressed as the sum of Ns and Os).
5. No more than 5 fused rings.

- Large molecules generally have poor cell membrane penetration (#1 and #5 above).
- The *Partition Coefficient*[♣] of the molecule should not be too high, otherwise the molecule will not be able to cross fatty cell membranes *and* travel through body fluids (#2 above).
- Hydrogen bond donors and acceptors are known to impair crossing of cell membranes (#3, #4 above).

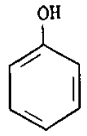
[♣] The Partition Coefficient is a ratio of the equilibrium concentrations of a dissolved substance in a two-phase system made of two immiscible solvents (water and n-octanol).

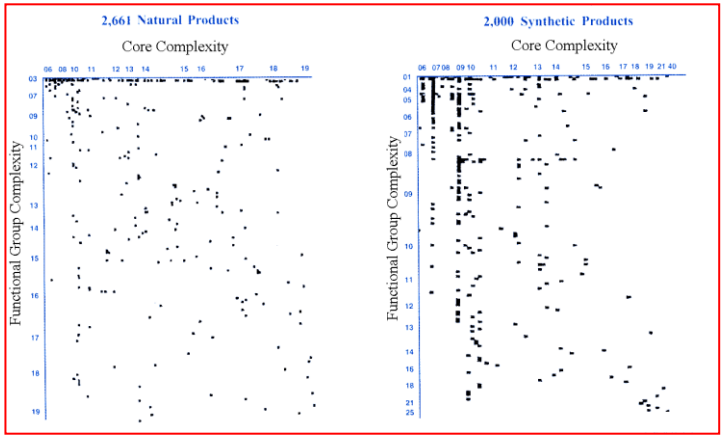
1.1.2.4. Scatter-graph of Drugs

So, is it entirely impossible to predict biological action of a chemical? One way to find out is to look at the chemical properties of a group of chemicals that all have some action on the body: drugs.
A basic way of describing drugs distinguishes *molecular core* from *functional groups*.

- *Core complexity* counts all possible ring sizes, from largest to smallest. This is a measure of the complexity of the molecules' basic core.
- *Functional group complexity* compiles all the possible ways of counting functional groups, from longest to shortest. This is a measure of the complexity of the molecules' functional groups.

In phenol, the *core* is benzene and the *functional group* is OH. If one draws diagrams (F1.4) positioning thousands of natural and synthetic drugs using axes representing *Core* complexity





F1.4. Scatter diagrams for natural and synthetic drugs.

and *Functional group* complexity, one does not see any groupings (F1.4 left).
The random scatter in natural products is thought to reflect unpredictability of physiological action, while the vertical concentrations in the synthetic products (F1.4 right) are thought to represent the practical preferences of synthesis chemists[12].

1.1.2.5. Antibiotics

The pharmaceutical industry has invested large efforts into the design of antibiotics (bacterial toxicants). Rather than focusing on specific structural chemical species, the strategy has generally been to address 4 major targets rather specific to bacteria:

- the cell wall, a structure found in bacteria, but not animals (β-lactam rings),
- protein production,
- DNA and RNA replication (bacteria divide very rapidly), and
- folate synthesis, a nutrient which donates one carbon unit in various biosynthetic pathways.

In the future, as a result of the development of molecular biology, drug identification may depend more on knowledge of genes, proteins and biochemical pathways.

1.1.3. Classifications of Toxicants

1.1.3.1. Classification by Function

Toxicants that have similar effects or uses can be grouped together, often reflecting applications. Frequently, compounds that are chemically related are used for similar purposes and form sub-groups within the general classifications.

Since bacteria are increasingly evading even the most potent front-line drugs, as resistance continues to rise, there is a clear need for new antibiotics. But the production of new drugs has slowed to a crawl since the 1940s and 1950s. In the last 50 years, just one antibiotic of an entirely new class has made it into clinical practice.

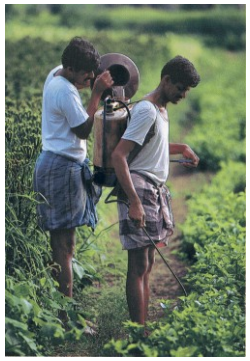
Looking for new antibiotics, some scientists turned to frog peptides, because most of our infections occur across mucus membranes. Because a frog's skin is essentially a moist mucus membrane, it presumably need something to protect it against pathogens. But despite decades of work, and thousands of candidate compounds, not a single amphibian antimicrobial peptides has turned into a marketable drug.

The compound that came closest to success (magainin), derived from the skin of the African clawed frog—passed through Phase II clinical trials for patients with diabetic foot ulcers, but the drug failed to get approval from the US FDA in 1999. Large peptides make poor drugs: some get metabolized too readily, while others trigger immune reactions. Their size also makes it difficult to synthesize them in large enough quantities.

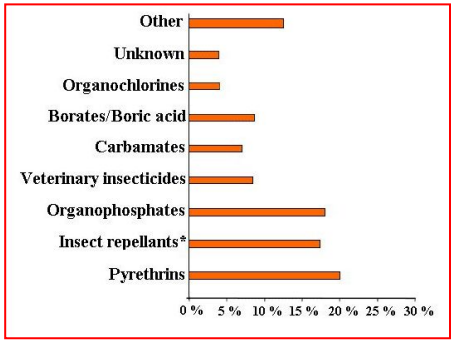
1.1.3.1.1. Pesticides

Insecticides

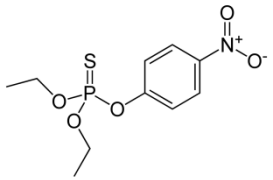
F1.5. Application of parathion insecticide in India.



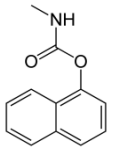
Most insecticides disrupt the nervous system. Under the same doses by weight as insects, humans show similar symptoms.



F1.6. Use of insecticides-pesticides in the US (1997-2000).

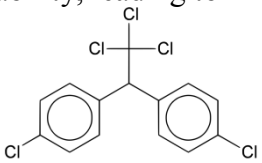


Organo-phosphates: parathion (shown, a pale yellow liquid), diazinon, malathion[ψ]. Inhibit cholinesterase. Peripheral and CNS toxicity. Degraded by the liver. Not usually persistent in the environment.

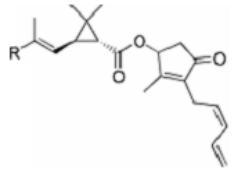


Carbamates: aldicarb, carbaryl (shown), propoxur. Inhibit cholinesterase. Peripheral and CNS toxicity. Rapidly degraded.

Organochlorines: dichlorodiphenyltrichloroethane (DDT, shown). Change cell membrane ion permeability, leading to altered firing rate. Readily stored in fat. Metabolized at a rate of 1 % per day. Bio-accumulates in the food chain. Banned in 1972 in the US.

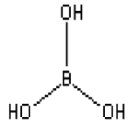


Pyrethrins: synthetic versions of the pesticide pyrethrin occurring in the flower chrysanthemum. Modified to increase their stability in the environment. Some synthetic pyrethroids are toxic to the nervous system.



Pyrethrin I = R-CH3 Pyrethrin II = R-CO2-CH3

Boric acid: (borax and boron-containing salts): low-toxicity minerals with insecticidal, fungicidal, and herbicidal properties. Do not evaporate or volatilize into the air or pose the same health concerns as synthetic pesticides. Slower acting than the synthetic pesticides like diazinon or pyrethrins, but highly effective over a long period of time. As an insecticide, boric acid acts as a stomach poison for ants, cockroaches, silverfish and termites,



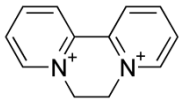
[ψ] Malathion is being phased out for many uses in Quebec in 2005. Methyl parathion is now banned for use on most fruits and vegetables in the US because of effects on the development of children's nervous system (see Chap. 7). It is used in lice shampoos.

and as abrasive to the insect exoskeleton. As an herbicide, boric acid causes desiccation or interrupts photosynthesis in plants. Ingestion of boric acid leads to a dermal rash called "boiled lobster syndrome".

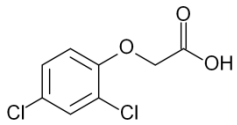
Herbicides

Mostly of low human toxicity, because of physiological differences between humans and plants. Interfere with plant hormones, inhibit photosynthesis, or promote water loss.

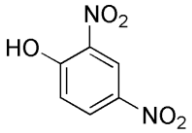
Bibyridyls: diquat (shown), paraquat. Desiccate plants. Ingestion of concentrated paraquat almost always leads to death due to lung failure (see Chapter 11).



Chlorophenoxy compounds: 2,4-D[Σ] (shown) and 2,4,5-T. Agent Orange, a defoliant used in the Vietnam war, was a 50-50 mixture of above compounds. Promote uncontrolled growth, leading to rapid plant death. Weakly toxic to humans, but a trace impurity of 2,4,5-T is dioxin (a teratogenic, carcinogenic compound causing chloracne).

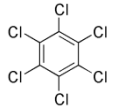


Dinitrophenol: 2,4 dinitrophenol (DNP). Inhibits ATP synthesis. Marketed as an anti-obesity agent between 1935 and 1937.



Fungicides

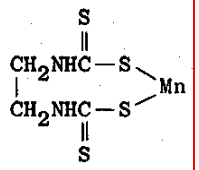
Hexachlorobenzene (shown) **and Organomercurials**: Are no longer marketed, but were used to treat seed grain.



In various incidents, *seed* was mistaken for *grain* in Turkey and Iraq, and was consumed by humans.

Phthalimides: use halted. Structure similar to thalidomide.

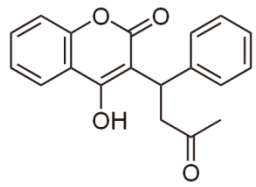
Dithiocarbamates: maneb (shown), zineb, mancozeb. These three fungicides are metallo co-ordination complexes with ethylenebis (dithiocarbamate), and exist as polymers. The manganese and zinc coordination complexes are known as maneb and zineb, respectively. In use, also used as insecticide.



Rodenticides

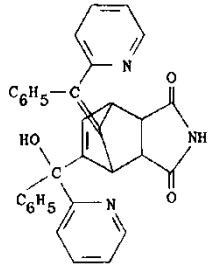
Most exposures are from accidents in industry, since they are directly applied to palatable animal baits.

Anticoagulants: warfarin (shown). Inhibits synthesis of vitamin K, leading to elimination of prothrombin and absence of clot formation. Animals bleed to death.

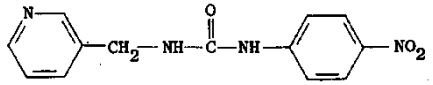


Inhibitors of cellular respiration: derivatives of fluoroacetic acid. Block many enzymes in the Krebs cycle, impeding ATP production.

Vasoconstrictors: norbormide (shown). Triggers a vascular reaction *specific to rodents,* leading to necrosis and death. Similar responses are not seen in humans with doses 30 times larger.



Diabetogenics: pyriminil (shown). Blocks nicotinamide metabolism, leading to death from paralysis and respiratory arrest. Supposed to be non-toxic to humans, but is noted to destroy beta-cells of the pancreas, leading to a diabetic state. Peripheral neuropathy and numerous Central Nervous System effects have been noted. Antidote is nicotinamide.



1.1.3.1.2. Metals

Many metals have variable oxidation numbers, which presents an opportunity for physiological use (ex, transport of oxygen by the iron of hemoglobin), but also for toxicity. They may

[Σ] Quebec is banning 2,4-D on the basis of possible carcinogenicity and endocrine and nervous system disturbances.

substitute one another (ex, lead for calcium) in terms of chemistry or size (at cell membrane pores), but carry subtly different characteristics that lead to toxicity. For example, lead has a broader chemistry than calcium. Calcium prefers oxygen ligands, while lead will also complex with the sulphydryl group, and form complex ions with OH^-, Cl^-, NO_3^- and CO_3^{2-}. Metals also interfere in neurotransmission and energy metabolism, stimulate reactions, interact with DNA and create free radicals.

Fig. 1.6a. Worker mining salt in Lake Katwe, Uganda. Any skin injury will allow salt, Cu, Pb, Cd and Zn to enter the body, preventing healing.

Metals may present in elemental, inorganic or organic forms.

- Some atomic forms of metals (in red in T1.7) are outright toxicants, having no useful metabolic function: Be, Ga, Cd, Hg, Tl, Pb, Bi, Ra, U.
- Some metals (in green in T1.7) are essential to humans: Na, Mg, K, Ca, Fe, Cu, Zn, Mo.
- Others (in yellow-green in T1.7) are also essential, but are toxic at high doses: V, Cr, Mn, Co, Ni.
- Still others (◎) have important long-term toxicity: Be, Cr, Mn, Ni, Cd, Hg, Pb.
- While others (◎) are carcinogenic in animal tests: Be, Cr, Co, Ni, Cd, Sb, Pb.

Molybdenum (atomic number 42) is the only second-row transition metal that is required by most living organisms, and the few species that do not require molybdenum use tungsten (W), which lies immediately below molybdenum in the periodic table[10].

T1.7. TOXICITY OF METALS displayed on the PERIODIC TABLE OF THE ELEMENTS

1 H																	2 He
3 Li	4 Be ◎◎											5 B	6 C	7 N	8 O	9 F	10 Ne
11 Na	12 Mg											13 Al	14 Si	15 P	16 S	17 Cl	18 Ar
19 K	20 Ca	21 Sc	22 Ti	23 V	24 Cr ◎◎	25 Mn ◎	26 Fe	27 Co ◎	28 Ni ◎◎	29 Cu	30 Zn	31 Ga	32 Ge	33 As	34 Se	35 Br	36 Kr
37 Rb	38 Sr	39 Y	40 Zr	41 Nb	42 Mo	43 Tc	44 Ru	45 Rh	46 Pd	47 Ag	48 Cd ◎◎	49 In	50 Sn	51 Sb ◎	52 Te	53 I	54 Xe
55 Cs	56 Ba	57 La *	72 Hf	73 Ta	74 W	75 Re	76 Os	77 Ir	78 Pt	79 Au	80 Hg ◎	81 Tl	82 Pb ◎◎	83 Bi	84 Po	85 At	86 Rn
87 Fr	88 Ra	89 Ac	104 Rf	105 Db	106 Sg	107 Bh	108 Hs	109 Mt									
				58 Ce	59 Pr	60 Nd	61 Pm	62 Sm	63 Eu	64 Gd	65 Tb	66 Dy	67 Ho	68 Er	69 Tm	70 Yb	71 Lu
				90 Th	91 Pa	92 U	93 Np	94 Pu	95 Am	96 Cm	97 Bk	98 Cf	99 Es	100 Fm	101 Md	102 No	103 Lr

January 22, 2013: after 4 years of negotiations, more than 140 nations signed the Minamata Convention, a treaty to reduce global mercury emissions by 2020. But limits on the use of mercury in coal power plants—one of the two main sources—won't go into effect for 5 to 10 years. Mercury is thought to impact the productivity of populations by reducing their ability to learn.

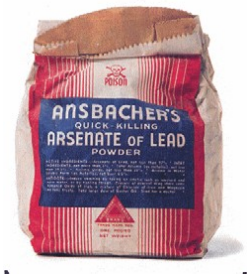
In the 1600s, women in Europe used white lead face paint, and mercury sulphide for rouge. The white lead made their hair fall out, which explains the fashion for high foreheads, as hairlines receded. Lead carbonate was used as a contraceptive in Rome (topical application).

Bacteria will grow over pieces of solid lead. Lead "resistance" in bacteria has been attributed to efflux pumps, P-type ATPases that are known to also transport Pb^{2+}, Cd^{2+} and Zn^{2+} across cell membranes [19].

These ATPases are present in bacteria and mammalian membranes, and handle cations such as calcium, sodium-potassium and protons.

In the Middle Ages, syphilis was treated with mercury, which was toxic to the bacterium (the spirochete *Treponema pallidum*), but also to patients.

Later, potassium iodide was used. In the early 1900s, arsenic compounds ($C_{12}H_{12}N_2As_2O_2$) were introduced♣. But occasionally, the liver would break down the organic arsenic compound into elemental arsenic, which is toxic to the liver. A protective measure to avoid this toxicity was to give patients injections of vitamin C.



Criminals favor arsenic because it is tasteless and odorless. Arsenic exploits cellular pathways and binds to critical proteins. Chronic administration produces weakness, confusion and paralysis. Acute ingestion (a few mg/kg) induces nausea, vomiting, diarrhea, low blood pressure and death.

In the early 1940s, penicillin replaced arsenic as the treatment of choice for syphilis. Arsenic derivatives are still used to treat African sleeping sickness and remains an effective chemotherapy for acute forms of leukemia.

Metals are often eliminated through the kidneys, which become targets themselves. Many metals are suspected of carcinogenicity, although we do not understand the exact mechanisms.

T1.8. Essential Metals.

Metal	Function	Excess Produces
Cr	A common ingredient in vitamin and mineral supplements. Formation of glucose tolerance factor and for many enzyme reactions.	Hexavalent chromium (+ 6) is a carcinogen (inhaled form).
Co	In vitamin B-12	Polycythemia, Cardiomyopathy.
Cu	Synthesis of hemoglobin	Microcytic Anemia.
Fe	Erythropoiesis (formation of RBCs)	Liver and Cardiovascular damage. If inhaled, Silicosis-like symptoms.
Mn	Mn Superoxide Dismutase is an important protector of mitochondria.	Manganese Pneumonitis, CNS disorders.
Mo	Aldehyde oxidase, Sulfite oxidase and Zanthine oxidase.	Anemia and Diarrhea (animal studies).
Se	Glutathione peroxidases neutralize hydrogen peroxide from metabolism.	Neuropathies, Dermatopathies, Decreased Fertility, Teratogenesis.

♣ Inorganic arsenic is a carcinogen (arsenite ions probably cause extra blood vessels to develop around tumors), while organic forms are less toxic, combat animal diseases and accelerate growth.

1.1.3.1.3. Organic Solvents

Organic solvents are widely used in industry and fall into a number of classes.
Aliphatic Hydrocarbons: methane, ethane, propane, butane, pentane, hexane, heptane, octane, ethylene, propylene, butadiene, and isoprene.
Aromatic Hydrocarbons: benzene, toluene, styrene, ethylbenzene, xylene and naphthalene.
Halogenated Hydrocarbons: methyl chloride, methylene chloride, ethyl chloride, chloroform, methyl chloroform, bromoform, carbon tetrachloride and vinyl chloride.
Esters: acetate, ethyl silicate, ethyl formate, and methyl formate.
Ketones: methyl ethyl ketone, methyl isobutyl ketone, and diisobutyl ketone.
Ethers: methyl ether, isopropyl ether, chloromethyl ether, and chloromethyl methyl ether.
Alcohols: methanol, ethanol, propanol, isopropanol, isobutanol, amyl[↓] alcohols, and allyl[♦] alcohols.
Aldehydes: acrolein, acetaldehyde, formaldehyde, furfural, and chloral hydrate.
Amines: methylamine, dimethylamine, trimethylamine, ethylamine, diethylamine, triethylamine, propylamine, butylamine, allylamine and cyclohexylamine.

Organic solvents, because of their solubility in cell membranes, often depress the Central Nervous System and irritate tissues and membranes. Many are anesthetics, penetrating well into nervous and fatty tissues. They are frequently nephro- hepato- and cardio-toxic.

[↓] a hydrocarbon radical, C_5H_{11}.
[♦] CH_2=CH-CH_2-

1.1.3.1.4. Food

Food can be toxic (mushrooms, rhubarb leaves), and more chemicals are released when food is cooked. Cooking kills micro-organisms that could make preserved food, in particular, toxic (botulism), and it also makes many foods more palatable. Since it helps breakdown cell membranes, it makes foods more digestible and more easily absorbed.
More than a thousand low molecular weight compounds are produced when food is heated, mostly at ppm levels, but the toxicity of these chemicals has not been established.
For example, a cola drink uses caramel color produced by heating sugar and ammonia, and contains 138 µg of 4-Methylimidazole, 4.8 times greater than California's 29 µg-per-day limit, computing to a lifetime risk of cancer of 5 out of 100,000 people.

Other potentially carcinogenic chemicals that have raised concerns in the past include polycyclic aromatic hydrocarbons, N-nitrosamines, aromatic amines and acrylamide.
However, there is no practical way to remove these chemicals from cooked or processed foods, and also there is not enough evidence that they present health risk to humans. In each case, after some time passes, public attention fades away, as (1) we must eat something, (2) we have been cooking for a long time (500,000 years), and (3) the risk is difficult to avoid.

1.1.3.2. Classification by Potency: Toxicity is in the Dose

Toxicants have effects that depend on the amount administered. As Paracelsus said, *the dose makes the poison*. 20 drinks of vodka, taken rapidly on an empty stomach, could kill you.

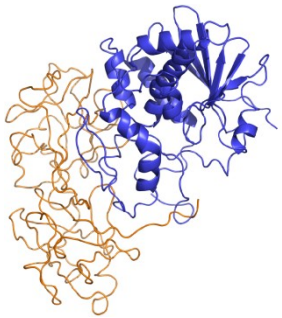
Toxicants have multiple effects. So, to

obtain a coherent toxicity classification, it is necessary to agree on the *final outcome by which toxicity will be judged*. The single Lethal Dose that kills 50 % of animals (LD_{50}) has historically been the landmark for the purposes of classification.

Although the determination of LD_{50} implies the death of animals (unethical), it should be considered that almost any chemical manufactured will inevitably intoxicate some human at some time. When this occurs, knowledge of the LD_{50} allows immediate evaluation of the gravity of the intoxication, a considerable advantage in clinical treatment.

Thus, toxicants can be classified according to their *potency*, the weight that will produce a given response per animal body weight, otherwise known as the *specific* dose (T1.9).

LD_{50} generally refers to death of an animal, but it could also refer to death of a cell. For example, a *single molecule* of ricin (shown, from castor seeds, LD_{50} = 2µg/kg) reaching the cytosol has been reported to kill a HeLa cell as a result of protein synthesis inhibition. Ricin causes lethal damage to the gut if ingested, and to the lungs if inhaled. A vaccine has been developed against this toxicant (antibodies can neutralize it).



A whole class of bacterial secretions, the bacteriocins, are lethal at a dose of a single molecule per cell. Interestingly, the producers of bacteriocins also synthesize a second protein that pairs up with the antibiotic, keeping it inactive until it reaches its target.

AGENT		LD_{50}, mg/kg*
Ethyl alcohol	SLIGHTLY TOXIC	10,000
Sodium chloride	MODERATELY TOXIC	4,000
Ferrous sulfate	MODERATELY TOXIC	1,500
Morphine sulfate	MODERATELY TOXIC	900
Phenobarbital sodium	VERY TOXIC	150
Picrotoxin	EXTREMELY TOXIC	5
Strychnine sulfate	SUPER-TOXIC	2
Nicotine	SUPER-TOXIC	1
d-Tubocurarine	SUPER-TOXIC	0.5
Hemicholinium-3	SUPER-TOXIC	0.2
Tetrodotoxin	SUPER-TOXIC	0.10
Dioxin (TCDD)	SUPER-TOXIC	0.001
Botulinum toxin	SUPER-TOXIC	0.00001

T1.9 Potency Rating Chart using acute LD_{50}s.
LD_{50} is the dosage (mg/kg body weight) causing death in 50 % of exposed animals.

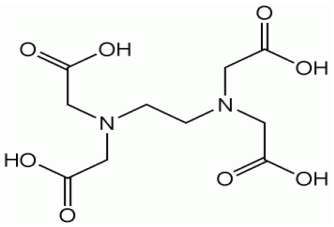
1.1.4. Limiting the Consequences of Intoxication

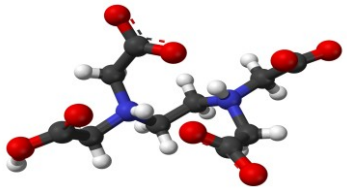
Intoxication is dealt with by
(1) removal of the toxicant,
(2) elimination of the toxicant from the body,
(3) treatment with an *antidote*, *antagonist, metabolic modulator* or *substrate*, and
(4) allowing the body's own regenerative powers to take over.

1.1.4.1. Eliminate the Toxicant from the Body

Once absorbed into the body, can toxicants be taken out?
If the skin has been exposed, it can be washed. The lung can take in toxicants, but it can also exhale them.

Emesis, the regurgitation of a poison taken via the oral route, can be induced by *syrup of ipecac*, but this must be done within tens of minutes to be effective.
If an ingested poison cannot be vomited, perhaps it can be bound within the *enterum* using a high performance *absorber* such as *carbon black*, which provides a binding tight enough that the toxicant is chaperoned out of the body in the feces. Typically, charcoal given at 10 g of charcoal per gram of toxicant will absorb 50 % of a poison, even 1 hour after ingestion. The list of toxicants that are bound by carbon black include: cyanide, malathion, parathion, diazinon, dichlorvos, DDT, carbamates, mercuric chloride, methanol, N-methyl carbamate, ethylene glycol, kerosene, turpentine, isopropyl alcohol and tolbutamide. It does not bind lithium and potassium, iron or lead.
If the toxicant has escaped from the digestive system into the blood, perhaps injection of a *chelator*, able to specifically bind the toxic agent in the blood and help carry it out of the body, is possible. For example, injections of the chelator ethylene-diamine-tetra-acetic acid (EDTA, shown) have been the method of choice for the treatment of lead intoxication.





In most cases, toxicants bound by *chelators* can be excreted from the body via the natural routes, mostly the liver and kidneys.

For many chemicals, the slow process of clinical recovery is closely related to elimination of the agent from the body, often monitored using blood plasma levels (Chapter 4, Toxicokinetics).
But some injuries cannot be cured by elimination of the toxicant. In burns, for example, the injurious agent dissipates completely following injury, but leaves behind manifestations such as edema, intravascular coagulation, small vessel thrombosis and ischemia, all this in the absence of the agent itself. This underscores that the *agent* itself and its *effects* can have different *half-lives*. More will be said on the elimination of toxicants in Chapters 4 and 8.
There are basically two types of biological monitoring in toxic reactions: following the evolution of the toxicant concentration over time, and assessing the evolution of various aspects of the toxic injury. These two aspects are often linked.

1.1.4.2. Treatments or "Antidotes"

Some toxicants act by inhibiting or over-stimulating the nervous system. In this case, if appropriate chemicals can be ingested or injected, the effect of the toxicant may be compensated or at least blocked long enough for the toxicant to be naturally eliminated. As well, some toxicants target molecular steps in metabolism: inhibition of enzymes, depletion of vital substrates, de-activation of critical molecular species. Occasionally, there are effective answers to such intoxications that can reverse the toxic effects. A number of examples are presented in Table 1.10.

1.1.5. Specificity of Toxicants

As a rule, simpler toxicants are likely to have more widespread effects among species and body systems.

T1.10. TOXICANTS and…	ANTIDOTES
	BY SPECIFIC BINDING TO THE TOXICANT
Botulism	*Botulinum* antitoxin
Scorpion, spider, rattlesnake	Specific Antivenins
	BY GENERAL BINDING TO THE TOXICANT (CHELATION)
Arsenic and mercury	Dimercaprol, D-penicillamine
Iron	Deferoxamine
Lead	Calcium ethylenediamine tetra-acetic acid (EDTA)
	BY BUFFERING OR NEUTRALIZATION
Chlorine gas	Nebulized sodium bicarbonate
Heparin	Protamine
	BY COUNTER-ACTING EFFECTS ON CELLS (ANTAGONISTS)
Organophosphates and carbamates	Atropine
Opiates	Naloxone, nalmefene (opiod antagonist)
Dioxin	Epigallocatechin gallate (green tea leaves)
Benzodiazepines	Flumazenil
	BY MODULATION OF METABOLISM
Acetaminophen	N-Acetylcysteine (increases glutathione)
Methanol	Ethanol (slows metabolism of methanol)
Methemoglobin-forming agents	Methylene blue (reactivation of Hemoglobin)
Methotrexate	Folate (vitamin B9, rebuild folate levels), leucovorin
Isoniazid, monomethylhydrazine	Pyridoxine (vitamin B6, rebuilds GABA levels)
Anticholinergics	Physostigmine (cholinesterase inhibitor)
Warfarin anticoagulants	Vitamin K (restores levels depleted by warfarin)
Ethylene glycol	Ethanol (slows metabolism of ethylene glycol)
Ethanol	Thiamine (vitamin B1, Wernicke-Korsakoff syndrome)
Hypoglycemic agents and insulin	Dextrose (restores blood sugar)
	BY CREATION OF AN EXCRETION ROUTE
Cyanide	Amyl nitrite, sodium nitrite, thiosulfate
Hydrogen sulfide	Sodium nitrite, thiosulfate

1.1.5.1. Wide-Ranging Toxic Effects

- For example, for embalming bodies, it is necessary to kill or halt the progress of a wide range of microorganisms. For some time, arsenic trioxide was used for this purpose, until it was replaced by formaldehyde (5-35 %), glutaraldehyde, methanol or ethanol (9-56 %).
- Insects are vulnerable to many of the traditional pesticides because most of their bodies or food supplies are being sprayed, while humans are hopefully exposed to relatively small quantities. The careless human handler can experience life-threatening consequences if given sufficient dose, just as the insect: here again, the toxic effects are relatively non species specific.

Other toxicants depend on particularities of tissues or species, and are species-specific. Protein toxicants are more likely to be specific.

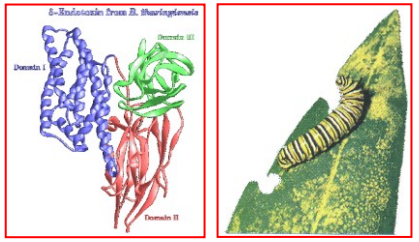
1.1.5.2. Specific Toxic Effects

- Aspirin can kill cats and acetaminophen snakes, while guinea pigs can consume strychnine, which is very poisonous to humans.
- Diclofenac was used to treat swelling and pain in cattle in India. As the drug became popular, the population of vultures decreased by 95 %, as the vultures eating cattle carcasses died of kidney failure[24]. Diclofenac is being replaced by another non-steroidal anti-inflammatory drug, meloxicam.
- *Allelopathic* plants such as the black walnut, sycamore and sassafras trees release substances in the environment through their roots, leaves or by evaporation that limit the germination of competitors.
- The toxicant specificity can involve very recognizable mechanisms: the spotted knapweed of the American west secretes catechin, which in tiny amounts induces apoptosis in other plants.

1.1.5.3. Bt-Toxin

Ideal pesticides would affect only a target species (the pest) while leaving the human handlers unharmed. Recently, a controversy erupted concerning the *Bt-toxin* from Monsanto, a molecule specifically lethal to corn borer caterpillars by perforating their gut. Derived from the bacterium *Bacillus*

thuringiensis, it was bio-engineered into a corn seed that makes its own pesticide (F1.11). An unexpected toxicity was discovered: in sufficient quantities, pollen from Bt-plants also kills larvae of monarch butterflies.
Most toxicants are also tissue specific, presenting more risk to certain organs than to others. Lead stored in bone, and organic solvents stored in fat do not cause functional damage, but both can seriously affect the nervous system, if they reach it.



F1.11. Structure of Bt-toxin. A monarch caterpillar dines on a milkweed dusted with corn pollen.

1.1.5.4. Side-Effects

The problem of agent specificity is also important in pharmaceuticals, where "side-effects" are common. Some side-effects such as rashes, fever, fatigue and nausea are mild, but some, like internal bleeding, reduction in white blood cell numbers and abnormal heart rhythms, are serious and can result in hospitalization and death.

- Class "A" side effects are an exaggerated therapeutic response.
- Type "B" side effects are unpredictable from known pharmacology.
- Type "C" side effects affect something else than the intended target and are often caused by a metabolite of the drug.

About 60 % of drugs known to trigger adverse reactions do so because the patient metabolizes them too slowly, lengthening the effective time of exposure.

In the case of drugs that *need* to be metabolized to be active, the patient may get no benefit if metabolism is too slow. Conversely, if metabolism is too fast, the patient overdoses.

1.1.6. Definition of Dose

Toxicologists assume that detection of adverse biological effects in animal models increases the chance that the same effects occur in humans. But do the effects start at the same threshold dose?
Although the most common way of specifying dose is weight of substance per weight of living subject (for example, mg/kg), this metric is not necessarily the most useful.

In 1883, Max Rubner pointed out that between animals of different size, mass increases according to Length^{3} (or Mass^{1}), while surface increases according to Length^{2} (or Mass$^{2/3}$).

Under the assumption of complete and uniform penetration of toxicants in a passive body, if one wished to obtain a constant toxicant concentration at the action sites, dose should be proportional to Mass to the first power (Mass^{1}).

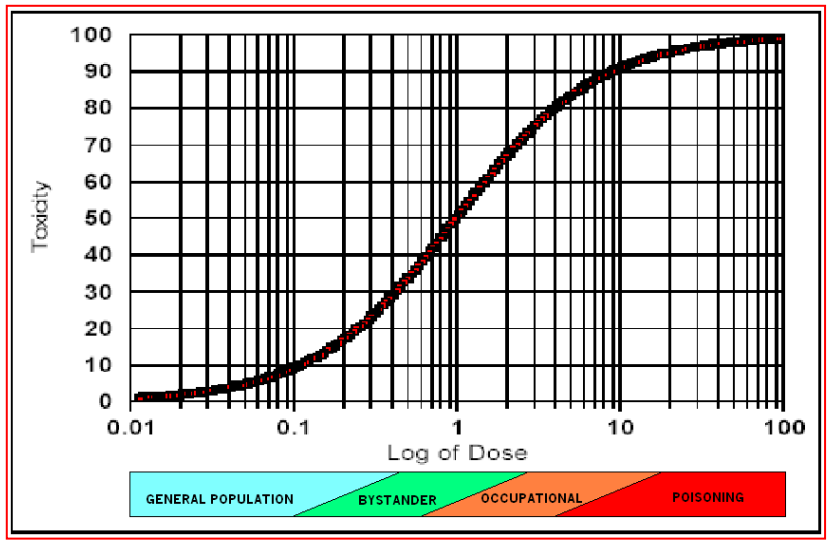
Imagine now the *dynamic* absorption of the toxicant through the body. The concentration at the action site will be primarily determined by transfer across body membranes, for example the inside of the lungs, digestive system, or the inside surface of the vascular system. This surface transfer scales according to surface or Mass$^{2/3}$.

In 1932, Max Kleiber looked at a broad range of data from many animal models and found experimentally that metabolic rate was related to Mass$^{3/4}$. The value of 3/4 falls between 1 and 2/3, and this range is probably appropriate for the bulk of toxic reactions.

Therefore, both geometry and metabolism seem to point to the importance of maximum local concentrations as opposed to long-term bulk averages in determining toxicity.

Refinements are possible in the definition of dose. Since the molecular weight of the toxicant is not a factor in the expression of toxicity, perhaps an even better metric would be the number of moles of toxicant (= a number of molecules) per mass of animal to the power 0.75.
Other scaling relations have been observed. An animal's lifespan scales as $Mass^{1/4}$. Heart rates scale as $Mass^{-1/4}$ (F1.14).

However, the widespread practice is to use a simple metric of mg/kg for dosage. It is simple, and toxicologists feel they have other more substantial inaccuracies to deal with in the conduct of their tests.



F1.12. Ranges of chronic toxic exposures in human populations. General Population, Bystander, Occupational, and Poisonous exposures cover broad ranges, with overlaps.

1.1.7. Levels of Exposure

1.1.7.1. High Exposures

Victims of accidental or suicidal poisonings, occupationally exposed workers, bystanders (workers nearby, those living close to an industrial facility emitting toxic agents) and the general public typically have different exposures to a particular agent, as shown in F1.14.

It is easiest to document toxic effects at the highest doses, where symptoms of toxicity are easily and rapidly detected. If no discernible adverse health effects can be detected acutely (within a day) at high exposures, it is conveniently assumed that no life-threatening effects will be observed at lower levels over short periods.

1.1.7.2. Low Exposures

Significant low-exposure impacts of pesticides, for example, are being discovered regularly. At a concentration of 15 ppb in the Sierra Nevada, the pesticide endosulfan, widely used and drifting from California, killed a number of frog species. In some frog species, the LD_{50} is only 0.3 ppb[25].

1.1.7.3. Ranging across Exposure Levels

Documenting acute effects, such as the **Lethal Dose to 50 % of animals** (LD_{50}) is the first step is assessing safety. This value is know for a wide range of chemicals, in part because experiments are not too expensive.
Although LD_{50} levels are very important in guiding dosage for progressively longer tests to be performed subsequently at lower levels, toxicologists are most concerned with identifying toxic reactions that occur at the smallest exposures (**Lowest Observed Adverse Effect Level** or LOAEL), because of the importance these levels have on ultimately determining safe limits over long periods of time in human populations.

1.2. The Principles of Toxicology

1.2.1. Determinants of Toxic Action

The basic principles of Toxicology attempt to give a theoretical framework for the understanding of toxic reactions, essentially defining the *factors that will influence the development of toxicity*.

1 - TOXICANT REACHING THE VULNERABLE SITE

Molecular size, hydrophobicity-hydrophylicity and ionization of the toxicant mostly determine repartition within body compartments. The toxicant exerts detectable effects if it reaches these body compartments.

2 - TIME-COURSE OF TOXICANT DELIVERY

Whether the same amount of a toxicant is administered as a single bolus, or spread over decades, will influence strongly the type and intensity of the toxic reaction. How the body handles the toxicant by mechanisms of elimination is the subject of *Toxicokinetics*[1].

3 - ACTIVATION OF DETOXIFICATION MECHANISMS

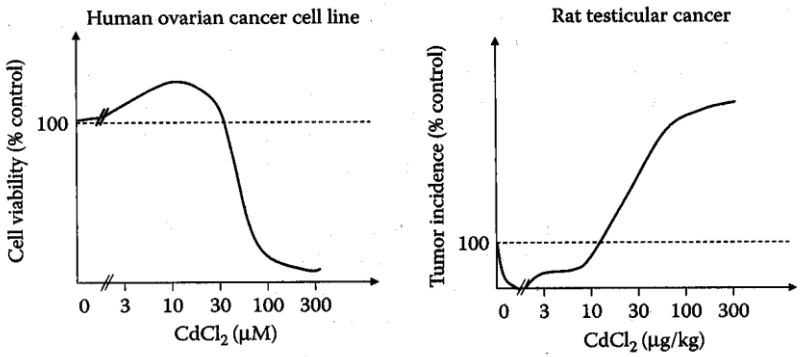
Living systems are adaptive, and deal with weak toxic exposures by maintaining *homeostasis*. Therefore, weak toxic exposures can be accompanied by a tissue reaction to intoxication for a certain period of time. These changes may be related to stress protein responses, DNA and cell repair mechanisms, and apoptosis (culling of marginal cells).

Hormesis

A 19th century view by Arndt is that weak stimuli slightly *accelerate* vital activity, middle-strong stimuli *raise* it, strong ones *suppress* it and very strong ones *halt* it. Schultz published at that time many examples of yeast growth *stimulation* by subtoxic concentrations of various poisons (iodine, bromine, arsenious acid). The *Arndt-Schultz law* says that every stimulus on a living cell produces an activity inversely proportional (within limits) to the intensity of the stimulus[5]. This modulation with dose of the vital reaction is the *inverse* of the conventional dose response.

Certainly, low doses of toxicants awaken responses in the immune system. A single dose of an antitumoral immunosuppressive substance (cisplatin) is able to induce *increased* lymphokine-activated killer activity[6], and low doses of cytostatic agents *stimulate* human granulocytes and lymphocytes growth[7]. Southam and Erlich[8], who reported the stimulatory effect of an antifungal when used at low doses, proposed the term "**hormesis**", defined as the *stimulatory effect of sub-inhibitory concentrations of toxic substances*. Stebbing[9] developed the concept with other scientists.
Hormesis, a non-specific phenomenon increasing the resistance and growth of all living organisms, may be frequent in pharmacology and toxicology. Hormesis relating to a number of endpoints, such as growth, learning, reproduction, birth defects and cancer is seen in organisms from bacteria to humans, caused by a variety of chemicals, including lead, cadmium, mercury and dioxin. Many *stimulants* are toxic at high doses.

[1] Toxicokinetics is the mathematical description of the elimination of toxicants.



F1.13. Protective response at low doses (hormesis) can be illustrated either as an increase in cell viability (left) or a decrease in tumor incidence (right). *Calabrese and Baldwin, 2001.*

The existence of hormesis raises questions[29] about

(1) the use of high-doses in tests to predict what happens at low-doses, specifically about using the maximum-tolerated-dose in testing for carcinogenesis,

(2) the use of certain chemotherapeutic drugs. The decay of a drug's concentration over time may produce a long tail at a concentration that stimulates the proliferation of the surviving cells. The tumor eradication potential of the drug may be partly attenuated by proliferation stimulation.

Action of Caffeine vs Dose

Caffeine has never been strictly approved as a pesticide, but it can be used as a 2 % solution to kill frogs. Caffeine consumption in pregnant women should be limited, to protect developing brains.

Stimulation (+)

Two cups of coffee, blood caffeine level of 1-10 μg/ml.

Seizures (-)

Have been reported at 50 μg/ml.

Death (- -)

Reported as low as 80 μg/ml.

Adaptation to Toxicants

This "action-reaction" mode of hormesis, coupled with the synthesis of specific defense molecules in reaction to mild intoxication, may explain why sub-lethal doses of toxicants trigger *adaptive responses* which maximize the innate poisoning resistance of living organisms[30]. When a strong challenge is subsequently applied, the toxic reaction is attenuated. Mithridate[φ] and Raspoutin are celebrated users of this technique.

This response, because it includes time for adaptation, is likely different from hormesis, but is similarly effective against toxic effects. Some believe that low doses of synthetic and natural chemicals are actually beneficial, and reduce cancer rates by potentiating defense responses.

In other cases, low doses may lead to a subtle tolerance that is unwanted. It is believed that the repeated mild exposures to drugs that anesthesiologists are subjected to while performing their functions (they are frequently in the breathing zone of patients, where intravenously administered drugs are exhaled by the lungs) may make them more vulnerable to developing drug abuse problems[23].

Tachyphylaxis designates a rapid decrease in the response to a drug after repeated doses over a short period of time. General explanations for the phenomenon: depletion of intermediates creating the drug's effect, or depletion of the drug's receptors in response to their saturation. The best known examples are from street drugs such as amphetamines, ecstasy and ephedrine.

[φ] King of Pontus, enemy of Rome, nibbled a mix of 54 ingredients to protect himself against poisoning. When he actually attempted to poison himself, as the Romans were closing in, he failed, and had to die by the sword.

A tempting view is that all intoxications are basically *non-threshold*, but that thresholds are observed experimentally because of lumped reactions that include detoxification mechanisms and hormesis together that add their contributions to the *non-threshold* curve.

4 - TOXICANT INTERACTIONS

Multiple toxicants in combination may have effects unpredictable from their individual actions. Primarily because of interactions documented in the liver (cytochrome enzymes), it is known that two toxicants can have combined toxic effects that add

- *synergistically* (more than the sum),
- *linearly* (the sum) or
- *antagonistically* (less than the sum).

For example, administration of ethanol is used to attenuate ethylene glycol (anti-freeze) toxicity. Toxicants that act by generating free radicals or causing cancer, such as dioxins, furans and PCBs are generally assumed to have additive risks, although it is relatively rare that solid data is available to confirm the assumption[21].

5 - TIME-COURSE OF TOXIC REACTIONS

Toxicodynamics investigates the changes over time in the physiology and structure of an organism as a result of toxicant exposure.

Positive toxicodynamic change: the rise in the level of CYP enzymes in liver cells, to allow faster clearance of a toxicant.
Negative toxicodynamic change: elimination of healthy cells from tissue as a result of necrosis or apoptosis.
Positive or negative toxicodynamic change: up- or down-regulation in the nervous system.

Toxic reactions will develop and repair at different rates, depending on whether the lesion is metabolic, structural, genetic or heritable.

Short time-frame: metabolic poisons such as cyanide.
Long time-frame: liver inclusions of fat and fibroblasts from exposure to alcohol.
Very long time-frame: genetic changes in the soma (cancer of individual) and in the reproductive cells (changes in the offspring).

Toxicities can cumulate over time, as a consequence of the time-course of toxicant delivery (Principle 2), or attenuate over time, as a consequence the activation of detoxification mechanisms (Principle 3).

Cumulation: heritable genetic damages from ionizing radiation.
Attenuation: increased number of detoxification pumps in the membrane of cells to enhance elimination of low levels of toxicants.

1.2.2. Modeling principles

For reasons of ethics and cost, toxicology is heavily dependant on models in securing toxicity data. While maintaining the representativity of the test, the toxicologist seeks:

- to use a lower species or test system,
- to obtain results faster, and at lower cost.

1 – FIND A RELEVANT MODEL

The intent of toxicology studies is often to determine Safe Human Doses such that *no adverse effect occurs in human subjects*.

Because of limits in our knowledge, it is not possible to reliably create toxicological models that exactly determine human toxic thresholds, and the corresponding safe toxicant exposures.

The next best strategy is to use toxicological representations that acknowledge less precision, but that include *Safety Factors* to compensate for our ignorance. The following factors contribute to the imprecision of toxicological models.

A- DESPECIATION

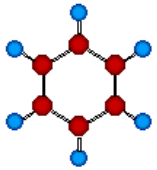
Species have enough similarities between them to make animal or cellular models from many species acceptable, but it is difficult to certify that the system chosen is appropriate for the determination of a particular toxic threshold.

Anatomical, micro-anatomical and molecular-genetic similarities must be considered in the choice of appropriate models for toxicity testing. Selected test species should ideally display the same natural diseases, syndromes or toxicities as humans. Models of tissue cultures, cell cultures, or even biochemical mixtures can meaningfully represent complete living systems only if they are chosen wisely based on previous physiological knowledge. Most of the differences are quantitative, but in a game of toxic thresholds, these quantitative differences can be important.

Specific variables among a broad range of mammals have been studied. Basal metabolic rate (BMR) dependance on body mass (M) in mammals is generally expressed as BMR = aM^b, but the value of b is uncertain. Surface area–to–volume ratio argues for geometric scaling (b = 0.67), while others claim a quarter-power scaling (b = 0.75) supported by theoretical analyses of nutrient supply networks. These simple views do not account for the shared evolutionary history of some species, who may share metabolic characteristics. b differs among lineages, ranging mostly between 0.67 and 0.75, and there may be no universal relationship between mammalian BMR and M.[28]

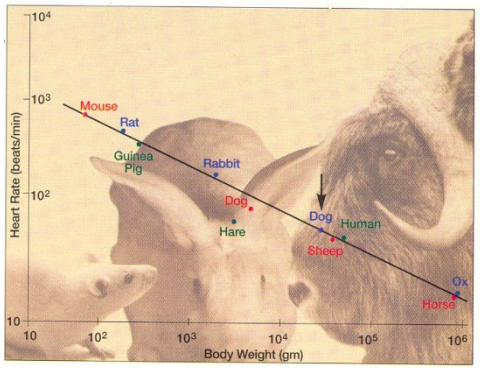
1.2.2.1. Good Animal Models

Animal models may need only **small adjustments** to compensate for inter-species variations. For example,



(1) the hematopoietic toxicity observed following exposure to benzene. The mechanism is the same in most mammalian species, but some models are more sensitive than others,

(2) some of the neuropathic conditions in humans attributed to pesticides and solvents are faithfully reproduced in certain animal species. The adult chicken is a good model for neuropathy from some organophosphorus ester insecticides and hexacarbon.



F1.14. Among animals, heart rate tracks weight with a 0.25 power law. A dog about 1/16th of the weight of a horse has a pulse twice as fast.

1.2.2.2. Poor Animal Models

Some animals models may be **inappropriate** because they lack the biochemical machinery that makes another species vulnerable (such as the lack of thyroid binding protein in rodents versus humans, see Chapter 10).

1.2.2.3. Inter-Species Barriers

About 60 % of infectious diseases are shared between humans and animals (zoonosis), a testimony to similarities between them: bird flu, **SARS**, **HIV**, **Ebola**, bubonic plague, **yellow fever**, **monkey pox**, bovine tuberculosis, Lyme disease, **West Nile** fever, **Marburg**, strains of **influenza**, **rabies**, **Hantavirus** pulmonary syndrome and **Nipah**. Most (in bold) are due to viruses. Smallpox does not have an animal reservoir, and this is why it could be eradicated.

Inter-Species barriers prevented migration of scrapie-like diseases to humans from sheep, but bovine spongiform encephalopathy (BSE) did migrate somewhat to mink and humans...These barriers manifest subtle differences between models that look superficially equivalent.

Species variations problems may be overcome by testing in numerous animal species, or by an *appropriately chosen* single model.

However, the consensus appears to point to an unavoidable imprecision in substituting a model for the real thing, this imprecision being set as a safety factor of 10 in human toxic threshold determinations.

B- CHOOSE RELEVANT VARIABLES

There are innumerable end-points that can potentially be chosen to quantify toxicity, and finally to determine toxic thresholds (smallest adverse effect level). They range from death of a subject (this would be used with extrapolation to lower doses) to, say, increases in the concentration of ornithine decarboxylase enzyme in cultured cells.

How can we be sure that the toxicity variables we have retained are appropriate to quantify the human toxic threshold, and that, for that matter, our choice of variables was wise in the context of all possible human adverse effects, especially the chronic ones? Does the test display effects on the most sensitive target within the organism? Most tests can find a connection between an exposure and an effect, but is this effect appropriate to determine the acceptable human exposure levels? In the absence of complete knowledge, many toxicologists believe that a safety factor of 10 is appropriate to cover this uncertainty.

C- CHOOSE APPROPRIATE TEST ENVIRONMENT

Once we have selected the proper species for representation of the human toxic threshold, and have decided on the variables to represent such an effect, how do we know that the *circumstances under which the tests will be conducted* will result in a safe human dose that will guarantee safety?

> Would you have predicted that in some animal models (but not in others) the baseline incidence of liver tumors can increase solely as a result of animals being fed a choline-methionine deficient diet?

Do we know that, within a given species, we have chosen the most appropriate strain, subspecies or genetic variant for this toxic threshold determination? Could our determination be influenced by age, sex, route of entry, the test's environmental parameters or the culture medium in the case of *in vitro* tests? In the absence of complete knowledge, many toxicologists believe that a safety factor of 10 is appropriate to cover this uncertainty.

It has not escaped your attention that all of these limitations of models (safety factors of 10) can weight heavily in the direction of reducing safe human doses to very small values, potentially making them difficult to implement in practice.

2 - LOW-DOSE EXTRAPOLATION

Toxicology is in a hurry to get results. Low exposures in humans are often simulated by high doses in an animal model, under the assumption that high doses in animals will produce the same toxic reaction as observed at low doses in humans, but with more statistical strength (*binding isotherm* view, see Chapter 2).

To reduce the number of animals necessary for a test, while still obtaining robust data, extrapolation from high to low doses is frequently used.

The reliability of toxicity testing therefore often depends on a monotonous relation between the log-of-dose of the agent and the toxic outcome. Although this assumption is almost universally used, there is substantial evidence that there are significant departures from it, as elevating exposure changes the diseases expressed in a model from the chronic class to the acute class.

1.2.2.4. Difficulties of Low-Dose Extrapolation

- Patterns of toxicant distribution and metabolism differ between low and high doses.

  Using high exposures in experiments tends to target the organ with the weakest elimination (short-term bioaccumulation). This organ then becomes the determinant of the TLV, although it may be the wrong target for chronic exposure.

- Defense mechanisms, active at low doses, may be disabled at higher ones.

  A low dose may be compensated by normal physiological mechanisms, only a small fraction of the toxicity being expressed in the model, as much of the toxicant is effectively neutralized in a healthy subject.

1.2.2.5. Improving the Models

If exposure must remain low in the model to conserve mechanisms, can the model itself become more sensitive to reduce testing costs?

Toxicity amplification, other than by increasing dose, may be possible.

- For example, by reducing the reserve of detoxification molecules and antioxidants. But it is not clear that the effects are equivalent to increasing dose with none of the disadvantages.
- In animals, we can enhance the sensitivity to chronic diseases by genetic knockout techniques (Chapter 10). This approach may be meaningful for genetic mechanisms, but not necessarily for toxicity generally.

Example of Low-Dose Non-Linearity

Ionizing radiation risk is in great part based on health outcomes from the Hiroshima and Nagasaki explosions, and assume that cancer risk is proportional to dose, even at low levels.

In careful experiments, researchers we able to irradiate the nuclei of cells in Petri dishes with one alpha particle each, creating mutations of a specific gene.

Irradiating 20 % of the nuclei produced 80 mutations per 100,000 cells.

Irradiating 5 % of the nuclei produced 57 mutations per 100,000 cells.

Bystander effects due to cell-to-cell communication are thought to account at least in part for the observed non-linearity[14].

3 – LONG-TERM EXTRAPOLATION

This is the assumption that brief tests (days to a year) can represent decades of human life.

Many testing procedures in various areas of technology attempt time-saving measures. In the testing of materials, higher temperature or temperature cycling are often used to increase the aging rate. In the testing of electrical cable insulation, the frequency of the applied voltage can be increased.

In inorganic chemical reactions, concentration (law of Mass Action) or temperature (Arrhenius equation) can be increased to augment reaction rates.

These tactics cannot be used in toxicological models.

Biological molecules have limited tolerance to heat and pH. Molecular components of living systems, particularly protein, react and denature differentially under altered conditions (temperature, pH, concentration).

1.2.2.6. Accelerating the passage of Time

There are however acceleration methods that are applicable to biological models:

- increasing the cell cycling (division) rate,

There is a generally higher susceptibility to toxicants by organisms hosting rapidly dividing cells (fetus, bone marrow, immune system).

- cancer initiation-promotion,

In cancer research, promoters can be introduced to illuminate and amplify the action of initiators, and vice-versa.

- genetic susceptibility.

Animal models can be created with genetic susceptibility to specific pathologies (for example, with one copy of p53 disabled, see Chapter 10).

However, most (brief) toxicological tests performed are *assumed* with relatively little proof to be representative of risks developing over much longer periods. Within the duration of a 2-year chronic animal test, animals go through maturation, gestation, adulthood and aging, generally assumed to correspond to segments of human life.

1.2.2.7. Choices in Toxicology

In the thinking of toxicologists, low-dose extrapolation is thought to amplify the risk because more mechanisms of toxicity are thought to be active at high doses than at low doses.

To the contrary, long-term extrapolation is thought to attenuate the risk because it does not take into account bio-accumulation of agents, or slowly emerging damage.

Compensating one factor with the other, toxicologists often simulate chronic low level exposures in humans using short high level doses in an animal model.

Taken together, low-dose extrapolation and long-term extrapolation pose formidable credibility problems for toxicology. Would an athlete look the same after training forcefully for 6 months as if he took it easy for 3 years ?

Therefore, a basic problem of Toxicology can be stated as:

Time does not respect what is done without it.

1.3. Dose and Time in Toxicity

High doses of toxicants generally have rapid effects, whereas effects of lower doses may become detectable only much later. This makes sense, since many physiological processes accelerate when concentrations increase.

Since acute toxicity determinations are relatively simple, fast and inexpensive, they form relatively little of the ongoing activity in Toxicology.

A class of natural compounds with considerable acute toxicity are venoms. They are rare in mammals, but very common in

lower life forms (snakes, spiders, anemones). The proteins and peptides of venoms act rapidly.
Venoms target the blood and blood vessels, inhibiting clotting and dilating vessels, leading to a loss of blood pressure. They also target nerves and muscles by acting on calcium, potassium and sodium-channel blockers, as well as sodium-channel activators and prolongers and antagonists of nicotinic and muscarinic receptors. Venoms are probably derived from the immune system's β-defensin molecules, and are usually represented in numerous types within the same animal to maximize effectiveness.

By contrast, considerable effort is expended on elucidating more subtle effects that occur slowly at lower exposures. These more common exposures, without being an immediate threat to survival, may produce subtle alterations, with delayed impacts on chronic survival or organ function. Teratogenicity and carcinogenicity are such "subtle" effects. Those may develop after some period of time, following a single high-level exposure, repeated moderate exposures or continuous exposures to low levels of agents for decades (T1.15).

T1.15. Acute or delayed toxicity may occur after a single high-level exposure. Repeated exposure at low levels may manifest in subtle, chronically developing adverse effects.

EXPOSURE	EFFECTS
Single, High	Acute, immediate symptoms, or delayed toxicity after some indeterminate time.
Repeated, Moderate	Covert (subtle) chronic symptoms, or slow onset of symptoms.
Continuous, Low-Level	Emergence of real risks after some time, or risks merge with general background risks.

1.3.1. Haber's Law

The relationship between *level* of exposure and *time* of exposure is quantified by *Haber's Law*:

$$Toxicity = Potency \times (Concentration - Threshold) \times Time$$

where *Toxicity* quantifies the toxic effect,
Potency represents the toxic potency of the agent,
Concentration is the concentration of the agent or dose rate,
Threshold is the detoxification term, and
Time is the duration of agent administration.

According to Haber's Law, any toxic effect is the product of the *Potency* of the agent, its *Time of application* and its *Concentration* minus a penalty term for metabolic detoxification, "*Threshold*".
Although Haber's Law is too simple to account for all aspects of toxic reactions (for example, "*Threshold*" is typically a function of agent concentration, rather than a constant, as we will see in Chapter 4), it explains the assumptions most often used by toxicologists, namely:

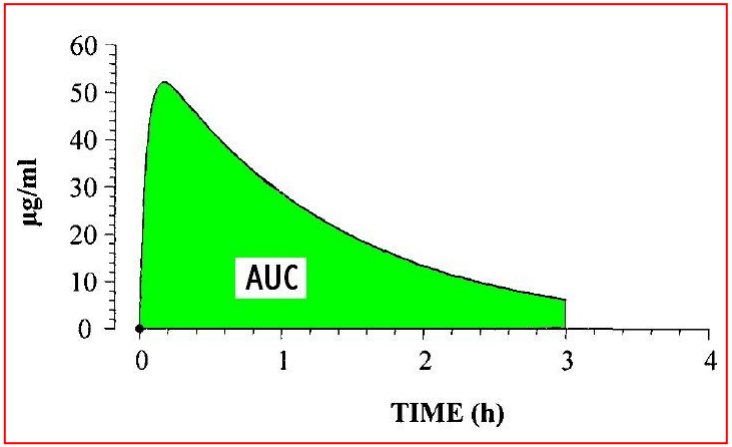
- The presence of "*Threshold*" in the equation justifies the existence of a safe exposure. The claim is that *no hazard "exists"* below a Threshold Limit Value (TLV).
- Use the "Area-Under-the-Curve" (AUC) to average exposures that vary over time, according to the integration of Haber's Law (below).

$$Time\ Integrated\ Toxicity = Potency \int \left[Concentration(t) - Threshold \right] dt$$

Haber's Law is not often referred to in the description of toxicology experiments, in great part because experiments properly measuring exposure over time (as blood

concentrations, for example) are more difficult to perform than the administration of a simple dose. This has lead many to use more descriptive and approximate (as opposed to analytical) methods [2].

1.3.2. Area under the Curve



F1.16. The "Area Under the Curve" in an exposure vs time plot can be used to quantify toxic effects.

1.3.3. Threshold Limit Values

Nevertheless, the *legal* protection of human health has taken the form of threshold limit values (TLVs) using Time-Weighted-Averages (TWAs), compatible with Haber's Law. Short-term exposure levels (STELs) have been added to take into account the more acute effects of exposure.

High exposures lead to frank effects on an organism that does not have time to adapt. Low exposures may barely show any effects until long into the future, with the added complexity that the organism may adapt and age as the test proceeds.

THE TIME-WEIGHTED AVERAGE METRIC ONLY APPROXIMATES TOXICITY

While the assumption that the Area under the Curve is the proper exposure metric to gauge toxic effects, it is obviously true only for slow-acting chemicals with straightforward physiological actions.

There are many known exceptions to this rule. For example, suppression of the immune system by diesel exhaust fumes is more effective if smaller doses are repeated at intervals than if one large single bolus is administered[22].

In contrast to the case above, most toxicants, if administered in many very small doses as opposed to a single large one, will have negligible effects (because of the Threshold term in Haber's Law).

1.3.4. Time Course of Toxic Reactions

Toxic reactions unfold over periods ranging from a minute to a lifetime. A toxicant reaches various sensitive sites within the body at various moments, and multiple toxic effects from a toxicant can each have secondary consequences. In some cases, toxicity can be altogether delayed because of the need for bioactivation. Because of basic as well as size differences, toxicities can also happen at different rates in animal models and in humans.

The acute effects may strike an organ or produce a disease totally unrelated to the one that will become apparent as a result of chronic exposure.

Acute effects are generally well known and chronic effects highly uncertain, because acute tests are cheap, while chronic tests are expensive.

1.3.4.1. Toxidromes

From the point of view of a victim, acute toxic reactions follow a certain development over time, depending on the poison concerned. This development is called a *toxidrome*. For a physician, history of the patient is paramount, but the toxidrome (i.e., appearance of symptoms) may provide indications on the compound ingested and on the dose.
In occupational and environmental health, the *cholinergic toxidrome* is the most important; other toxidromes (anticholinergic, sympathomimetic, opioid, sedative/hypnotic, hallucinogenic) are most often associated with overdoses of drugs or medications. Interpretation can be complicated if multiple substances are involved.

Imagine a worker exposed to organophosphate pesticides (malathion, diazinon), carbamates (physostigmine, carbaryl), certain mushrooms or the warfare agent sarin. He is a victim of the *cholinergic toxidrome*. Since there are various classes or receptors in the body that could be stimulated in this toxidrome, the symptoms could include any of those described in Table 1.17.

T1.17. Cholinergic Toxidrome

MUSCARINIC	NICOTINIC	CENTRAL
Pupil contraction	Pupil dilatation	Agitation
Slow heartbeat	Fast heartbeat	Confusion
Mucus in lung, obstructed breathing	Bronchodilation	Lethargy
Vomiting, diarrhea	High blood pressure	Coma
Abundant saliva/tears	Perspiration	Seizure
Urinary incontinence	Weakness (resp. arrest)	Death

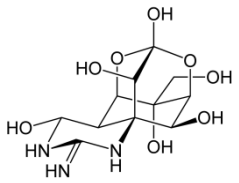
If a specific toxidrome is identified, the physician should consider toxin-specific treatments, such as an *antidote*.

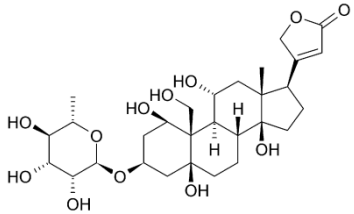
Antidotes are usually given after the patient is stable, preferably within a few hours of ingestion, and may require multiple doses because of short durations of action.
Initial laboratory testing may include bicarbonate level, electrolytes, serum urea nitrogen, and serum creatinine levels to evaluate for renal failure and electrolyte imbalance; blood glucose levels for hypoglycemic ingestion; electrocardiography for cardiotoxicity; prothrombin time for coagulopathy; pulse oximetry for hypoxia; serum acetaminophen level for acetaminophen toxicity; and urine human chorionic gonadotropin levels in female patients of childbearing age.

1.3.4.2. Cell Membrane Toxicity – Minutes

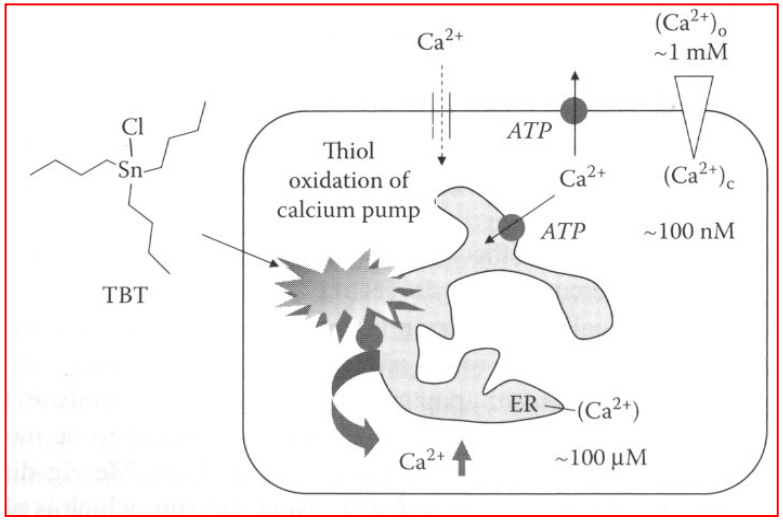
Toxic substances released in extracellular fluids rapidly meet cell membranes. Alterations to the membranes come from altering the state of membrane receptors (neurotransmission, hormones, cytokines), dissolving or altering the lipid matrix, or from binding or cross linking membrane protein. Such toxicities can be established very rapidly, especially for some agents that are quickly absorbed through the lungs.

Acetylcholinesterase inhibition, which over-stimulates membrane receptors, is typical of warfare nerve gases such as sarin. Neurotoxicants (DDT, pyrethroid insecticides, tetrodotoxin-shown) either block or alter the function of specialized protein channels for inorganic ions or neurotransmitters.
For example, ouabain (shown) inhibits the cell membrane's Na^+/K^+ pump (ATPase enzyme), eliminating its electrical polarization.





Organic solvents (ethanol, benzene) and anesthetics expand lipid membranes and increase their fluidity and permeability. Oxidation of membrane lipids (overwhelming the protection provided by glutathione) occurs from herbicides such as paraquat and diquat and from antibiotics such as adriamycin and bleomycin.
Calcium in the blood is at a concentration of 0.002 M, but the intracellular resting concentration of free calcium is 10^{-7} M (a ratio of 10,000). Inside the cell, low calcium concentrations are maintained by sequestering calcium in mitochondria, the smooth endoplasmic reticulum and by protein binding (to regulators such as calmodulin). Carbon tetrachloride, acetaminophen and tri-n-butyltin are examples of substances that act by releasing calcium within cells, leading to loss of cell functions and cell death (F1.18).



F1.18. Tri-n-butyltin chloride mobilizes Ca^{2+} from the endoplasmic reticulum, flooding the cytosol. The dark circles are calcium transporters. Apoptosis results. *Boelsterli, 2007.*

1.3.4.3. Cell Metabolism Toxicity - Hours

Metabolism uses external materials to produce living substance: lipids, protein and nucleic acids. Toxic action occurs by inhibiting materials absorption or bio-synthesis (T1.19).

A few metabolic toxicities occur extremely fast, because they target bottlenecks of cell metabolism. Oxygen deprivation by carbon monoxide suppresses ATP synthesis in the mitochondrion, which can damage the brain within minutes. Cyanide and H_2S also block energy utilization and are extremely fast acting. Milder reductions of mitochondrial metabolism are obtained from the drugs salycilate and tetracycline. Extensive studies have been done on the reaction of mitochondria to a variety of toxicants (T1.20).[2]

Typically, metabolic toxicity develops a little more slowly than in the extreme examples above, as

(1) blocking one pathway usually leaves alternatives for cells to compensate with, and
(2) cells usually have some reserve which allows them to pull through brief episodes of intoxication.

Many components of cells nevertheless need to be replaced, as they wear out though normal metabolism. For example, RNA is synthesized and replaced in cells approximately every day.

1.3.4.4. Cell Proliferation Toxicity - Days

Although most cells in vivo are in the resting (non-dividing) phase, some are not. Body tissues with high growth rates are

[2] About 1 in 5,000 women have mitochondrial mutations that lead to fatal disorders that affect the muscles and the brain. They are passed from mother to child, as mitochondria are maternally inherited. During *in vitro* fertilization, it is possible to move the nucleus of an egg to the cytoplasm of an egg with healthy mitochondria.

bone marrow, small intestine, skin, hair and nails. Any substance toxic to the mitotic apparatus will display cell proliferation toxicity. Methotrexate, an anticancer drug which inhibits DNA replication, is not toxic to cells in the resting phase, but is highly toxic to proliferating cells. This is why people undergoing chemotherapy are subject to hair loss, digestive problems and low blood counts.
Any compound able to alter cell proliferation rate is a potential teratogen, as the shaping of embryos is critically dependant on speed of cell division.

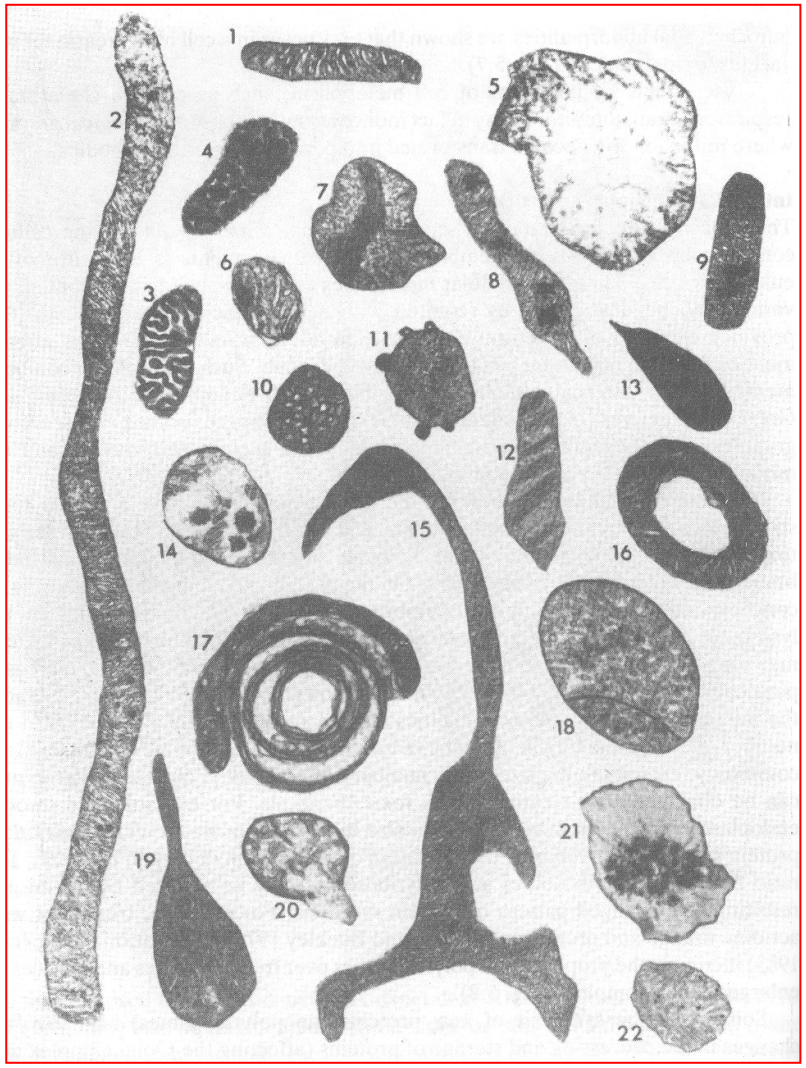
T1.19 . Inhibitors of Cell Metabolism.	
RNA only	Actinomycin D
Inhibit protein and RNA synthesis	Streptomycin Ethionine Dimethylnitrosamine Carbon Tetrachloride Galactosamine
Protein only	Cycloheximide Chloramphenicol Puromycin

1.3.4.5. Cell Oncogenicity - Years

Long-term experiments on toxicity teach us that the longer you study toxicity, the more surprises there are.
Cancer is known as “a multiple step process”, and these steps typically do not all occur within a very short period of time. Even after a tumor cell is created, functionally significant tumors need to grow in order to affect the organism, and be detectable. So, cancer typically develops over years or decades. In rat models, this period of cancer development is compressed within a year.

T1.20. Changes in Mitochondria caused by Toxic substances.		
1	Control (no exposure)	
2	Methotrexate	Elongation
3	Oligomycin	Condensed
4		Twisted
5		Swollen
6	20,25-diazacholesterol	Enlarged matrix granule
7	Isonicotinic acid hydrazide	Swollen, collapsed cristae
8	Sodium fluoride	Asteroid inclusions
9	Diethylenetriaminetetraacetic acid	Aggregated matrix granules
10	Puromycin	Vesicular cristae
11	Acetone	Outer membrane blebs
12	Fluoroacetate	Perpendicular stacks of cristae
13	Iodoacetate	Dense matrix
14	Ouabain	Mineral granule accumulation
15	Cyanide	Branching
16	Excess ferric ion	Ring formation
17	Excess calcium ion	Nesting cup formation
18	Dicyclohexylcarbodiimide	Coagulated matricial protein
19	Actinomycin D	Clear matricial area with tail formation
20	Puromycin	Disruption
21	Ouabain	Mineral granule accumulation
22	Cadmium ion	Scalloped outer margin

Mitochondria are former bacteria, and many antibiotics can lead to mitochondrial failure through Reactive Oxygen Species generation. N-acetyl-L-cysteine (NAC) can reduce the damage while maintaining bactericidal action.



Mitochondrial Micrographs, x 25,000. See T1.19.
Brikhauser Verlag, Basel.

In New Guinea, the central nervous system disease called "kuru" (connected with mad cow and Creutzfeld-Jacob diseases) was related to the practice of ritual cannibalism that included the eating of human brains, which was banned in the late 1950s. According to epidemiological data, this prion-based disease could have an incubation time of 34-41 years[27].

1.4. Scope of Toxicological Data

A rounded approach to evaluating toxic risks includes:

- physical and chemical characterization of the toxicant,
- *in vitro* studies,
- *in vivo* studies, and
- epidemiology.

The types of studies chosen and how they are performed will depend on the **intent of the toxicity study**. Because of cost, acute (short) studies tend to be available in the literature and reasonably well standardized, while chronic (long) studies tend to be few, with more variation in the methods. The evidence available for any toxic agent has accumulated over a period of time, consequently the historical changes in methods and emphasis bring unexpected diversity to an agent's file.

The most inexpensive way to assess the toxicity of a chemical is to do a **literature review**. For example, a review of accidental or suicidal intoxications and of occupational exposures may allow recognition of the acute hazards of the agent.
If literature cannot be found, a modest investment in lab work will yield acute studies of lethality (LD_{50}), dermal sensitization, genotoxicity and cytotoxicity in some models. Such models are well controlled, but there is a problem in extrapolation from the various models to human cases.

Some ***in vitro*** experiments are highly relevant to cancer risks (cell proliferation studies, genetic determinations), while **computer modeling** may be important to compartmentalization (solubility and Toxicokinetics) and gross assessment of toxicity.
The contributions of **computer modeling** and ***in vitro*** studies are primarily in confirming and documenting basic effects and mechanisms. Systematic efforts are being made to expand the scope of these studies to provide more complete information relevant to human risks.
Further experimental work and substantial resources can yield *in vivo* genotoxicity, sub-acute, sub-chronic, chronic studies, reproduction studies, etc. Chronic animal experiments are very useful in assessing teratogenic and cancer risks.

All experimental studies have in common the difficulty of establishing appropriate **levels of exposure** for tests. Doses are larger for acute studies than for chronic ones, and there is always a risk in a chronic study of being "off the mark" due to improper dosing.

The usefulness of many **epidemiological studies** is limited by two factors:
(1) the duration and level of exposure: retrospective studies of worker health typically involve complex exposures, for example as a result of particularities in industrial processes and as occupational health and safety regulations change over the years,
(2) the simultaneous exposure to a mixture of agents, leading to a difficulty in attributing observed health effects to any individual component.

On the other hand, when epidemiological studies are performed using environments very similar to those that are of interest, their conclusions can be very revealing of real human risks.

1.5. Toxicity Databases

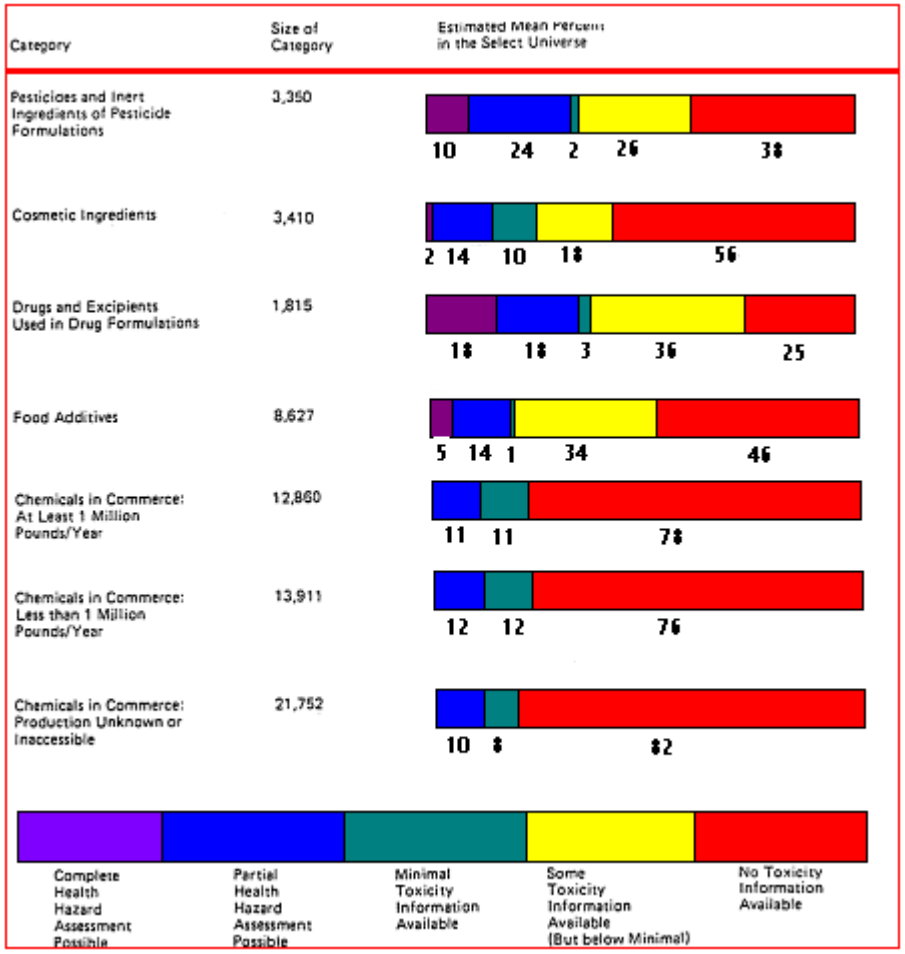
1.5.1. Availability of Toxicity Information

Toxicology will not become decrepit for lack of new chemicals, added at a rate of 36,000 new chemicals to the Chemical Abstracts Service database every day…

Some 65,000 basic chemicals are used in industry to create 5 million compound products. Most of them have been in use for many decades. Few have been re-assessed using the present, more stringent requirements of regulatory bodies[15]. These *grandfather* chemicals cause most of the problems.
A study by the US National Academy of Sciences reveals that between 76 and 82 % of chemicals in commerce have too little information to conduct a health-hazard assessment: virtually no toxicity information, as shown in F1.21.

"Adequate" toxicology exists for only 2-3 % of the chemicals. In 1976, the US *Toxic Substances Control Act* established that 60,000 chemicals would be "grandfathered", and that the Environmental Protection Agency would be responsible to perform scattered tests on 100 chemicals, and to restrict the use of 7 % of the chemicals introduced until 1979. This situation has arisen due to:

- the absence of demands for testing,
- the lack of adequate testing protocols,
- the large numbers of chemicals introduced since the 1950s.



F1.21. Availability of health hazard assessments in seven categories[15].

Only a quarter of the 82,000 chemicals in use in the US have been tested for toxicity. Each year, the US EPA reviews 1700 new compounds to be introduced. They are tested only *if evidence of potential harm exists*. 90 % of the new compounds are approved without restrictions.
Most of the chemicals in use will have some information on acute toxicity, but virtually none on reproductive toxicity, teratogenicity or carcinogenicity. In the absence of a specific "need to know", the costs and the time required discourage retro-testing.

"Restriction of Hazardous Substances (RoHS)" Compliance

Any RoHS compliant electronic and electronic component is tested for Lead, Cadmium, Mercury, Hexavalent chromium, Polybrominated biphenyls (PBB), and Polybrominated diphenyl ethers (PBDE).
For Lead, Polybrominated biphenyls (PBB), and Polybrominated diphenyl ethers (PBDE), there must be no more than 0.1% of the material, when calculated by weight at raw homogeneous materials.
For Cadmium and Hexavalent chromium, there must be less than 0.01% of the substance by weight at raw homogeneous materials.
Any RoHS compliant component must have 100 ppm or less of mercury, and the mercury must not have been intentionally added to the component.

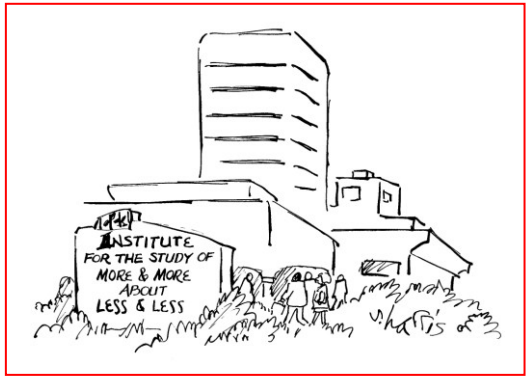
One may derive the impression, from the above, that there is never enough toxicity information. In some ways, this is true. For toxicological science, it would be desirable to have complete results on all substances. However, after the understanding that the gathering of all potentially useful information is impossibly expensive, the focus shifts to gathering information of good quality and to obtaining the *relevant* information (as opposed to the superfluous).

The strategy is to perform only the tests that need to be done to recognize health risks. A major task of experimental toxicologists is therefore to determine what data is critical to safety, and how this data should be interpreted.
A vital aspect of resource use in toxicology is the use of broad assumptions to qualify categories of chemicals, essentially removing them from expensive experimentation lists because some information is available on similar or connected substances. Although such inferences are not always correct and can lead to spectacular errors, Toxicology often plays the differential science card, avoiding costly experimentation based on acceptance that substances closely related in their physical and chemical properties may share similar toxicity.

1.5.2. Reliability of Toxicity Information

Agreement among experts is not always a guarantee of safety. Science often proceeds as a self-centered peer-review process (F1.22), which makes it vulnerable. Toxicology, for practical reasons, is more sensitive to toxicities that have obvious manifestations, and that develop over the short term.
Many sciences suffer from this vulnerability:
microbiology is biased towards the study of organisms that proliferate well in current culture media, and chemistry favors molecules that are easy to isolate, and are conveniently stable.

F1.22. Sometimes, science can lose perspective.
American Scientist, Nov-Dec 2003.



When new deleterious effects are detected, we often find that they spring from a new mechanism, involve a new variable, or occur over a longer time frame than previously investigated.
An important line of defense is vigilance on the part of occupational physicians, hygienists, toxicologists and workers themselves, which has contributed significantly to identifying overt as well as covert hazards. The reinvestigation of particular chemicals is frequently driven by a chance observation, a bizarre effect on an exposed individual, or an epidemic intoxication.
In the introductory chapter to a New York Academy of Sciences volume "celebrating" the passage of 200 years since Percival Pott's paper linking soot with scrotal cancer in chimney sweeps, Wagoner made a critical observation[16].
Despite the fact that we know about occupational carcinogenicity associated with coal and oil combustion products, radioactivity, inorganic arsenic, aromatic amines, asbestos, etc., we still have workers exposed to these agents, and they are dying at rates far higher that the normal population. What has been accomplished in these 200 years, if excess exposures still occur?
There is little concern about many of these chemicals, because they have been in use for a long time and are widely distributed. Often, the agent is not connected to the disease, or the disease is difficult to diagnose.

Consider the uranium miners from East Germany and their lung cancers resulting from poor to negligible occupational health practices during decades[17]. Lung cancer and disease killed at least 20,000 miners out of a workforce of 450,000 as a consequence of exposure to radiation and dusts.
Consider the neurotoxic disorders, one among a list of 10 leading work-related diseases identified by NIOSH[18].
Neurotoxicity can be severe and debilitating, with a large numbers of workers at risk. A vast array of commonly used

chemicals are associated with diverse neurological signs and symptoms, resulting in peripheral and central nervous system damage[18].
Consider the toxicity of lead (Pb) with effects on the nervous system (neuropathy and behavioral-learning deficits), the liver, the kidney and the hematopoietic system.

1.5.3. Regulations on Toxicity Information

Public and worker concerns, encouraged by the media, are demanding more information about chemicals manufactured or imported, distributed across the country by rail, truck or ship, used in various industries, in the home and garden, and disposed of when no longer needed.
National and international regulatory agencies are requiring more extensive data for each chemical entering the marketplace (T1.23). Under current Canadian, US and OECD/EC regulatory requirements, new chemicals must be accompanied by a basic package of certain toxicity tests (varying with the volume being imported or manufactured per year).
The list below is the information requirement for countries of the European Community.

Similar data is required for new chemicals under the Canadian Environmental Protection Act of 1999 (http://laws.justice.gc.ca/en/C-15.31/text.html).
Also included at the end of this table is information concerning disposal of the agent, designed both for industry and the individual user, in keeping with the "cradle-to-grave" responsibility of industries for their products.

T1.23.INFORMATION REQUIREMENTS IN SOME EEC COUNTRIES FOR NOTIFICATION AND HAZARD ASSESSMENT OF NEW CHEMICALS

IDENTITY OF THE SUBSTANCE

- Name
- Names in the International Union of Pure and Applied Chemistry nomenclature
- Other names (usual name, trade name, abbreviation)
- Chemical Abstracts Service number (if available)
- Empirical and structural formula
- Composition of the substance
- Degree of purity (%)
- Nature of impurities, including isomers and by-products
- Percentage of (significant) main impurities
- If the substance contains a stabilizing agent or an inhibitor or other additives, specify: nature, order of magnitude:... ppm;....%
- Spectral data (Ultra Violet, Infra Red, Nuclear Magnetic Resonance)
- Methods of detection and determination
- A full description of the methods used or the appropriate bibliographical references

INFORMATION ON THE SUBSTANCE

- Proposed uses
- Types of use

  Describe: the function of the substance, the desired effects
- Fields of application with approximate breakdown
  - ♦ closed system, industries, farmers and skilled trades,
  - ♦ use by the public at large, open system, industries
  - ♦ farmers and skilled trades, use by the public at large
- Estimated production and imports for each of the anticipated uses or fields of application
- Overall production and imports in order of tons per year [1, 10, 50, 100, 500, 1,000 and 5,000]
  - ♦ first 12 months
  - ♦ thereafter
- Production and imports, broken down in accordance with fields of application, expressed as a percentage,
  - ♦ first 12 months
  - ♦ thereafter
- Recommended methods and precautions concerning:
  - ♦ handling
  - ♦ storage
  - ♦ transport
  - ♦ fire (nature of combustion gases or pyrolysis. where proposed uses justify)
  - ♦ other dangers, particularly chemical reaction with water.
- Emergency measures in the case of accidental spillage
- Emergency measures in the case of injury to persons (e.g. poisoning)

PHYSICO-CHEMICAL PROPERTIES OF THE SUBSTANCE

- Melting point
- Boiling point (...°C at .. Pa)
- Relative density (D_4^{20})
- Vapour pressure (...Pa at ...°C)
- Surface tension (N/m at...°C)
- Water solubility (mg/litre at...°C)
- Fat solubility
- Solvent-oil [to be specified] (mg/100g solvent at...°C)
- Partition coefficient (n-octanol/water)
- Flash point (...°C in open cup and closed cup)
- Flammability
- Explosive properties
- Auto-flammability (...°C)
- Oxidizing properties

TOXICOLOGICAL STUDIES

- Acute toxicity

  Substances other than gases shall be administered via two routes at least one of which should be the oral route. The other route will depend on the intended use and on the physical properties of the substance. Gases and volatile liquids should be administered by inhalation (a minimum period of administration of four hours) in all cases, observation of the animals should be carried out for at least 14 days. Unless there are contraindications, the rat is the preferred species for oral and inhalation experiments. The experiments shall be carried out on both male and female subjects.

- Administered orally (LD50 in mg/kg)
  - ♦ Effects observed, including in the organs
- Administered by inhalation (LC50 in ppm)
  - ♦ Duration of exposure in hours
  - ♦ Effects observed, including in the organs
- Administered cutaneously, percutaneous absorption (LD50 in mg/l)
  - ♦ Effects observed. including in the organs
- Skin irritation
  - ♦ The substance should be applied to the shaved skin of an animal, preferably an albino rabbit.
  - ♦ Duration of exposure in hours.
- Eye irritation
  - ♦ The rabbit is the preferred animal
  - ♦ Duration of exposure in hours
- Skin sensitization
  - ♦ To be determined by a recognized method using a guinea pig
- Sub-acute toxicity (28 days)
  - ♦ Effects observed on the animal and organs according to the concentrations used, including clinical and laboratory investigations
  - ♦ Dose for which no toxic effect is observed
  - ♦ A period of daily administration (five to seven days per week) for at least four weeks should be chosen. The route of administration should be the most appropriate having regard to the intended use, the acute toxicity and the physical and chemical properties of the substance. Unless there are contra-indications, the rat is the preferred species for oral and inhalation experiments.
- Mutagenicity (including carcinogenic pre-screening test)
  - ♦ The substance should be examined during a series of two tests one of which should be bacteriological. with and without metabolic activation. and one non-bacteriological.

ECO-TOXICOLOGICAL STUDIES

- Effects on organisms
- Acute toxicity for fish (LC50 in ppm)
  - Duration of exposure
  - Species selected (one or more)
- Acute toxicity for Daphnia (LC50 in ppm)
  - Duration of exposure
- Degradation - biotic and abiotic
- The Biological Oxygen Demand and the Biological Oxygen Demand/Chemical Oxygen Demand ratio should be determined as a minimum.

POSSIBILITY OF RENDERING THE SUBSTANCE HARMLESS

- For industry/skilled trades
  - Possibility of recovery
  - Possibility of neutralization
  - Possibility of destruction:
    - controlled discharge
    - incineration
    - water purification station
    - others.
- For the public at large
  - Possibility of recovery
  - Possibility of neutralization
  - Possibility of destruction:
    - controlled discharge
    - incineration.
    - water purification station
    - others.

1.5.4. Material Safety Data Sheets

Much of the information above can be incorporated into the standardized *Material Safety Data Sheet*. The MSDS is a complete, up-to-date set of information for each product that a company manufactures or uses which, by law, must be available at all times in the Health or Safety office. The US Occupational Health and Safety Agency requires MSDSs for at least 600 chemicals. Companies are not required to perform tests, but to *list what they know*, including:

T1.24. COMPONENT PARTS OF THE MATERIAL SAFETY DATA SHEETS

SECTION I. Identity
Product name, family/chemical name, product type, Department of Transport category, hazard rating, fire rating, reactivity, emergency phone response number, prepared by...

SECTION II. Hazardous Ingredients
Ingredients with ACGIH, OSHA TLVs PELs and CAS no. plus source of information. Flags the hazardous ingredients.

SECTION III. Physical Data
Physical state, specific gravity, density, boiling point, percent volatiles, evaporation rate, odor, appearance.

SECTION IV. Fire and Explosion Hazard
Flash point, flammable limits, autoignition temperature, extinguishing media, fire-fighting procedures.

SECTION V. Health Hazard Data
Primary route of exposure (dermal, oral, eye, inhalation), over-exposure effects (irritation, sensitization) for acute and chronic effects, special toxicity (mutagenicity, teratogenicity, carcinogenicity, reproduction/fertility). Emergency and first aid procedures.

SECTION VI. Reactivity Data
Stability, incompatibility (materials to avoid), conditions to avoid, hazardous decomposition products, hazardous polymerization.

SECTION VII. Spill or Leak procedures
Spill control: how to do it, and what with. Waste disposal methods.

SECTION VIII. Special protection information
Respiratory protection (type of equipment needed), protective clothing (gloves, suits, eye, shoes).

SECTION IX. Special precautions
Handling, shipping and storage precautions, special warnings.

SECTION X. Regulatory information.

- the ingredients and quantities in the formulation,
- the physicochemical properties of each ingredient,
- the safety procedures and emergency first aid treatments,
- a section (Section V) dealing with toxicity data.

A quick review of most MSDSs reveals rather limited toxicity information. Section V usually relates to adverse health effects from acute, high level exposure through the oral, ocular, cutaneous or respiratory pathways, usually expressed as LD_{50} (in mg/kg body weight) as determined in a rodent model.
An example of a rather rich MSDS, for benzene, is available at: Principles of Toxicology DVD:\ Benzene MSDS.pdf.

The delayed effects are usually more sparingly documented, even if, for certain classes of chemicals (halogenated hydrocarbon solvents, pesticides), the delayed toxicity is of greater concern. Any longer term (subchronic) toxicity test is generally also in the form of a rodent study.

The MSDS database is essential because workers can be exposed accidentally to chemicals during manufacture, transportation, and ultimate use. Invariably, accidents will occur despite training, precautions, etc.

1.5.5. Poisoning Databases

Acute toxicity studies can go far beyond the determination of the LD_{50}, for example into the measures needed to manage acute poisonings in humans.
The first emergency centers were created in 1949 in Copenhagen and Budapest. Information became systematized and computerized more thoroughly in the 1950s, when centers aimed at pediatric and general population poisonings started to grow. Most were operated as part of the emergency or pharmacy of a hospital, with little training and no full time staff. There has been a tendency since the 1970s to reduce the number of poison centers and to provide them with better trained staff. Children under the age of 5 are responsible for 60 % of calls to poison centers.
The most used databases for poison centers are POISINDEX, DRUGDEX, TOMES and TOXNET.

For example, TOXNET is a computer system run by the National Library of Medicine that includes a number of toxicological databases managed by the Environmental Protection Agency, the National Cancer Institute, and the National Institute for Occupational Safety and Health. For more specific information, go to http://www.toxnet.nlm.nih.gov

The databases included in TOXNET are:
CCRIS (Chemical Carcinogenesis Research Information System),
DART (Developmental and Reproductive Toxicity Database),
DBIR (Directory of Biotechnology Information Resources),
EMICBACK (Environmental Mutagen Information Center Backfile),
GENE-TOX (Genetic Toxicology),
HSDB (Hazardous Substances Data Bank),
IRIS (Integrated Risk Information System, from EPA), http://www.epa.gov/iriswebp/iris/index.html
RTECS (Registry of Toxic Effects of Chemical Substances), and
TRI (Toxic Chemical Release Inventory).

For example, HSDB contains chemical-specific information on:

- manufacturing and use,

- chemical and physical properties,
- safety and handling,
- toxicity and biomedical effects,
- pharmacology,
- environmental fate and exposure potential,
- exposure standards and regulations,
- monitoring and analysis methods,
- and additional references.

1.5.6. Hygiene and Research Databases

1. A booklet for hygiene and for general toxicology work, NIOSH's Pocket Guide to Chemical Hazards (2005) is a short toxicity summary for hygiene purposes (T1.25). A condensed version is provided at Principles of Toxicology CD:\NIOSH Pocket Guide to Chemical Hazards 2007.pdf or at http://www.cdc. gov/niosh/npg/.

2. For in-depth toxicity work, Sax's *Dangerous Properties of Industrial Materials* provides more information (1.26). It delivers in extremely abbreviated form the results of 45 toxicity studies for benzene, a real time-saver when doing research work.

3. The Internet. See Principles of Toxicology DVD:\Web Pages\WWW Risk Assessment Resources.pdf. Anyone wishing information about an agent has access to Internet resources, and may take the time to read basic research articles.

As a toxicologist, you will be called upon to interpret this database for your employers, the employees, those considering litigation against a company, etc.

Benzene

Synonyms & Trade Names Benzol, Phenyl hydride

CAS No. 71-43-2	**RTECS No.** CY1400000	**DOT ID & Guide** 1114 130
Formula C_6H_6	**Conversion** 1 ppm = 3.19 mg/m³	**IDLH** Ca [500 ppm] See: 71432

Exposure Limits

NIOSH REL : Ca TWA 0.1 ppm ST 1 ppm See Appendix A
OSHA PEL : [1910.1028] TWA 1 ppm ST 5 ppm See Appendix F

Measurement Methods
NIOSH 1500, 1501, 3700, 3800;
OSHA 12, 1005
See: NMAM or OSHA Methods

Physical Description Colorless to light-yellow liquid with an aromatic odor. [Note: A solid below 42°F.]

MW: 78.1	**BP:** 176°F	**FRZ:** 42°F	**Sol:** 0.07%	**VP:** 75 mmHg	**IP:** 9.24 eV
Sp.Gr: 0.88	**Fl.P:** 12°F	**UEL:** 7.8%	**LEL:** 1.2%		

Class IB Flammable Liquid: Fl.P. below 73°F and BP at or above 100°F.

Incompatibilities & Reactivities Strong oxidizers, many fluorides & perchlorates, nitric acid

Exposure Routes inhalation, skin absorption, ingestion, skin and/or eye contact

Symptoms irritation eyes, skin, nose, respiratory system; dizziness; headache, nausea, staggered gait; anorexia, lassitude (weakness, exhaustion); dermatitis; bone marrow depression; [potential occupational carcinogen]

Target Organs Eyes, skin, respiratory system, blood, central nervous system, bone marrow

Cancer Site [leukemia]

Personal Protection/Sanitation (See protection codes)
Skin: Prevent skin contact
Eyes: Prevent eye contact
Wash skin: When contaminated
Remove: When wet (flammable)
Change: No recommendation
Provide: Eyewash, Quick drench

First Aid (See procedures)
Eye: Irrigate immediately
Skin: Soap wash immediately
Breathing: Respiratory support
Swallow: Medical attention immediately

Respirator Recommendations
(See Appendix E)

NIOSH

At concentrations above the NIOSH REL, or where there is no REL, at any detectable concentration:
(APF = 10,000) Any self-contained breathing apparatus that has a full facepiece and is operated in a pressure-demand or other positive-pressure mode
(APF = 10,000) Any supplied-air respirator that has a full facepiece and is operated in a pressure-demand or other positive-pressure mode in combination with an auxiliary self-contained positive-pressure breathing apparatus

Escape:
(APF = 50) Any air-purifying, full-facepiece respirator (gas mask) with a chin-style, front- or back-mounted organic vapor canister
Any appropriate escape-type, self-contained breathing apparatus

Important additional information about respirator selection

See also: INTRODUCTION See ICSC CARD: 0015 See MEDICAL TESTS: 0022

T1.25. NIOSH's Pocket Guide to Chemical Hazards.

BENZENE

CAS RN: 71432 NIOSH #: CY 1400000
mf: C_6H_6; mw: 78.12

Clear colorless liquid. mp: 5.51°, bp: 80.093°-80.094°, flash p: 12°F (CC), d: 0.8794 @ 20°, autoign. temp.: 1044°F, lel: 1.4%, uel: 8.0%, vap. press: 100 mm @ 26.1°, vap. d: 2.77, ulc: 95-100.

SYNS:

(6)ANNULENE
BENZEEN (DUTCH)
BENZEN (POLISH)
BENZOL
BENZOLENE
BENZOLO (ITALIAN)
BICARBURET OF HYDROGEN
CARBON OIL
COAL NAPHTHA
CYCLOHEXATRIENE
FENZEN (CZECH)
MINERAL NAPHTHA
MOTOR BENZOL
NCI-C55276
PHENYL HYDRIDE
PYROBENZOLE

TOXICITY DATA: 3

skn-rbt 15 mg/24H open MLD
eye-rbt 88 mg MOD
eye-rbt 2 mg/24H SEV
cyt-rat-scu 12 gm/kg/12D-I
mnt-mus-ipr 500 uL/kg
cyt-mus-orl 100 uL/kg
cyt-mus-ipr 100 uL/kg
dlt-mus-ipr 5 mg/kg
cyt-rbt-scu 8400 mg/kg
scu-mus TDLo:2700 mg/kg/(13D preg):TER
ihl-hmn TCLo:100 ppm/10Y-I:CAR
orl-rat TDLo:52 gm/kg/52W-I:CAR
skn-mus TDLo:1200 gm/kg/49W-I:NEO
scu-mus TDLo:600 mg/kg/17W-I:ETA
par-mus TDLo:670 mg/kg/19W-I:ETA
ihl-hmn TC:400 ppm/8Y-I:ETA
ihl-man TC:2100 mg/m3/4Y-I:CAR
orl-rat TD:10 gm/kg/52W-I:CAR
orl-hmn TDLo:130 mg/kg:CNS
ihl-hmn LCLo:20000 ppm/5M
ihl-hmn TCLo:210 ppm:BLD
ihl-rat TCLo:670 mg/m3/24H (15D pre/1-22D preg)
ihl-rat TCLo:56600 ug/m3/24H (1-22D preg)
ihl-rat TCLO:50 ppm/24H (7-14D preg)
ihl-rat TCLO:150 ppm/24H (7-14D preg)
scu-mus TDLo:1100 mg/kg (12D preg)
scu-mus TDLo:2700 mg/kg/(13D preg) TFX:TER
orl-mus TDLo:9 gm/kg (6-15D preg)
orl-mus TDLo:12 gm/kg (6-15D preg)
orl-rat TD:10 gm/kg/52W-I TFX:CAR
ihl-hmn TCLo:100 ppm:CNS
unk-man LDLo:194 mg/kg
orl-rat LD50:3800 mg/kg
ihl-rat LC50:10000 ppm/7H
ipr-rat LDLo:1150 mg/kg
orl-mus LD50:4700 mg/kg
ihl-mus LC50:9980 ppm
ipr-mus LD50:990 ug/kg
orl-dog LDLo:2000 mg/kg
ihl-dog LCLo:146000 mg/m3
ihl-cat LCLo:170000 mg/m3
ivn-rbt LDLo:88 mg/kg
ipr-gpg LDLo:527 mg/kg
scu-frg LDLo:1400 mg/kg
ihl-mam LCLo:20000 ppm/5M

CODEN:

AIHAAP 23,95,62
AMIHAB 14,387,56
28ZPAK -,23,72
GTPZAB 17(3),24,73
ENMUDM 2,43,80
ENMUDM 2,43,80
ENMUDM 2,43,80
TPKVAL 15,30,79
PSDTAP 15,275,74
AMBNAS 17,285,70
TRBMAV 37,153,78
MELAAD 70,352,79
BJCAAI 16,275,62
KRANAW 9,403,32
KLWOAZ 12,109,33
BLOOAW 52,285,78
NEJMAG 271,872,64
MELAAD 70,352,79
AHYGAJ 31,336,1897
29ZUA8 -,-,53
27ZXA3 -,341,63
HYSAAV 33,327,68
HYSAAV 33,112,68
JHEMA2 24,363,80
JHEMA2 24,363,80
TOXID9 1,125,81
AMBNAS 17,285,70
TJADAB 19,41A,79
TJADAB 19,41A,79
MELAAD 70,352,79
INMEAF 17,199,48
85DCAI 2,73,70
TXAPA9 19,699,71
28ZRAQ -,113,60
TXAPA9 1,156,59
HYSAAV 32,349,67
JIHTAB 25,366,43
AGGHAR 18,109,60
HBAMAK 4,1313,35
HBTXAC 1,324,56
HBTXAC 1,324,56
JTEHD6 -(Suppl.2),45,77
HBTXAC 1,42,56
HBAMAK 4,1313,35
AEPPAE 138,65,28

Aquatic Toxicity Rating: TLm96:100-10 ppm WQCHM* 2,-,74. Carcinogenic Determination: Human Suspected IARC** 7,203,74.

TLV: Air: 10 ppm DTLVS* 4,37,80. *Toxicology Review:* ARPAAQ 11,434,31; EVHPAZ 11,163,75; AEHLAU 22,373,71; PAREAQ 4,1,52; FNSCA6 2,67,73; MUREAV 47(2),75,78; AMSVAZ 118,354,44; ZHPMAT 166,113,78; JTEHD6 -(suppl.2),69,77; PHRPA6 41,1357,26; CTOXAO 11,531,77; BNYMAM 54, 413,78; KRANAW 9,403,32; 27ZTAP 3,22,69. OSHA Standard: Air: TWA 10 ppm; CL 25 ppm; Pk 50 ppm/10M/8H (SCP-U) FEREAC 39,23540,74. DOT: Flammable Liquid, Label: Flammable Liquid FEREAC 41,57018,76. Occupational Exposure to Benzene recm std: Air: CL 10 ppm/60M NTIS**. Currently Tested by NTP for Carcinogenesis by Standard Bioassay Protocol as of December 1980. "NIOSH Manual of Analytical Methods" VOL 1 127, VOL 3 S311. Reported in EPA TSCA Inventory, 1980. EPA TSCA 8E NO:12770027-Followup Sent as of April, 1979.

THR: Poisoning occurs most commonly through inhal of the vapor, though benzene can penetrate the skin, and poison in that way. Locally, benzene has a comparatively strong irr effect, producing erythema and burning, and, in more severe cases, edema and even blistering. Exposure to high conc of the vapor (3000 ppm or higher) may result from failure of equipment or spillage. Such exposure, while rare in industry, may result in acute poisoning, characterized by the narcotic action of benzene on the CNS. The anesthetic action of benzene is similar to that of other anesthetic gases, consisting of a preliminary stage of excitation followed by depression and, if exposure is continued, death through respiratory failure. The chronic, rather than the acute form, of benzene poisoning is important in industry. It is a recog leukemogen. There is no specific blood picture occurring in cases of chronic benzol poisoning. The bone marrow may be hypoplastic, normal, or hyperplastic, the changes reflected in the peripheral blood. Anemia, leucopenia, macrocytosis, reticulocytosis, thromocytopenia, high color index, and prolonged bleeding time may be present. Cases of myeloid leukemia have been reported. For the supervision of the worker, repeated blood examinations are necessary, including hemoglobin determinations, white and red cell counts and differential smears. Where a worker shows a progressive drop in either red or white cells, or where the white count remains below 5,000 per cu mm or the red count below 4.0 million per cu mm, on two successive monthly examinations, he should be immediately removed from exposure. Following absorption of benzene, elimination is chiefly through the lungs, when fresh air is breathed. The portion that is absorbed is oxidized, and the oxidation products are combined with sulfuric and glycuronic acids and eliminated in the urine. This may be used as a diagnostic sign. Benzene has a definite cumulative action, and exposure to relatively high conc is not serious from the point of view of causing damage to the blood-forming system, provided the exposure is not repeated. On the other hand, daily exposure to conc of 100 ppm or less will usually cause damage if continued over a protracted period of time. In acute poisoning, the worker becomes confused and dizzy, complains of tightening of the leg muscles and of pressure over the forehead, then passes into a stage of excitement. If allowed to remain in exposure, he quickly becomes stupefied and lapses into coma. In non-fatal cases, recovery is usually complete and no permanent disability occurs. In chronic poisoning the onset is slow, with the symptoms vague; fatigue, headache, dizziness, nausea and loss of appetite, loss of weight and weakness are common complaints in early cases. Later, pallor, nosebleeds, bleeding gums, menorrhagia, petechiae and purpura may develop. There is great individual variation in the signs and symptoms of chronic benzene poisoning. Benzene is a common air contaminant. Exper MUT, CARC, TER, ETA, NEO.

Fire Hazard: Dangerous, when exposed to heat or flame; can react vigorously with oxidizing materials, such as BrF_5, Cl_2, CrO_3, O_2NClO_4, O_2, O_3, perchlorates, ($AlCl_3$ + $FClO_4$), (H_2SO_4 + permanganates), K_2O_2, ($AgClO_4$ + acetic acid), Na_2O_2.

Spontaneous Heating: No.

Explosion Hazard: Mod, when its vapors are exposed to flame. Use with adequate ventilation.

Disaster Hazard: Dangerous, highly flammable.

To Fight Fire: Foam, CO_2, dry chemical.

Incomp: diborane.

For further information see Vol. 2, No. 4 and Vol. 3, No. 3 of *DPIM Report.*

T1.26. Sax's Dangerous Properties of Industrial Materials.

1.6. Toxicologists in Society

What do toxicologists do ?
Some are employed by the government (Health Canada), others by the private sector. They can be involved in the protection of workers (Occupational Health) or of the general population (Public Health).
The may be concerned with pharmaceuticals (clinical toxicologists, poison specialists), household products, industrial and agricultural chemicals and food additives.
They perform tests in laboratories (research and development, evaluation of test results), risk analysis (literature reviews) and develop policy and regulations for government or corporations.

They may serve on committees determining Safe Human Doses or product safety and labeling regulations.

Environmental toxicologists are concerned with the persistence of chemicals in the environment, food chain accumulation and with the consequences of toxicants to the biota.

They may be involved in public relations, in scientific writing for the public and in legal proceedings, as expert witnesses.

The Society of Toxicology lists the following specialties:

- Biological Modeling,
- Carcinogenesis,
- Comparative and Veterinary toxicology,
- Dermal toxicology,
- Food Safety,
- Immunotoxicology,
- In Vitro toxicology,
- Inhalation toxicology,
- Mechanistic toxicology,
- Metals toxicology,
- Molecular toxicology,
- Neurotoxicology,
- Epidemiology,
- Occupational Health,
- Regulatory and Safety evaluation,
- Reproductive and Developmental toxicology,
- Risk Assessment,
- Toxicological and Exploratory pathology…

Of course, toxicologists may also pursue academic careers in universities.

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Risk Assessment

2· Risk Assessment

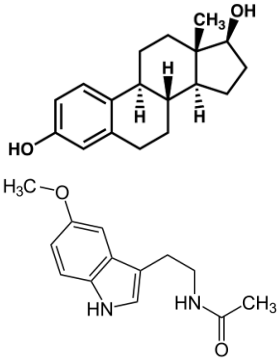
2.1. Complexity of Toxic Effects

All toxic agents have multiple effects occurring at different doses, and within specific time intervals, as shown in F2.1.
A critical decision in the practice of Risk Assessment is focusing on one or a few toxicity variables to estimate the Safe Human Dose. Which critical toxicity variable or disease rate is most affected by the agent, and to what extent should exposure be limited to protect human or animal health ?

Extensive data for a particular toxicity variable or test system may be available simply because a laboratory was funded to conduct the research, or because the measurement is convenient or inexpensive (ex, Ames test). The toxicologist aims to compensate for these biases, looking to obtain from restricted evidence a toxicity determination that is as comprehensive as possible.

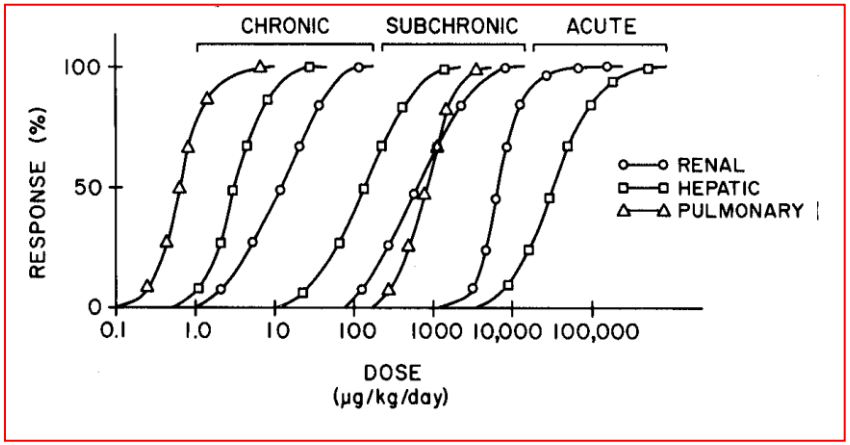
2.1.1. Low-Dose Toxic Effects

The appreciation of the full toxicity of an agent often proceeds historically through a series of successive discoveries. For example, it has been known for a long time that arsenic kills rapidly in high doses (8 mg/kg in the rat).
But it is relatively recent knowledge that at doses of 7 ppm in water, it leads to narrowing of the carotid artery[1] and that it can act as an endocrine disruptor[3,5]. Further, it interferes with the action of glucocorticoids at an environmental concentration of 10 ppb.
More subtle toxic effects are often uncovered over time. Environmental hormones, compounds that can alter physiology at low concentrations, are prime candidates for low-dose risks. Five years ago, almost all cases of environmental hormones (ex, nonylphenol, phthalates[6]) involved estrogen (estradiol is shown). More recently, agents substituting melatonin (shown), anti-androgens and thyroid hormones have emerged.



2.1.2. Significant vs Trivial Risks

Protective limits for an agent should take into account its lowest-dose *significant* risk. The decision of which risk to retain as *significant* is one that must be made on the basis of the deepest knowledge of physiology and epidemiology available.
Perhaps the greatest weakness of Risk Assessment is that its view is limited by the evidence available. It is impractical to test *all* body systems in sufficient detail to dispel *all* doubts about the safety of an agent.



F2.1 Plurality of Effects for a Toxicant.
In Vitro Methods in Toxicology, Jolles and Cordier, 1990

Files compiled for an agent typically contain a large number of studies. A first triage is usually made on the basis of

- species (human, rodent, cells),
- route of administration (inhaled, cutaneous, oral exposures) and
- time-frame (acute, chronic).

If biological exposure indices such as blood or plasma levels are available, this helps to make comparisons between studies more enlightening, particularly if the targets of the toxicant are known.

An important practical problem is that Safe Human Doses are intended to limit lifetime exposures, while few laboratory studies ever provide this time frame. Epidemiology often provides a more chronic perspective than experiments, but typically its results are much more difficult to interpret.

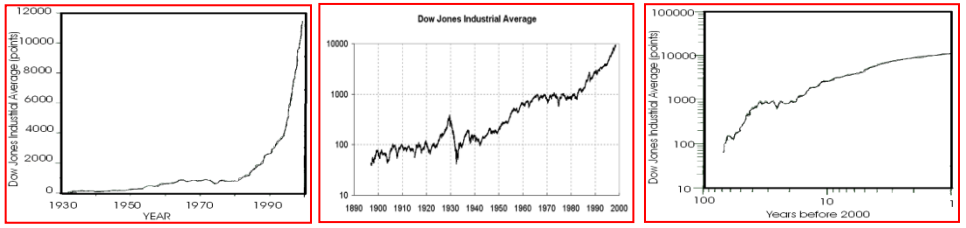
2.1.3. Tox File Diversity

The diversity of scientific knowledge can become an obstacle to obtaining a clear assessment of risk. Some studies often promote particular interests and methods, and high political stakes often tend to disintegrate a unified picture of risk. This can give the impression of an absence of coherence or reliability in science. The real problem is that risk is itself fairly complex in that it can apply to different organs, populations and situations. It is therefore often difficult to come to a single limit that will satisfy all points of view. Often the Safe Human Dose will be determined by focusing on a few *strategically chosen* studies.

2.2. The Choice of Graphs

The choice of graphic representations for toxicity data as a function of dose is important because graphs have a suggestive influence. One frequently quoted motive to use *logarithmic* axes is to view more clearly data spanning a very wide amplitude range (orders of magnitude). On a linear scale, the relative changes occurring at low values would be obscured, compressed in the early part of the graph.

F2.2 shows the same financial data (Dow Jones industrial average) presented in 3 different ways. One gets quite a different impression about the evolution of the economy, according to the axial metric chosen...



F2.2. The leftmost graph (linear-linear) gives the impression of a rapidly expanding economy in recent years. The middle graph (log – linear) suggests a steady evolution. The rightmost graph (log-log) shows a tendency to saturation. So, how is the economy *actually* evolving ? *American Scientist, 89:210*

Note the following about log scales.

-There is no "0".

-A log scale displays normal number labels that are positioned according to a log scale. The grids along the log scale change abruptly from intervals of 10 to intervals of 100, for example.

-You cannot re-number these labels, but you can choose the position of the decimal points.

-Equal-sized steps along a log scale correspond to a multiplication factor.

Toxicology uses specific conventions in its choice of graph axes, and these conventions can be justified using Michaelis-Menten kinetics or statistical arguments, as we shall see later in this chapter. The graphic conventions (log axes) used in Toxicology are set to ease detection of specific dependencies anticipated from experimental results. However, one should use log scales cautiously…

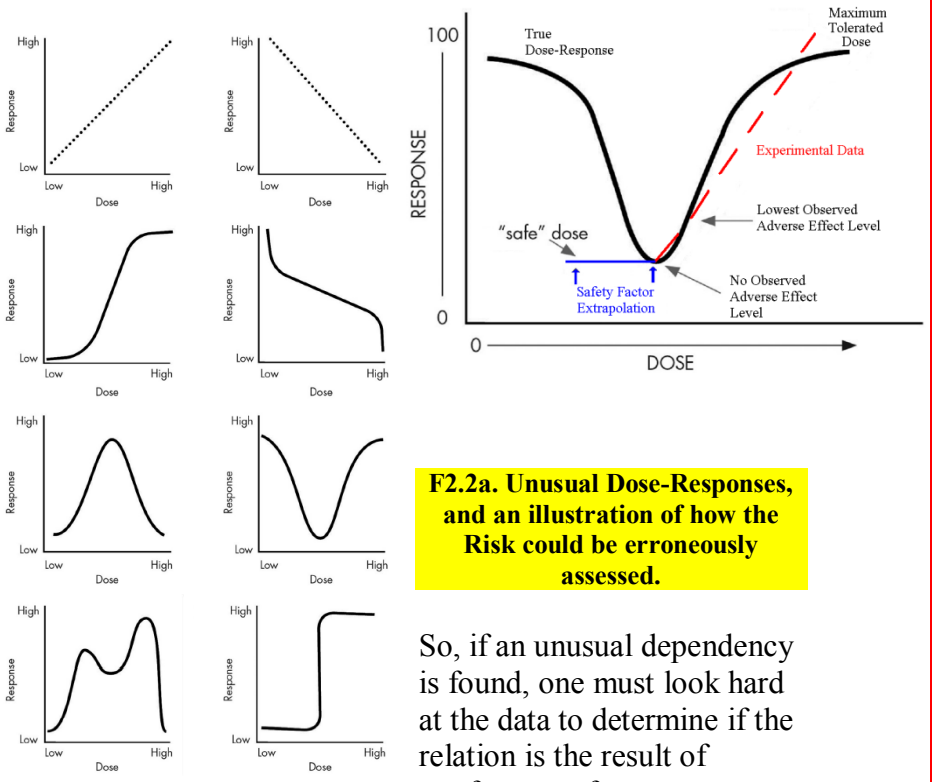
Do not let tricky graphs cloud your thinking about problems they are intended to illuminate.

2.3. Dose-Response Relations

Quantitative Toxicology relates exposure (usually expressed as toxicant concentration x exposure duration) and the toxicity produced (changes in liver, renal, neurological, pulmonary function, etc.), in other words, the dose-response relationship.

There is a great deal of emphasis in Toxicology on documenting fairly conventional, progressive dose-response relationships similar to F2.3. Obtaining a "proper" relation gives the experimenter or the epidemiologist confidence that his data is sound. However, this frequently observed curve type is not the only one possible, as was shown by the class of chemical known as "endocrine disruptors"[10], for example, and one should be prepared to accept unusual dose-response dependencies. Agents can be more toxic at lower doses than at higher ones, as can be seen in T2.2a and F2.2a. Examples include polluants that resemble hormones, drugs[4], electromagnetic radiation and ionizing radiation.

T2.2a. Unusual Dose-Effect Curves		
Agent	**Observation**	**Ref.**
di-2-ethylhexyl phthalate (DEHP, a plastic-softening agent)	Low DEHP dose suppresses brain activity of aromatase, critical for male development, while higher doses stimulate it.	9
X-rays and Gamma radiation	1 Gy increases lifetime risk of cancer by 5 percent, while 0.1 Gy defends against cancer.	8
Electromagnetic Radiation	Calcium efflux in nerve cells is stimulated within an intensity window only.	7



F2.2a. Unusual Dose-Responses, and an illustration of how the Risk could be erroneously assessed.

So, if an unusual dependency is found, one must look hard at the data to determine if the relation is the result of artefacts or of true physiology. Unusual dose-responses are less likely in more complex systems. Therefore, epidemiologists scrutinizing large populations are less likely to observe them than laboratory experimenters.

Emphasis on documentation of the dose-response tends to obscure other aspects of toxicity, specifically:

- whether the outcome variable chosen is the most relevant one to determine a Safe Human Dose for the agent,
- the influence of time: most tests are too short (to conserve resources), thereby blinding the testing process to the

evolution and reversibility of toxicity.

"**DOSE**" refers to the mass administered (ex, 10 mg of diazepam) and does not account for the size or weight of the animal. Generally, *body burden* is also expressed as an absolute mass of substance.
"**DOSAGE**" refers to the mass per body weight of the individual (ex, 0.2 mg/kg of body weight).
"**TOTAL DOSAGE**" takes into account the duration of exposure: a dosage of 10 mg/kg per day for 10 days translates to a Total Dosage of 100 mg .day / kg.

2.4. Characteristics of Dose- Response Curves

Many dose-response curves can be characterized according to the following characteristics: Efficacy, Potency, Spread and Statistical Dispersion.

2.4.1. Efficacy

The curve between dosage and resulting toxicity defines whether a given agent (say, caffeine) is *effective* at producing a given toxicity (say, a rise in heart rate).
The *efficacy* of an agent relates to the maximum effect that it can produce at any concentration (see F2.8).
Often, it is also appropriate to express the *efficacy* of an agent by comparing it to the maximum effect that can be obtained on a living system by the most effective chemical known. The ratio of the particular agent's result to the system's maximum response thus determines *efficacy* in a range from 0 to 1.

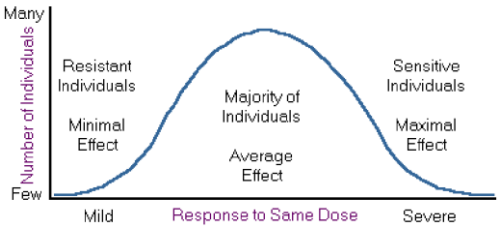
2.4.2. Potency

An agent's efficacy tells us something about its maximum effect, but not about how much of the agent is needed to obtain a given effect. A "reading" of how much of the agent (mg, concentration or total dosage) is required to obtain half of the maximum effect (efficacy = 0.5) is commonly used to derive a measure of toxicant *potency*[∅].
A drug that requires a lower dose than another drug to obtain the same half-maximum effect is therefore considered to be more *potent*. F2.7 illustrates 3 curves of different potency.

2.4.3. Spread

Some dose-response curves are very steep, while others need larger changes of dosage for the same change in toxic effect. F2.4 shows curves with different *spreads*. *Spread* can be specified by the toxicant dosage ratio between 10 % and 90 % efficacy. In F2.4, the curve on the left has a 10-90 % *spread* of 21.9, while the curve on the right has a 10-90 % *spread* of 3.88.

2.4.4. Statistical Dispersion



If a group of animals in a laboratory study is given exactly the same dose of a chemical, the response from each animal will be at least slightly different.

There is also an uncertainty as to the dose actually received by each animal. For example, in the example above, the toxicant may be mixed with food, and each animal does not eat exactly the same amount. In laboratory studies, the inaccuracy in the

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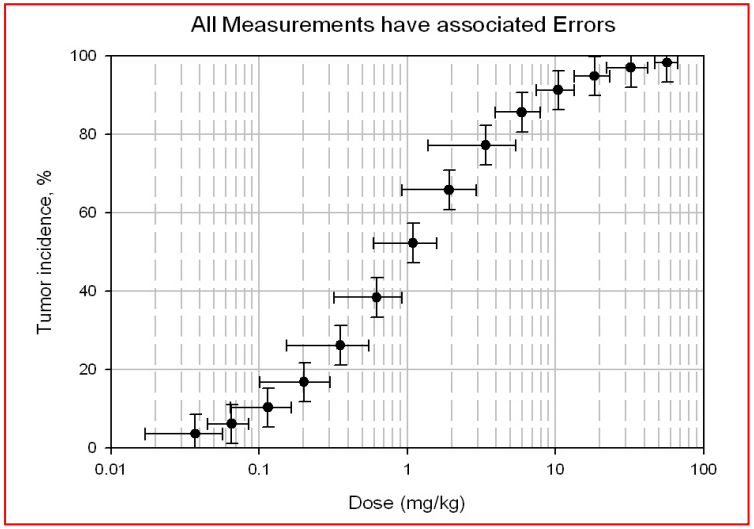
[∅] Drug expiration dates specify the manufacturer's guarantee of *full potency* and safety. **It is safe** to take drugs past their expiration dates. Even 10 years past the date, most drugs have a good deal of their original potency. So, if your life depends on an expired drug and you *must* have 100 % of its original strength, you should toss it, but if not, use it and see what happens. 90 % of drugs are safe and effective as far as 15 years past their expiration dates. Exceptions: nitroglycerin, insulin and some liquid antibiotics.

dose (horizontal) is usually fairly small, but the dispersion of the toxic reaction (vertically) cannot be controlled.
For example, if one uses a group of 6 animals, yielding 6 individual toxicity responses at a given dose, these 6 outcomes can be used to determine a mean response, as well as a standard deviation for this outcome at that dose.
Even in the case where the outcome is rated as all-or-nothing, such as presence of cancer, the number of animals used in any given study limits the reliability of the probability of getting cancer, particularly if the risk is small.
Although laboratory experimenters mostly attempt to reduce the dispersion in their data by keeping tight control over animals (to the point of using animal clones) and test conditions, *dispersions in toxicity reactions are part of the data,* if the data is to be relevant to a complex environment.
In epidemiological studies, there are uncertainties associated with gathering data from populations, and also uncertainties in the estimates of their exposures.



F2.3. Horizontal and Vertical dispersions.

In any dose-response graph, such as F2.3, both the variable describing the toxicity (here, tumor incidence), as well as that describing dose (horizontal), show statistical dispersions. Therefore, obtaining data dispersions representative of real-world situations may be legitimate objectives in laboratory experiments as well as in epidemiological investigations.

2.5. Analysis of Dose-Response Curves

2.5.1. Multi-Target Model

We study toxic dose-responses by building simple mathematical models of toxicity. Let us imagine that we initially have a group of N_0 cells exposed to a toxic dose (D). A number of cells (N) survive the exposure, while the others die. If we perform the experiment repeatedly at various doses, we can obtain dose responses such as in F2.4.
Considering the set of experiments on the blue curve, the supplementary number of cells dying (dN) per exposure increment (dD) is proportional to the number of cells (N) living at the lower dose multiplied by the "toxic slope" variable, "t". This assumes the same toxic slope at all levels of toxicity.

$$-\frac{dN}{dD} = t\ N$$

After exposure "D", the cell number declines from N_0 to N:

$$\int_{No}^{N} \frac{dN}{N} = -\ t \int_{0}^{D} dD$$

$$\ln \frac{N}{N_0} = -\ t\ D$$

The proportion of surviving cells is:

$$\frac{N}{N_0} = e^{-\ t\ D}$$

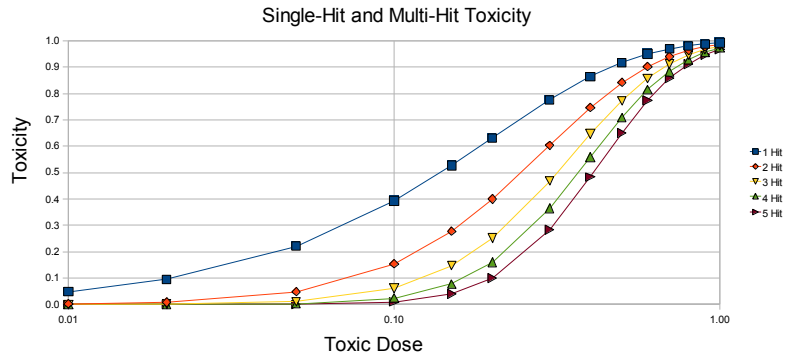
In this simple model, an infinite dose is needed to kill all the cells.
We can define Toxicity (T) as the fraction of dead cells:

$$Toxicity = \frac{No - N}{No} = \frac{No - No\ e^{-t\,D}}{No} = 1 - e^{-t\,D}$$

At this point, we modify the model slightly. The death of a cell can occur from a single toxic hit (F2.4, blue curve), or it can occur from numerous "hits" (below) to a single cell. The probability that *n* independent events of probability P occur is P^n. In this Multi-Hit model, the probability of death decreases, because "n" random hits must accumulate for one cell's death:

$$Toxicity = (1 - e^{-t\,D})^n$$

A semi-log graph of Toxic Dose (D) vs Toxicity is drawn for n = 1 to 5 hits in F2.4.



F2.4. Multi-Hits make dose-responses steeper.

The Single-Hit curve (blue) displays the condition "-dN/dD = tN", while the higher n-curves tend toward a threshold form. Threshold dose-responses are frequently observed in Toxicology. The 5-Hit curve's shape results from the fact that 1 to 4 hits do not produce toxicity in the left part of the curve, while in the right part, there is a relative richness of vulnerable targets (with accumulated hits), coupled with high toxicant concentration. The multiple hit curves are functionally equivalent to a low-dose detoxification mechanism. Note the 10-90 % spread in these curves.

T2.4a. 10-90 % Toxicity Spread					
1-Hit	2-Hit	3-Hit	4-Hit	5-Hit	10-Hit
21.9	7.82	5.4	4.42	3.88	1.91

When is the Single-Hit model applicable ?
The plant toxin ricin follows a retrograde transport route: from cell surface to endosomes to the Golgi complex, to the endoplasmic reticulum and finally the cytosol, where it binds to and inactivates ribosomal RNA, thereby inhibiting protein synthesis. A single ricin molecule entering the cytosol can inactivate over 1,500 ribosomes per minute, and kill the cell.

When is the Multi-Hit model applicable ?
The inactivation of membrane receptors is an example, because they are usually available in larger number than necessary. Toxicity emerges at the toxicant concentration where the remaining active receptor proteins are *required* for cell survival or function.

If absorbed in small enough quantities, most toxicants can be neutralized by normal physiological mechanisms such as Phase II metabolism (Chapter 3). This neutralization would shift the dose-response curves to the right.

2.5.2. Toxicant to Receptor Binding

Increasing the stimulation on a physiological system generally produces climbing curves that saturate at higher doses. *Equilibrium binding* can be applied to toxicant-receptor kinetics.

When *toxicants* interact with biological *receptors*, binding follows the *law of mass action*. The equation below describes the reversible binding of *toxicant* to *receptor*, quantified by binding and dissociation constants (K_x).

$$[toxicant]+[receptor]\underset{K_{diss}}{\overset{K_{bind}}{\rightleftharpoons}}[toxicant \bullet receptor]$$

The rate of bound complex (•) formation in moles/minute = [*toxicant*] x [*receptor*] x K_{bind}. Consequently, the binding rate constant K_{bind} is expressed in units of 1/(moles x min).
The rate of complex dissociation (+) is [*toxicant* • *receptor*] x K_{diss}. Consequently, the dissociation rate constant K_{diss} is expressed in units of 1/min.
At equilibrium, the dissociation reaction equals the binding reaction, so

$$[toxicant]\times[receptor]\times K_{bind} = [toxicant \bullet receptor]\times K_{diss}$$

or

$$[toxicant \bullet receptor] = [toxicant]\times[receptor]\times\frac{K_{bind}}{K_{diss}}$$

Define the Michaelis-Menten constant, K_M, equal to K_{diss}/K_{bind}, in moles units, which yields:

$$[toxicant \bullet receptor] = \frac{[toxicant]\times[receptor]}{K_M}$$

Low values of K_M indicate that the complex holds together very tightly. Since all *receptors* are either free or bound, we can express [*receptor*] as the total number of receptors minus the number bound:
[*receptor*] = [$receptor_{tot}$] − [*toxicant* • *receptor*].
Substituting this definitions into the previous equation,

$$[toxicant \bullet receptor] = \frac{[toxicant]\times([receptor_{tot}]-[toxicant \bullet receptor])}{K_M}$$

which can be rearranged into the *binding equation* as:

$$[toxicant \bullet receptor] = \frac{[receptor_{tot}]\times[toxicant]}{K_M+[toxicant]}$$

In toxicity testing, we vary the dose (or *toxicant* concentration) and measure toxic effects proportional to [*toxicant* • *receptor*], the left side of the equation above. Both [$receptor_{tot}$] and K_M are constants.
F2.5, created from the preceding equation, represents a *binding* curve. If you plot the same data using a semilog layout (the X-axis is *log* of dose), it becomes a soft sigmoid curve, as shown in F2.6.
The 10-90 % spread in these curves is about 100, independent of K_M.
This curve is the expected form of toxicity relationships between log of dose and continuous toxicity variables, if compatible with simple receptor-ligand kinetics.

This new *binding curve* has a central range where the slope changes relatively little, one practical reason for adoption of these axes. An estimate of the amount of the toxicant that produces a 50 % response is obtainable from the data points that contribute to the estimation of this central slope. Deviations from this form signal that more complex phenomena contribute to toxicity.

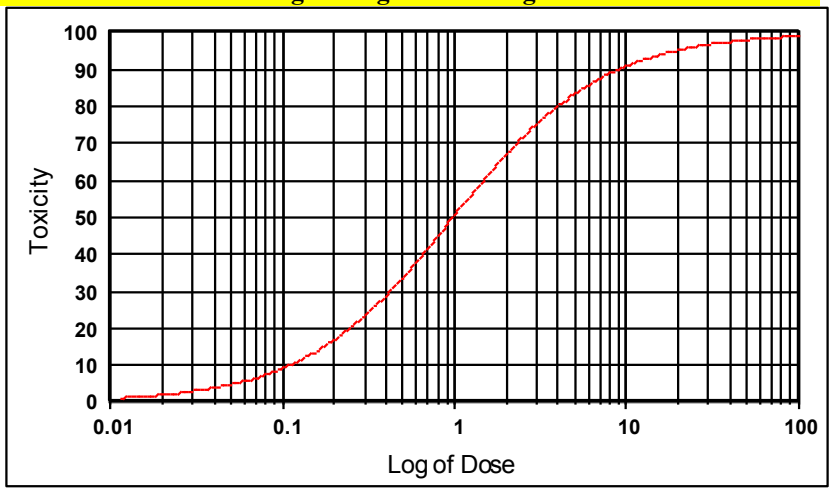
Further, if we change the value of the Michaelis-Menten constant (K_M) in the binding equation by a factor of 100 each way, identically shaped toxicity curves (F2.7) display variations in *potency*. These curves could be perceived as showing a threshold or not, depending on the range of dose chosen for the experiment.
The value of the Michaelis-Menten constant, K_M, represents the speed of chemical reactions in general, and is known for several enzymes (T2.4b).

If we wish to represent analytically changes in efficacy, the term "+[toxicant]" in the binding equation can be modified by addition of an factor corresponding to inclusion of two binding steps as opposed to one, the two successive steps cascading to contribute to a final expression of toxicity. The result is illustrated in the lower curve of F2.8, showing that two binding steps reduce the efficacy of the reaction.

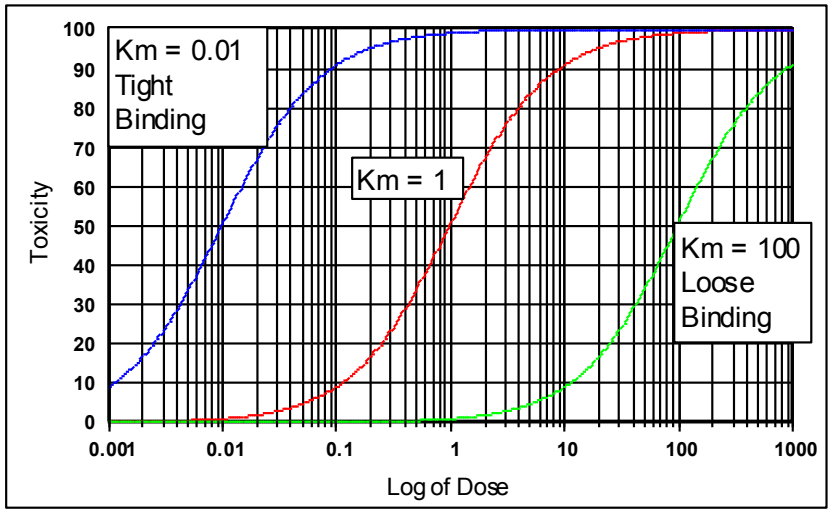
T2.4b. Michaelis-Menten constants, Km		
Enzyme	**Substrate**	**K_m (Molar)**
Catalase	H_2O_2	1.1
Chymotrypsin	Gly-Tyr-Gly	0.108
Carbonic anhydrase	CO_2	0.012
β-Galactosidase	d-Lactose	.004
Acetylcholinesterase	Acetylcholine (ACh)	0.00009
β-Lactamase	Benzylpenicillin	0.00002

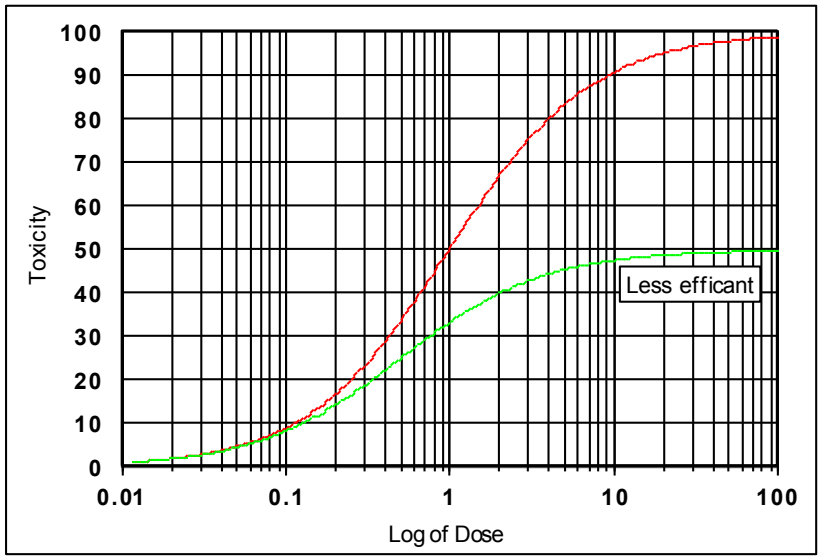
F2.5. Binding Curve.

F2.6. Log scaling of a Binding Curve.



F2.7. Changing K_M alters Toxic Potency.





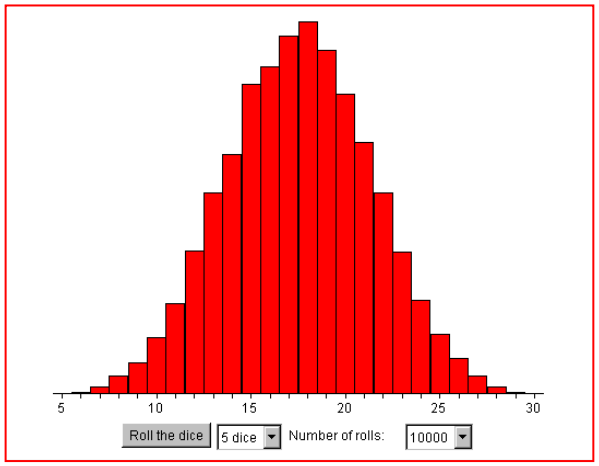
F2.8. Increasing number of binding steps reduces Efficacy.

2.5.3. Dose-Response of Complex Systems

In laboratory tests, the toxicologist almost always prefers to evaluate groups of animals or cells, basically because all animals or cells do not behave identically. In epidemiology, differences between human subjects lead to the determination of probabilities, as opposed to certainties.
To protect toxicological results against individual variations, a statistically significant sample of animals or cells is needed for testing. This plurality of tests objects is almost universally implemented, often according to budget limitations, even if no *a priori* information is available on the variability of the toxicity investigated.

2.5.3.1. Central Limit Theorem

To view how test-subject variations influence toxicological results, we take a brief tour of simple statistics...
If one rolls one die repeatedly, the values 1,2,3,4,5,6 will come out the same number of times, resulting in a flat histogram.



F2.9. Distribution generated by rolling 5 dice.
http://www.stat.sc.edu/~west/javahtml/CLT.html

However, as the number of dice increases, the distribution of the total results assumes the shape in F2.9. Even if the distribution for the individual die is flat, the sum of the readings of 5 dice will show a most frequent value (18) between the extremes (5 and 30), because there are fewer solutions for extreme values of the roll (low=1,1,1,1,1 or high=6,6,6,6,6) than there are for intermediate values.

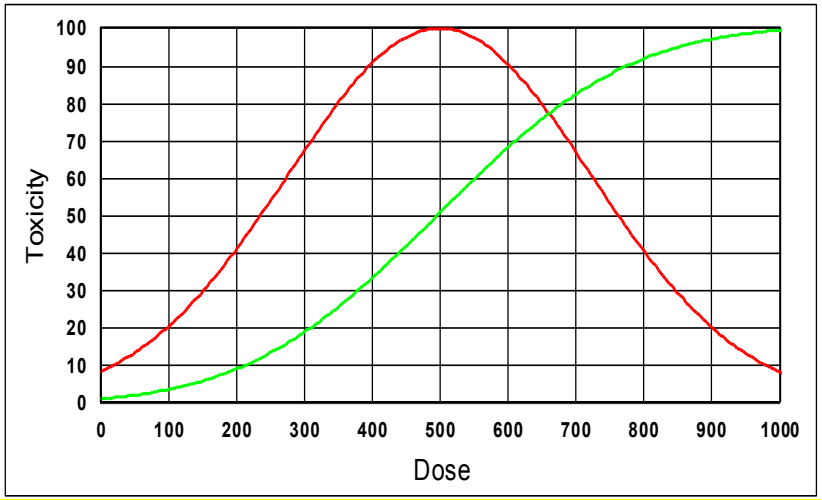
Each of the 5 dice represents a variable which can have 6 toxic sensitivity levels (1,2,3,4,5,6). All 5 variables (dice) contribute to the subject's overall toxicity dose-response (total score).

In order to obtain *extreme* values of toxicity sensitivity, either low or high, all 5 variables must contribute *extreme* characteristics, while more moderate thresholds can be obtained using *many more possible combinations of the individual variables*. The *normal distribution* that applies to the dies (F2.9) also applies to integrated toxicity, if a sufficiently large number of factors determine toxicity outcome.

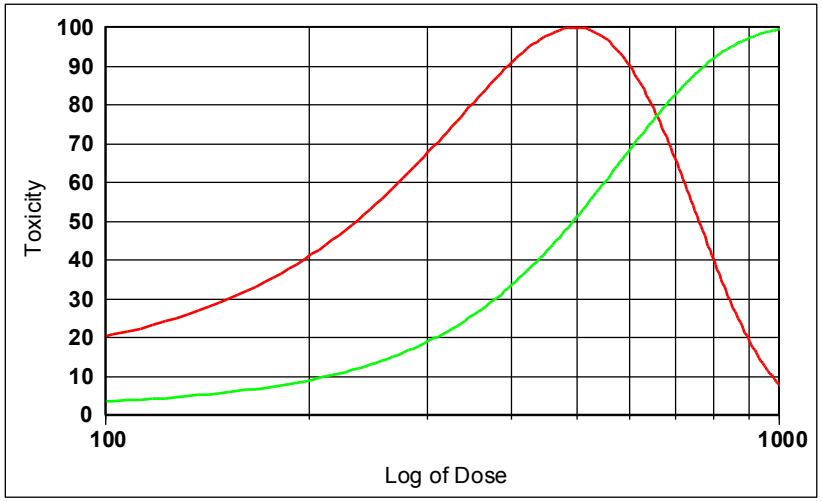
If integrated toxicity is *normally* distributed with respect to dose, few animals exhibit toxicity at extreme doses, while many show toxicity at intermediate values (dose of 500, as shown by the red line in F2.10).
If we are interested in the proportion of all animals that have reached the toxic threshold at a given dose, we integrate the red-line distribution (Gaussian) into the green curve (F2.10).
Further, if we graph the two previous curves over a log abscissa, we obtain the *Gaussian response* graph in F2.11.
We note here that the green curve shows, as in the example of the toxicant-receptor model (F2.6), a soft sigmoid curve with a central range where the slope changes relatively little.

The *Central Tendency Theorem* says that if we average more and more independent random quantities that have a common distribution (and if that common distribution is not too *pathological*), then the distribution of their means approaches a Gaussian.

Departures from this Gaussian (normal) probability distribution suggests that special variables are strong determinants of toxicity.
The variability within toxicant targets widens the spread of dose-response curves with respect to those obtainable from the simplest experimental systems.
Therefore, the physiology lab working on simple systems should display narrower spreads than those observed in epidemiology, or in groups of animals.
However, whether dealing with a continuous variable or threshold expression of toxicity in a group, in the physiology lab or in epidemiology, dose-responses are likely to display similar shapes over a log scale.



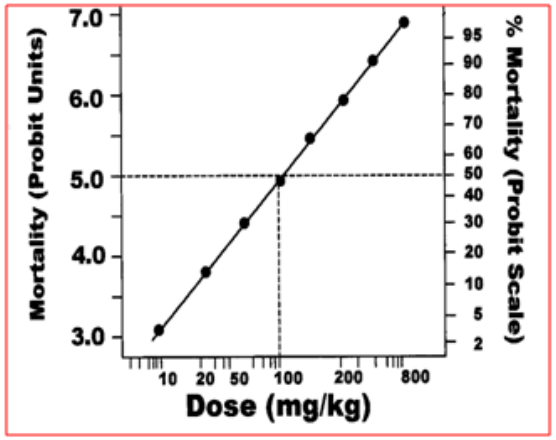
F2.10. Gaussian toxicity distribution.



F2.11. Log scaling of Gaussian toxicity distribution.

2.5.3.2. Probit Scale

An effort has been made to straighten completely the curve obtained from randomly determined (Gaussian) toxicity dependencies over dose. In this procedure, the proportion of subjects responding to each dose is transformed using a computed table into a number called a *probit*. The table is built assuming that the experimental events are distributed exactly according to a Gaussian. The graph of probit against log of dose often produces points that are linear over a wide region of dose in actual experiments (F2.12). The ideal probit plot is a straight line, with a slope equal to $1/\sigma$.



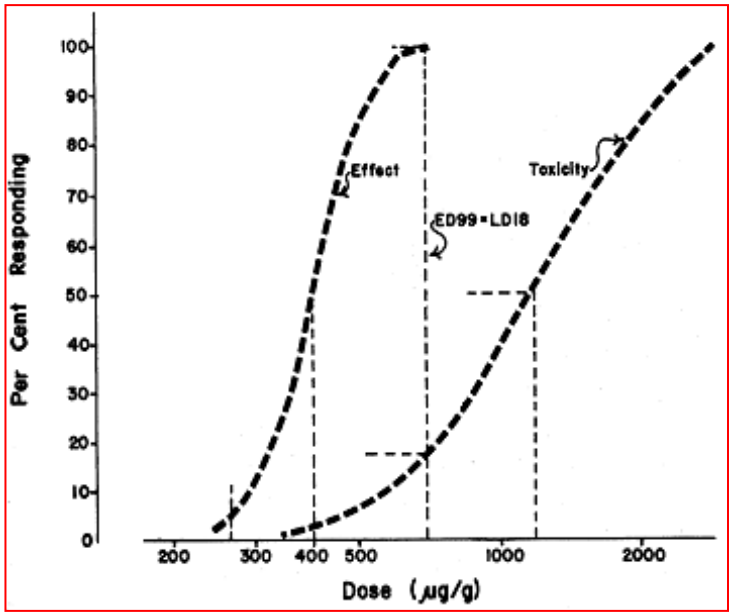
F2.12. A probit curve.

The processes of linearization used in toxicology allow for easier quantitative assessment of experimental results, and also facilitate the detection of extraneous contributions within simple results, as deviations from a straight line.

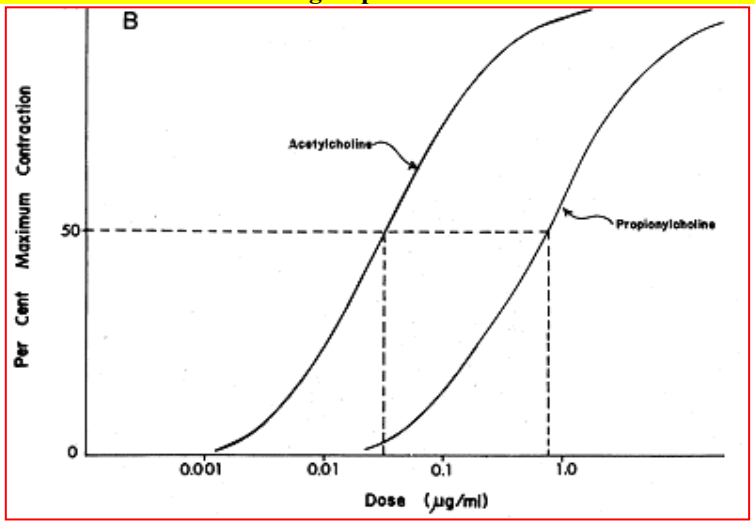
2.6. Dose-Response Examples

2.6.1. Example 1: Rat Ileum

F2.13 shows continuous dose-responses with administration of various concentrations of two drugs in the guinea pig ileum. These curves have a shape similar to the *binding curves* displayed previously. The 10-90 % spreads are 79 and 100.



F2.13. Acetylcholine and propionycholine in the rat ileum.
F2.14. Loss of righting reflex (left) and cessation of breathing (right) in groups of mice.



2.6.2. Example 2: Cancer in Rats

One common task in toxicology is to assess what *proportion of test animals* will show a response at various strengths of exposure. In the determination of lethal doses, there are only two outcomes: either the animal dies, or it lives (a *quantal* response). Similarly, in cancer studies, either there is a tumor or there is not. Cumulative curves similar to the ones in F2.13 can be documented for rat carcinogenicity.

2.6.3. Example 3: Anesthesia

Studying loss of righting reflex ("Effect") and cessation of breathing ("Toxicity") in groups of 20 mice yielded the graphs in F2.14. These curves are similar to the *Gaussian response* curves displayed previously. The 10-90 % spreads are 1.8 and 4.
As might be expected, a number a parameters can be adjusted within the mathematical expressions of the theoretical dose-response characteristics displayed previously, to fit experimental data. Observations cover the full range of 10-90 % spreads discussed theoretically in the earlier sections.

2.7. Hazard & Risk Assessment

2.7.1. Components of Risk Assessment

Some definitions…

The **"HAZARD"** produced by an agent is the injury that will occur as a result of exposure.
A **"RISK"** combines the **hazard** with **exposure**, yielding a **probability** that the injury will occur. For example, the probability of dying in an aircraft crash (hazard) is one per 8 million flights (risk).
Risk Assessment is made of components often occurring in succession.

Hazard Identification: the identification of known or potential health effects associated with a particular agent.

Hazard Quantification (*Toxicology*): the quantitative evaluation of the hazards associated with an agent. The aim is to collect all relevant human, animal and *in vitro* studies of toxicity.
Abstracted databases such as Sax's *Dangerous Properties of Industrial Materials* can be invaluable in such work. From this database, for each species, relationships between the dosage administered (mg/kg/day, mg total dose, etc.) and the effects produced can be developed in order to establish:

- the animal species or *in vitro* system most representative of humans for the *endpoints of toxicity retained*, and
- the dosage range and the shape (linear, convex, concave) of the dose-response relationship.

If the dosage range used in animal studies is different from that encountered in exposed humans, there is need to extrapolate to the human situation.

Exposure Quantification (*Hygiene*): determination, hopefully by actual measurement, of the amount of the toxicant to which individuals may be exposed. This will include air, water, soil, food analysis, the nature and size of potentially exposed populations, the duration of exposure (usually in the past), the routes by which the agent might be acquired.

Risk Setting: One of the issues in risk assessment is how *acceptable* risk levels are decided. There are standardized levels of risk that attempt to balance the acceptable risk with a particular situation.

A **risk of 1 per Million** is considered acceptable for by-standers and volunteers. This low risk is attributed because they are considered to be unsuspecting and naïve potential victims.

A **risk of 1 in 100,000** is considered acceptable for general patients receiving drugs, since they are expected to get a benefit from the drugs they are using.

A **risk of 1 in 10,000** is considered acceptable in workplace safety, since the subjects earn a salary, are likely aware of the exposure, and make the production of all goods possible.

A **risk of 1 in 1,000** is considered acceptable for patients that are victims of life-threatening diseases, since drug administration may represent their only chance for survival.

Risk Quantification: integration of hazard and exposure quantification into an estimation of the adverse effects likely to occur in a given population, including data uncertainties.
A critical aspect is whether the dose-response relationship is:

- threshold: there is a level below which toxicity (in animals or humans) does not occur, or
- non-threshold: there is always some toxicity, regardless of how small the exposure is.

It is not uncommon that risks associated with certain agents need to be updated due to new evidence or new analysis of old evidence. One of the most powerful methods of manipulating risk quantification is by expert assessment of publications, establishing the relative credibility of specific studies (expert assessment can be biased).

For example, in 2007, the Occupational Safety and Health Administration (OSHA) amended the occupational standard of

exposure to hexavalent chromium (Cr(VI)) because workers faced a significant risk of lung cancer, asthma, and damage to the nasal epithelia and skin at the current exposure limits. The new rule establishes an 8-hour time-weighted average (TWA) exposure limit of 5 $\mu g/m^3$ of Cr(VI). The previous limit was 100 $\mu g/m^3$ (a division by a factor of 20).
The new standard regulates separately general industry, construction, and shipyards in order to tailor requirements to the unique circumstances found in each of these sectors.
The new standard reduces the risk posed to workers to the maximum extent that is technologically and economically feasible.
Legal exposure limits reduce risks to levels that are considered acceptable by the standards of the time.
For example, the old standard meant that a worker with a 45-year maximum exposure to Cr(VI) had a cancer risk between 10 and 35 % (added to all other cancer risks), while the new standard is expected to induce cancers at rates of 1 to 4.5 %

Risk Decision: The weighing of policy alternatives to accept, minimize or reduce risks, and to select and implement appropriate options. Risk decision may involve, for example, implementation of protective measures within an industrial plant. If you are a member of a national committee, it may involve settling for a value of the Threshold Limit Value.
For the Environmental Protection Agency (EPA) and the International Agency for Research on Cancer (IARC), who assess the carcinogenic potential of agents based on the "weight of evidence"[1] approach, risk decision is the assignment of an agent to a specific class (see Chapter 13).

Risk Communication: Issues often occur in relation to dissemination of toxicity information, or use of biased information. If there is conflict, such as in a court case, all background data is usually shared between parties as documents are tabled. From the same information, the parties then develop their interpretation, and attempt to sway the tribunal in their favor.
If the controversy gets into the media, and it will, if it is contentious, your interpretation of the potential hazard should be presented rationally and convincingly.

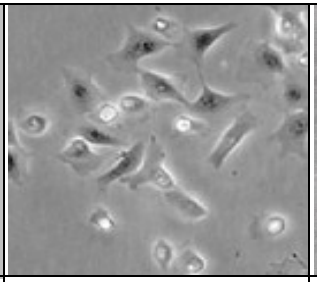
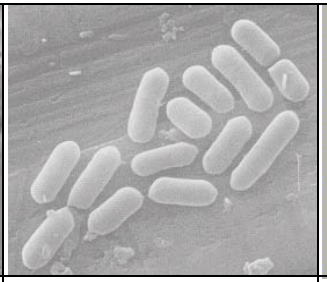
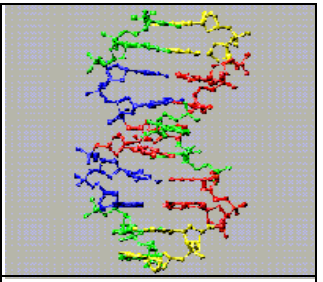
Next page: F2.15. Types of Evidence used in Risk Assessment.

[1] This is a decidedly, and perhaps deliberately, vague concept.

TYPES OF EVIDENCE USED IN RISK ASSESSMENT

INCREASING SIGNIFICANCE TO PHYSIOLOGY (MECHANISMS)

INCREASING SIGNIFICANCE TO HUMAN HEALTH

					
	Humans (*in populo*)	**Animals (*in vivo*)**	**Higher Cells (*in vitro*)**	**Lower Cells (*in vitro*)**	**Molecular Biology (*in vitro, in silico*)**
METHOD	Cross-sectional or longitudinal studies of prevalence or incidence.	Particularly rodents, because of their low cost.	Human or mammalian cells and tissues.	Lower unicellulars, such as bacteria.	Molecular experiments or computer simulations.
EXPOSURE	Sampled or estimated in epi., approx. controlled in clinical studies.	Approximately controlled.	Well controlled.	Well controlled.	Tightly controlled or defined.
TOXICITY	Human diseases.	Tumors, biopsies.	From cell function.	From cell function.	Inferred.
TIME FRAME	From acute poisonings up to lifelong (80 yrs for cancer incidence).	Up to lifelong (2 yrs) or even longer in reproductive studies.	Up to a week.	Days.	Hours.
TYPICAL TESTS	Epidemiology and Clinical studies.	Carcinogenicity study in rats.	Cytotoxicity (survival and proliferation of cells).	Ames test for genotoxicity.	Structure-Activity Relationships.

2.7.2. "Strength of Evidence" Criteria

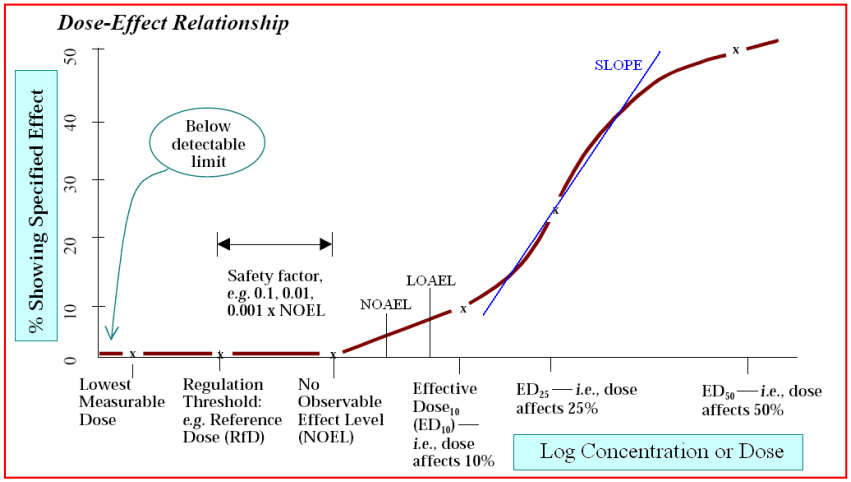
To confirm that *associations* are *causal* between agent tested and the effect found, we use **"Strength of Evidence"** criteria.

Time: toxicity indexes should grow after exposure starts (latency) and (generally) fall after exposure stops, in a time-frame compatible with toxicant elimination, or recovery from toxicity.
Strength of toxic reaction: a strong toxic effect is less likely than a weak one to be due to baseline noise in an animal model or to uncontrolled factors (that also unwittingly influence the toxic reaction studied). Therefore, a stronger reaction is less likely to be artefactual than a smaller one.
Dose-response: finding a threshold, as well as a simple dose-response, increases the chance that the data describes a toxic reaction. While it is not necessary that the dose-response follows a classic curve, data points along the dose curve should support, rather than discredit each other.
Consistency: toxicities are most convincing when they can be observed across species (despeciation), and when they can be confirmed using many laboratory techniques or toxicity indicators.
Plausibility: is there basic molecular knowledge that can explain the toxicity data observed ? Is an alternate explanation for the observations possible ?
Compatibility: are the new toxicity observations compatible with existing knowledge, or do they leave us with an incompatibility with the established databases ?

2.7.3. Threshold-Relationships

Most of the chemical-induced toxicities you will encounter fall in the classification of "threshold relationships". In those toxicities, the detoxification term in Haber's Law is not zero.

This means that below some concentration of exposure, no toxic effect will be detected or detectable.
Having developed a dose-response relationship for an endpoint of toxicity in the most susceptible species, what useful quantities might be recovered from the relationship (F2.16)?



F2.16. Dose-response relationship with the parameters that may be recovered from the curve: NOEL, NOAEL, LOAEL, ED_{50}, Slope, FEL.

- **FEL:** the frank effect level, where most of the individuals demonstrate the anticipated toxic effect.
- **ED_{50} :** as in the acute toxicity studies, where the LD_{50} was determined for lethality, this value gives the dose hazardous to 50 % of the individuals. In cancer, this means induction of tumors in 50 % of animals.
- **SLOPE** of the dose-response relationship, indicating whether a change in dosage produces a small or large alteration in toxic effect.
- **LOAEL:** Lowest Observable Adverse Effect Level, a dosage that still causes some adverse health effect, frequently being the lowest dosage administered in a 3-dose experiment.

- **NOAEL:** the No Observed Adverse Effect Level, the dosage that may cause some changes in status (reduction in body weight, organ function, etc.) but which are *not considered detrimental to general wellbeing*. In many cases, this may also be the lowest dosage administered in a 3-dose experiment.
- **NOEL:** the no observed effect level - a dosage administered that has *no measurable effect* on the test individuals, the subjects looking and behaving (both physiologically and biochemically) exactly like the controls. This parameter is not easily obtained from studies, the lowest dosage given usually producing a NOAEL or LOAEL.

2.7.4. Safety Factors

Under the assumption of a threshold relationship for toxicity, a *low-toxicity level* is quantified from experiments, yielding one or more of the above parameters: NOEL, NOAEL or LOAEL, in decreasing order of preference.
The *low-toxicity level* is then divided by a *Safety Factor*, often chosen in multiples of 10. For example, a Safe Human Dose may result from dividing the NOEL by a safety factor of 100. Such a *Safety Factor* takes into consideration:

- that the human may be up to 10-fold more sensitive than the animal species in which the study was done (inter-species difference), and

- that there may be a 10-fold range in sensitivity between the very young, the adult and the elderly within any human population (intra-species difference).

If a NOEL cannot be obtained from the dose-response relationship, then the assessor may work with a NOAEL or LOAEL, and may increase the magnitude of the *Safety Factor* from 100- to 1000-fold.
This addresses the problem that the threshold observed represents uncertain toxicity.
One may also use fractions, say 250 or 500 fold, depending upon your assessment of the quality of the information available. With any *Safety Factor* greater than 1000-fold, one begins to suspect a flimsy database.

The result of the computation represents a *Safe Human Dose*, an acceptable level of exposure or concentration via the normal human absorption route.

Safe Human Doses are also labeled:

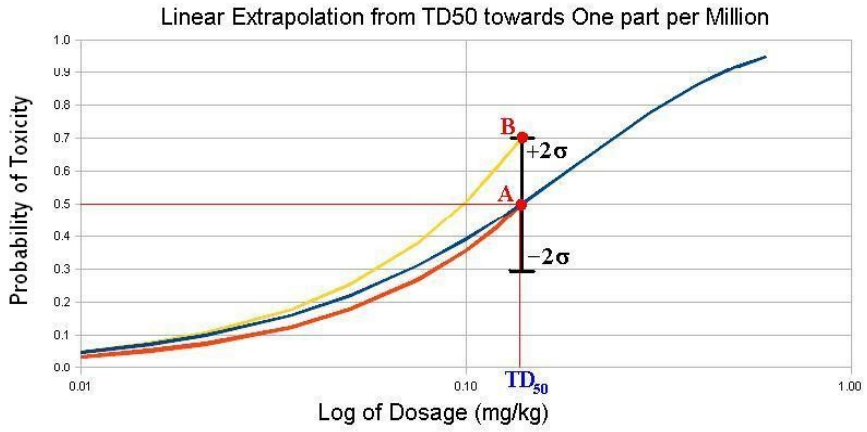
- maximum allowable concentration (MAC),
- reference dose (RfD),
- virtually safe dose (VSD),
- estimated population threshold for humans (EPT-H),
- acceptable daily intake (ADI), or
- tolerable daily intake (TDI).

A committee of the National Academies of Science pored over 50 years of data on survivors and descendents of survivors of the Hiroshima and Nagasaki atomic bomb attacks, as well as accumulated data from dozens of studies on exposures to workers in the nuclear industry and other high-exposure sites. Their conclusion is that any exposure to natural or man-made radiation, even at a very low level, carries a cancer risk. In the past, the nuclear industry and some independent experts have suggested that a safe low threshold for radiation exposure exists. Even at the highest end of what experts consider low-dose lifetime exposure, 100 millisieverts, about 1 person in 100 would develop cancer. To put that number in context, 42 other

lifetime cases of cancer can be expected in that same population of 100, due to such factors as smoking, environmental toxins or other influences. The impact of such conclusions related to one of the most tightly documented agents, ionizing radiation, could have impacts on Toxicology as a whole.

2.7.5. Non-Threshold Relationships

When dealing with carcinogenicity, where one molecule of an *ultimate carcinogen* could produce a cancer (Single-Hit), it is often assumed that the dose-response relation is *non-threshold*. This means that there is residual risk as long as there is *any* exposure.



F2.17. Linear extrapolation methods from 50 % probability to 1/1,000,000 probability. From A, a line is drawn starting at the mean value of TD_{50} towards a probability of 1/1,000,000. From B, a line is drawn starting at the upper 95 % confidence limit of TD_{50}. A linear extrapolation does not show as a straight line on a linear-log graph, and there is no zero on the "LOG. DOSAGE" scale. Extrapolations could also be made from the TD_{10} levels.

In F2.17, one chooses a Threshold Dose point (point A) on the dose-response curve (TD_{50}), and "linearly" extrapolates towards zero, to some conventional level of risk, such as 1/1,000,000 (or 1/100,000).

A more protective estimate can be obtained if one uses the upper limit (+2 standard deviations) of the TD_{50} (point B). The $+2\sigma$ value can be statistically determined from the experimental data.

Assessors typically use two or more of these starting points, obtaining multiple scenarios, and finally choosing among them later on.

The use of linear models for the assessment of carcinogen hazards arose from early work on radiation, where effects on cells were *expected* to relate linearly to particle emissions detected by Geiger counters (1940's - 1950's). This concept was transferred *in toto* to mutagenic chemicals, which did not emit anything, but could be shown to interact with nucleic acids at relatively low concentrations.

Arguments rage even today around whether or not there is a threshold level for carcinogens, but analysis has demonstrated a non-linear, Gaussian (sigmoid-shaped) relationship between radiation and biological effects.

It is easy to draw straight lines, but the exact relation between dose and effect at low doses is usually a matter of speculation, because of the lack of reliable experimental results. People and assessors naturally get very nervous when they suspect that they might be underestimating risks. To this end, complicated and sophisticated models based on a variety of toxicological assumptions have been developed.

The level retained as the reference point for risk calculation (A or B) is then extrapolated to an *acceptable risk of cancer,* arbitrarily established usually as 1 case/1,000,000 or 1 case/100,000 of population. Such a level would go undetected by routine epidemiology, eliminating the scenario where human deaths could be proven from anything but the most sophisticated examination of health files. Furthermore,

experiments to reliably prove such low risks would be prohibitively expensive.
If, for example, incidence of cancer rose by 10 % at a dose of 1 mg/kg/day, the Safe Human Dose would be 100 ng/kg/day, assuming extrapolation to a risk of 1/100,000.

There has been a recent trend in the USA in particular to unify cancer risk assessment with other risk assessment techniques, resulting in a Unified Safe Human Dose approach for carcinogens *and* non-carcinogens.
The proposed assessment may start from a NOAEL and apply to this level 10x Safety Factors to a maximum of 10,000.

Beyond the normal Safety Factors classically applied, such as:

- need to extrapolate from animal tests to humans, and
- existence of sensitive individuals,

the following factors are commonly considered:

- exposure of children (Reproductive Toxicology),
- limited availability of chronic data, and
- any special "modifying factors".

For cancer data, a 10 % excess of disease above baseline determines the "Benchmark Dose" or "Effective Dose" at 10 % (ED_{10}). The Safe Human Dose for the carcinogen would be obtained as LED_{10} / 10,000 or other safety factor. "L" in LED_{10} refers to a lower statistical limit (1 sigma) of the Effective Dose.

2.7.6. Integration of Multiple Exposures

Philosophical differences between risk assessment methods become apparent when integrating effects of multiple agents.

The *non-threshold* method, commonly applied to carcinogens, provides a simple method of integration. The basic observation in a non-threshold model is, for example, that 1 mg/day of agent "A" produces a lifetime cancer rate of 1 in a million.

Environmental Limits vs Occupational Limits

The U.S. EPA, in seeking to protect general populations from carcinogens, tolerates cancer rates of 1/1,000,000 to 1/10,000 for most of their guidelines. The U.S. Occupational Safety and Health Administration, in accomodating economic feasibility, puts workers at much higher risk than EPA, as shown in the Table below (highest EPA risk is 0.1/1000 workers).

Excess Cancers per 1000 workers at OSHA *Permissible Exposure Level*	
Ethylene Oxide	1.2-2.3
Asbestos	6.7
Benzene	10
Formaldehyde	0.0056-2.64
Methylenedianiline	0.8
Cadmium	3-15
1,3 Butadiene	1.3-8.1
Methylene Chloride	3.6
Chromium VI	10.45

Most OSHA limits are to some extent obsolete, because of time-consuming committee work, leading to inevitable discussions in court.

This is a slope of 1/1,000,000 cancer-day/mg. If agent "B" is added with a similar risk, the cancer rates can simply be added to 2/1,000,000 cancer-day/mg:

$$Integrated\ \ Risk = \sum_i D_i \times Slope\ of\ Carcinogenicity\ Curve_i$$

For *threshold* risk assessment, the integration is more vague because the concepts relate to the "Safe Dose", as opposed to a specific disease rate. When combining the effects of two or more agents, we obtain a compounded "Risk Ratio", which sums the ratios between actual doses of agents (D_i) and doses considered "safe".

$$Risk\ Ratio = \sum_i \frac{D_i}{Safe\ Dose_i}$$

"Safe" is an absolute and elusive concept, not a measured disease rate.
Both of the integrations above assume no interactions between the individual agents.

2.7.7. Precautionary Principle

A major difficulty in risk management is the public's unwillingness to accept a low (estimated "safe") level of carcinogens in air, water, food, etc. At one extreme, the public requires zero risk, an impossibility in this day and age, when our analytical capabilities can measure minute (ppt, ppq) amounts of contaminants. In the public's psychology, if the agent has a concentration number, it should not be there, no matter how small the number may be.
Such an attitude is difficult to reconcile with technological feasibility. However, the notion that risks attendant to various agents are discovered by science *after* exposure has occurred is very strong in the public's eye, and partly justified. The *final* experiment is that of human exposure…

Various philosophies are used in risk management, but one that has gained favor, particularly in the European Union (it is already law in Germany and Sweden), is the *precautionary principle*, also known as *prudent avoidance*.

> *"When an activity raises threats of harm to human health or the environment, precautionary measures should be taken even if some cause and effect relationships are not fully established scientifically."*

In effect, the Precautionary Principle shifts the burden of proof away from the protection advocates and towards industry. It also dictates the approach when evidence is lacking or is inconclusive.
The Precautionary Principle might have effects soon in influencing the inclusion of new chemicals on the UN's list of Persistent Organic Polluants (POPs). The list presently includes 12 polluants: dioxins and furans, PCBs, aldrin, chlordane, DDT, dieldrin, endrin, heptachlor, hexachlorobenzene, mirex and toxaphene.

2.7.8. Hazards of Risk Assessment

2.7.8.1. Toxicology vs Risk Assessment

The procedures of toxicology, with standardized tests and hopefully careful inferences, are perceived as more scientific than the deliberations of Risk Assessment. Hazard & Risk Assessment[4] is a semi-scientific process usually involving many segments of society, and leading to decisions.

2.7.8.2. Objectivity in Risk Assessment

Weaknesses inherent in Risk Assessment techniques, for example

(1) dealing with incomplete data,
(2) focussing on specific sets of data,
(3) the choice of Safety Factors and statistical methods,
(4) relying on status quo (previous decisions or traditions),

underlie its vulnerability to politics, making exposure to toxicants a matter of *choice*.

1 - The lack of information creates opportunity for individuals to devise models, even quantitative models, that are susceptible to political influence.

As incomplete science meets a demand for decisions, small segments of science can be given too much importance.

All scientific work is incomplete-whether it be observational or experimental. All scientific work is liable to be upset or modified by advancing knowledge. That does not confer upon us a freedom to ignore the knowledge we already have, or to postpone the action that it appears to demand at a given time.
(Bradford Hill)

2 - Toxicity evidence may sometimes appear incoherent. Restoring coherence can be done by arbitrarily weighing the importance of data, giving more credibility to some, less to others, while still ignoring others entirely. The choices may depend on how hazards and risks are *perceived.*

Selection of scientific data can be used to support hidden economic and political agendas.

3 - The choice of quantitative technique, for example threshold vs non-threshold dose-response, can have a serious impact on the level of protection.

Statistics is an excellent servant and a bad master.

4 - Canada has viewed asbestos as a natural resource for decades, and is still exporting large amounts of asbestos to poor countries in Asia.

The recipients of profits have little incentive to objectively examine their products for adverse health consequences.

Many polemists represent these conflicts as a contest between financial interests (money) and human health.

Managers and politicians may place public interest behind self-interest

But "money" concerns are sometimes legitimate. Although we need to protect human health, the efforts that we marshal to protect it should not create more grief than the problems we are trying to solve. To strike a balance, objectivity is necessary, or perhaps more accurately, adoption of a point of view that balances the interests of everyone.

The cost of safety vs the desire for zero risk.

Gold standards of objectivity are hard to attain in difficult questions. In the distant past, objectivity was thought to issue from social position or high education. Today, the need for impartiality incites the use of more resources (committees) and technical means such as statistics (vs vague statements) and images (vs drawing or recounts) to enhance credibility.

Quantitative methods help, but they must be used adequately to produce the best results.

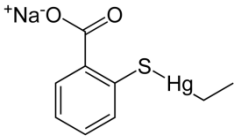
F2.18. From the June, 1947 issue of Time.

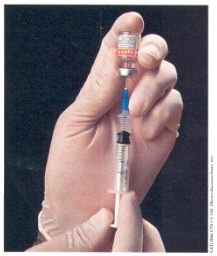
2.7.8.3. Example 1: DDT

Dichloro-diphenyl-trichloro-ethane (DDT) was used extensively in the period from 1940 to 1960 to control insect populations. It was cheap, effective, persistent in the

environment and made a significant dent in the world incidence of malaria. It was applied in large quantities to cotton crops. But western public mood changed when unexpected environmental toxicities were found. Some concerned the fragilization of the eggs of wild birds, particularly the bald eagle, a sensitive subject in the US, where DDT was banned in 1971. Today, DDT is still surrounded in controversy, particularly because developing countries have not found inexpensive substitutes.
Whether DDT is acceptable could be decided using mountains of specific toxicity data. But the matter is even more easily settled depending on whether your country is currently infested by mosquitoes and malaria. There are at least 300 million acute cases of malaria each year, with more than a million deaths.

2.7.8.4. Example 2: Thimerosal





Thimerosal, which contains ethyl mercury, has been used to prevent bacterial contamination in vaccine vials over the last 50 years, apparently with a "good safety record".

Work is going on to eliminate thimerosal from vaccines, in an effort to minimize exposure to mercury, particularly in children (see the document DVD:\Thimerosal Debate.pdf).
Thimerosal prevents the conversion of dietary forms of vitamin B_{12} into the active form. This blocks methionine synthase, which interferes with DNA methylation (an epi-genetic control mechanism).
Whether thimerosal is acceptable depends on the availability of refrigeration for the conservation of vaccines, or on whether the cost of individual vials, as opposed to large multi-dose vials, is acceptable.

These two examples show that the use of a chemical usually has both positive and negative impacts. The balance between them can be compiled by a procedure of *Hazard and Risk Assessment*.

2.7.8.5. Factors Influencing Risk Assessment

The determination of how objectionable a given risk is depends on a number of factors, many intimately related to human fear:

- Is the injury heritable ?
- Is the injury permanent ?
- How debilitating is the injury ?
- Does a hypersensitive population exist ?

- Is the presence of risk recognized by the subjects (environmental vs occupational) ?
- Can exposure be avoided ?
- Are only a few individuals exposed, or is everyone ?
- Is the source natural or industrial ?
- Is the source controllable ?
- Is the source familiar ?
- Is the risk larger than other familiar risks ?
- Are there alternatives to the agent ?
- Is there a high potential for misuse ?

2.7.8.6. Manipulation of Risk Assessment

Biases in Risk assessment can result from improper scientific techniques. Here are a few examples.

- The investigative power of a study can be limited by using groups too small for the level of risk expected, insuring that the conclusions will have no statistical significance
- A study can be too short to see the full development of disease incidence.
- The selection subjects or the exposure systems can be poor so as to include non-exposed among the exposed, or

incorporating large uncertainty margins in the exposure, weakening the potency of the study
- Alter routes of exposure that do not affect targets as strongly as they should
- Perform histology on samples not well preserved, so that effects of agents become undetectable

2.7.9. An Example of Risk Assessment

(modified from Williams & Burson)

This example sets a drinking-water standard for "chlorinated ethylene", designating a group of compounds for which hypothetical but realistic data, largely derived from *perchloroethylene*, are used. It illustrates how various Safe Human Doses can be derived, according to different assumptions or data sources. The various SHD values (mg/day) obtained are highlighted in red. Emphasis was added to various parts of the text by your professor.

2.7.9.1. Introduction and Historical Perspectives

Chlorinated ethylene (CE), a volatile compound, is used as a degreaser for machinery and as a solvent for organic products. CE has been used in consumer products such as glues, rug cleaners, as a propellant for aerosol products, and as an extraction solvent for decaffeinated coffee prior to the late 1970s. Near that time, the Food and Drug Administration (FDA) prohibited its use as a direct or indirect food additive because of suspicions that CE was a carcinogen. Prior to the FDA ban, CE was measured in foods:

ITEM	ppm
Dairy products	1-10
Meat	10-20
Oils and fats	1-19
Hot beverages	160
Mollusks	0-0.250
Fish	0-0.48

Besides food sources, it was estimated that some 5000 medical, dental, and hospital personnel were exposed to CE and that about 60,000 additional persons were subjected to it annually when it was used as an anesthetic in medical situations. Concentrations in hospital rooms ranged from 0.3 to 103 ppm.
Based on its widespread use and occurrence in our environment, it is not surprising that levels of 1-32 ppb were reported post-mortem in human tissues.
The FDA therefore also moved against any medical uses and banned all anesthetics containing CE. Furthermore, CE was disallowed in cosmetic products, animal and pet foods, animal drug products, and as an oil-extraction solvent for seed products.
The Environmental Protection Agency (EPA) quickly followed the precedent of the FDA and issued notice that CE was a candidate for denial of renewed registration on the basis of its possible carcinogenicity.
The OSHA guidelines now limit air concentrations of CE in areas where employees work to **100 ppm** (500 mg/m^3) during an eight-hour time-weighted average period. The NIOSH (National Institute for Occupational Safety and Health) guidelines, however, recommend that no occupational exposure to halogenated anesthetic agents be greater than **2 ppm** (10 mg/m^3) for a one-hour sampling period.

2.7.9.2. Animal Metabolism and Kinetics

Absorption. CE is readily absorbed by all routes of exposure, as would be predicted by its physical and chemical properties. Its historical use as an anesthetic indicates its rapid rate and high percent of absorption via inhalation. Measurements taken after oral administration of CE to rats indicate that at least 80 percent is absorbed from the GI tract.

Elimination. The elimination of CE in mammals is fairly rapid. After inhalation of CE at 300 ppm for 8 hr, CE was undetectable in the expired air of rats in one study. However, another study indicated approximately 80 percent would be eliminated via the lungs. The estimated half-life for CE for most tissues is about 100 min, while for fat tissues the half-life is 200 min. Thus, eight hours after administration some 95 percent of the CE absorbed should be eliminated.

Metabolism. Much of the early toxicity research with CE was thought to support the idea that metabolism generated a reactive metabolite, probably the epoxide form, which was hepatotoxic and possibly capable of binding DNA and initiating cancer. However, recent work has changed much of the current thought on CE metabolism by demonstrating that the alcohol metabolite is not dependent upon nor derived from the epoxide metabolite form. This in turn suggests that species differences in hepatotoxicity and carcinogenicity are not related to species differences in the conversion rate of the epoxide to the less reactive alcohol metabolite. In addition, it has been shown that covalent protein binding of CE intermediates does not correlate with the formation of the epoxide metabolite. However, research has demonstrated that species differences do exist for covalent binding of reactive metabolites in rats and mice and that these differences in covalent binding parallel the different carcinogenic responses observed in these species.

Mechanistic Differences between Species. Interestingly, human microsomes form DNA adducts at a low rate similar to that found in the noncarcinogenic model, Osborne-Mendel rats. Both rats and humans appear to have substantially lower rates of microsomally generated DNA-adduct formation when compared to the B6C3F1 mouse, a species that is positive for liver cancer after chronic CE exposure. Researchers have also reported a species difference in microsomally generated covalent protein binding, which was likewise consistent with the results from the carcinogenicity tests. A sex difference in covalent binding in the B6C3Fl mouse has

also been demonstrated which is consistent with the higher liver cancer incidence observed in male mice.

Differences between Rats and Mice. Observations of rats versus mice show differences in metabolism, both quantitative and qualitative. It has been found that the B6C3F1 mouse metabolized CE to a greater extent and generated even more macromolecular binding than expected when compared to the Osborne-Mendel rat. Also noted in the mouse was a lack of DNA alkylation[⇓], an increased hepatotoxicity, and an increase in DNA synthesis. This led to the proposal that CE was probably not initiating liver cancer in the mice through alkylation of DNA, but was increasing tumor formation through a recurrent injury (i.e., a cytotoxic type of mechanism). The importance of this proposed mechanism is that chronic, hepatotoxic doses would be required to induce cancer in any species.

Acute and Chronic Toxicities of CE. The acute and chronic toxicities of CE have been adequately summarized in a number of reviews and the data used here are drawn largely from them. The acute lethal effects (LD_{50}) of CE occur at moderately high concentrations (g/kg):

Oral in Rats	5-7
Oral in Dogs	6
Intraperitoneal Injections in Mice	3
Intraperitoneal Injections in Dogs	2.8

The NOAEL vapor concentrations after 7 hr/day, for 5 days/week, for 6 months are:

- 100 ppm (0.5 g/m^3) in guinea pigs.
- 200 ppm (1 g/m^3) in rats and rabbits.
- 400 ppm (2 g/m^3) in monkeys.

Likewise, 30 exposures each of 8-hr duration to 700 ppm (3.5 g/m^3) or continuous exposure to 200 mg/m^3 for 90 days produced no visible signs of toxicity in rats. dogs, monkeys, guinea pigs, or rabbits.

Like other chlorinated aliphatics, when administered at acutely toxic levels CE is principally a central nervous system depressant. It also produces some liver and kidney damage, but at doses far higher than those at which carbon tetrachloride produces similar damage. In rodents, liver injury occurs after a single dose at about 2 g/kg. CE, like many chlorinated solvents, may sensitize the heart to epinephrine such that stress or excitement may produce cardiac arrhythmias.

Teratogenicity and Reproductive Toxicity. CE produced no teratogenic effects in either rats or mice exposed for 7 hr/day at 300 ppm (1.5 g/m^3) on days 6-15 of gestation. In this study the indices monitored for adverse effects were the number of implantations, litter size, incidence of resorptions, fetal sex ratios, fetal body measurements, and morphologic anomalies. It was concluded that CE was not significantly maternally toxic, embryotoxic, fetotoxic, nor teratogenic in this study. Another study has reported that male mice exposed to 2000 ppm (0.2 percent in air) for 4 hr/day for 5 days had 2.4 percent abnormal spermatozoa compared to a 1.4 percent abnormal sperm count in controls. While this was a statistically significant difference, no change was observed at the 200 ppm exposure and the relevance of the 2000 ppm exposure in mice to expected human exposures, if any, is minimal.

Mutagenicity. CE has been reported as weakly positive or positive in only a few mutagenicity tests, but negative in a number of others. The role that might have been played by contaminants in some of the positive tests has been suggested, but not proved. Therefore, evidence of the mutagenic potential of CE is considered to be inconclusive and confounded by the presence of mutagenic contaminants in a number of studies using the technical grade compound. Pure CE is apparently not mutagenic.

Carcinogenicity. The National Cancer Institute published results of its bioassay in 1978. B6C3Fl mice had been administered CE 5 days/week for 78 weeks. The results are in the table below.

Hepatocellular Carcinoma Rates among Mice, according to Dose

Male		Female	
1.2 g/kg day	2.4 g/kg day	0.9 g/kg day	1.8 g/kg day
26/50	**31/48**	**4/50**	**11/49**
In control animals the rate was 1/40 when both sexes were combined.			

In Osborne-Mendel rats exposed to doses of either 0.6 or 1.2 g/kg day, no liver cell tumors occurred and there was no statistically significant increase for any other type of tumor. The rat data are complicated somewhat by the poor survival of all groups during the course of this particular bioassay. Likewise. the positive result in the B6C3FI mouse is somewhat undermined by the fact that about 0.3 percent of the CE consisted of the mutagenic contaminants common to technical grade preparations. Subsequent studies in these strains of rodents yielded essentially the same results.

An inhalation study performed by an independent laboratory reported that CE again produced positive results in the B6C3F1 mouse but was not carcinogenic in the Osborne-Mendel rat. A third study using rats also yielded negative results. Dermal and oral administration of CE to ICR/Ha Swiss mice has also yielded negative results. Finally, no increased incidence of tumors was found with an 18-month inhalation exposure to 100 ppm (500 mg/m^3) or 500 ppm (2.5 g/m^3) of nonepoxide-stabilized CE in hamsters, rats, and mice. Thus, even when using technical grade CE with mutagenic contaminants present, CE was found to be positive in only one strain of mice.

[⇓] A radical with the general formula C_nH_{2n+1} such as methyl, ethyl…

2.7.9.3. Summary of the Toxicology of CE in Humans

The acute toxicity of CE in humans is remarkably similar to that observed in animals and will not be discussed here for the sake of brevity.

Chronic Toxicity Produced in Humans. Of two epidemiologic studies examining the cancer mortality in occupationally exposed persons, one study reported a lower-than-expected total mortality (49 versus 62) and cancer mortality (11 versus 14.5*)* for 600 male workers exposed to 50 ppm of CE. The other study reported a cohort of 2117 CE-exposed workers. Again, both total mortality and cancer mortality were lower than expected based on rates established for the general population. Out of these two studies some 1050 workers could be identified who had been exposed to CE for at least 15 years at an exposure level of about 50 ppm or greater.

2.7.9.4. Extrapolation of the Animal and Human Data

Both threshold and non-threshold models might be considered for the animal data. Since a significant human database is available, risk estimates can be generated from these data as well, and then used in comparison with the animal extrapolations and as a guideline for final recommendations.
For non-cancer toxicity, or threshold situations, models for extrapolating to no risk are reasonably straightforward and basically are similar to the suggested no-adverse-response level (SNARL) developed by the National Research Council of the National Academy of Sciences or the no-observable-effect limit (**NOEL**) of the EPA. Calculations are applied that make the conversion from a no-effect level in animals to a **safe upper limit of exposure to humans** (i.e., the human threshold dose). The conversion must also take into consideration such factors influencing species response as absorption, metabolism, and excretion. Finally, a **safety** or uncertainty **factor** is applied, which is derived from an evaluation of the extent, reliability, reproducibility, and interspecies variability of the available data from animal and, when available, human studies.

2.7.9.5. Safe Human Dose Threshold Model using Non-cancer Animal Data

A review of the animal data on CE toxicity listed earlier, shows that the *best estimate* for an animal-derived no-observable-effect level (TD_0) is 35 ppm. This can then be used as a threshold dose in a risk assessment formula for human exposure to CE. The particular formula we will use here is

$$ADI = \frac{ThD \times BW \times AF \times ER \times t½}{SF}$$

ADI = acceptable daily intake;
ThD = the threshold dose for which no observable effect was produced in the animal species;
BW = body weight of human (average assumed to be 70 kg);
AF = absorption factor, the percentage of the dose absorbed via the designated route of exposure in the animal species divided by the percentage of the dose absorbed by humans via the designated expected route;
ER = exposure ratio, the dosage regimen (i.e., rate of exposure) in the animal species divided by the anticipated exposure in humans;
t½ = a ratio of the elimination half-life in the animal species to the elimination half-life for humans;
SF = a safety factor included to dampen individual variability in responses to chemicals; SF depends upon the reliability of the database used for the extrapolation. A value of 10, 100, or 1000 is selected as shown below:

10 = When the extrapolation is derived from a good database of human origin and there exists a substantial animal data base.
100 = Human data are scanty. inconclusive, or absent; long-term, reliable animal data are available. No long-term human data are available; animal data are scanty, or interspecies sensitivity varies greatly.

The BW factor is given a value of 1.0 rather than 70 because it is assumed that for an inhalation exposure at steady state the concentrations in critical tissues will be similar for all species and there will be no size adjustment needed.
The AF is assumed to be 0.70 even though this value is probably similar for all species and would therefore be 1.0 when making animal-to-man comparisons. It is included as 0.70 in order to provide an additional margin of safety in this calculation.
The ER is 1.0 in as much as exposure situations are set to be the same in animals and humans.
The ratio t½ is assumed to be 1.0.
The SF is set at 100 and is derived from a factor of 10 because good human data exist that suggest a ThD of 35 ppm does not lead to chronic illness in humans, but since chronic animal data were not utilized, and as the actual ratio for t1/2 cannot be calculated because the *human half-life is not known*, a second factor of 10 is included to insure safety for a heterogeneous human population.
Thus, substituting in the equation:

$$ADI_{\text{air continuous exposure}} = \frac{35 \text{ ppm} \times 1.0 \times 0.7 \times 1.0 \times 1.0}{100}$$

or, ADI = 0.245 ppm in air, or 1.225 mg/m^3.

To convert this value to a safe **water concentration** it is assumed that a 70-kg man will inhale about 24 m^3 of air per day and consume 2 liters of water per day. Thus the ADI becomes

$$ADI_{\text{water}} = \frac{ADI_{air} \times 24 \text{ m}^3}{2 \text{ l/day}} = \frac{(1.225 \text{ mg/m}^3)(24 \text{ m}^3)}{2 \text{ l/day}} = 14.7 \text{ mg/l} = 14.7 \text{ ppm}.$$

This means a daily intake of ~ 30 mg/day.

2.7.9.6. Safe Human Dose Threshold Model using Non-cancer Human Data

This second approach uses human data. An Estimated Permissible Concentration (EPC) can be calculated by using safe occupational air concentrations as a guideline. Essentially, the EPC for air is calculated by converting the amount of CE a worker theoretically can be exposed to safely during a 40-hr week to the same total amount of CE but derived from a continuous daily air exposure (i.e., 40 hr/168 hr per week). A safety factor of 100 is recommended in calculating an EPC. The EPC for water is then derived by applying the same conversion as was made for the ADI calculation in the preceding section.
The occupational threshold limit value (TLV) for CE is 100 ppm (500 mg/rn^3) for an 8-hr shift; thus the EPC for air is calculated to be:

$$EPC_{air} = \frac{(500\ mg/m^3)(40/168)}{100} = 1.19\ mg/m^3.$$

Using the same extrapolation as before to convert safe air exposures to safe drinking-water exposures we get

$$EPC_{water} = \frac{(1.19\ mg/m^3)(24\ m^3)}{2\ l/day} = 14.3\ mg/l = 14.3\ ppm.$$

Note that the techniques used to extrapolate risk from the animal and human data produce very similar values.

2.7.9.7. Safe Human Dose Non-Threshold Model using Cancer Animal Data

For the nonthreshold (i.e., cancer) risk estimates, several different models can be chosen and each will derive a slightly different number. The linear interpolation model proposed by Gaylor is selected here because it probably represents the most conservative approach and essentially derives the upper bound of the risk estimates that would be arrived at in other ways in other models. The formula for deriving the risk estimate is

$$SHD = \frac{Rd_e}{UCL}$$

where
R = the conventional *acceptable* probability that cancer will occur;
d_e = the experimental dose chosen as reference in the animal studies;
UCL = the upper confidence limit cancer incidence observed at the reference;
SHD = the safe human dose.

If the acceptable risk (R) is set at 10^{-5} or 1/100,000, and if we use the *lowest positive dose* for male mice from the NCI cancer bioassay data and the upper bound of the 95 percent confidence interval (0.50), the safe human dose can be calculated as

$$SHD = \frac{(10^{-5})\ (1200\ mg/kg.day)}{0.50} = 0.024\ mg/kg.day.$$

Extrapolating to a 70-kg man, and assuming a human lifetime exposure of 70 years versus 2 years for a rat, the safe human daily intake (ADI) becomes:

$$ADI = \frac{(24\ \mu g/kg.day)\ (70\ kg)}{35} = 48\ \mu g/day.$$

If humans are assumed to consume 2 liters of water per day, then the acceptable water concentration, based on animal data for the extrapolation purposes, is:

Water Concentration Limit (2 l/day) = 24 µg/1 or 24 ppb.

2.7.9.8. Safe Human Dose Non-Threshold Model using Cancer Human Data

In the case of CE, the human data do not show an observable increase in the cancer rate. But using "0" in calculations is not practical. Statistically, the 99 percent upper confidence interval for a test population of 1000 individuals is 0.45 percent.
That is, the actual cancer rate could be as high as 0.45 percent (0.0045) or 4.5/1000 persons exposed and, statistically, once in a hundred times, it will be mistakenly identified as zero.
Assuming this worst case when no cancer risk is detected in 1000 exposed individuals, the actual undetected risk could be as high as 4.5 x 10^{-3}.
There exist two epidemiologic studies based on occupational exposures for CE. In both, the total mortality and cancer mortality were less than expected, so the risk observed can be considered to be zero. If we combine the populations from both studies, which had approximately the same CE exposures, we may make the following assumptions and calculations:

- There were 1050 persons exposed to about 50 ppm in an occupational setting.
- The average daily exposure was 1.4 g/day (about 2.0 g/day for a 5-day work-week; conversion to a 7-day week will need to be made for daily environmental exposure).
- The 99 percent upper confidence limit on the risk for this population is approximately 4.5/1000 or 4.5 x 10^{-3}.
- It was assumed that the average exposure interval was only 15 years; thus, this estimate will need to be converted to a lifetime of 70-yrs.

- The ADI is contained in 2 liters of water, which is the amount of liquid a person consumes each day.

Since the risk for the 1050 persons exposed to 50 ppm of CE is equivalent to $4.5 \times 10^{-3} = 1.428$ g/day, a 10^{-3} risk will be

$$\frac{1428}{4.5} = 317 \text{ mg/day}$$

Since the 10^{-3} risk = 317 mg/day after only 15 years of exposure to CE, a 10^{-3} risk for a lifetime exposure (70 yr), given the same dose, would be

317 mg/day x (15/70) = 70 mg/day;

and a 10^{-5} risk, with daily exposure for a lifetime, would be 700 µg/day. Therefore, assuming 2 l are consumed per day, the safe water concentration for a 10^{-5} risk estimate is 350 µg/l (or ppb) if human epidemiologic data are used for extrapolation purposes.

Note that our hypothetical risk has been calculated from a substantial body of negative data by making worst-case assumptions that reflect the actual data. A hypothetical risk has therefore been calculated to help set an exposure guideline even though no such risk has been identified in humans.

2.7.9.9. Recommended Groundwater Standard

Possible water Standards, using threshold model:

	Safety factor of 100	Safety factor of 1000
1. Threshold derived from animal data	14.7 mg/l	1.47 mg/l
2. Threshold derived from human data	14.3 mg/l	1.43 mg/l

The values are very similar; the similarity stems from coincidental use of nearly equivalent inhalation levels obtained from animal experiments and time-established human occupational guidelines. These rather high values for the CE content of water reflect the fact that CE is not a potent toxicant at acute exposures. The few studies that reflect its noncancerous chronic toxicity likewise suggest that it is a relatively unremarkable toxicant at other than high concentrations. As was seen earlier, while there is animal evidence demonstrating that CE increases the tumor incidence in the B6C3F1 mouse, these studies are mitigated by the fact that CE is not carcinogenic in the rat, or hamster, or in another strain of mice. The positive studies are also confounded by the fact that contaminants in technical-grade CE are mutagenic and may have contributed significantly to the response observed in the susceptible mouse.

Studies comparing metabolic and biochemical differences between susceptible and nonsusceptible species for CE-induced oncogenicity *suggest that the susceptible strain of mouse is not a relevant animal model for predicting human risks*. In addition, a ranking of CE using the system proposed by R. A. Squire indicates that CE would score approximately 31 points, which is equivalent to the score for saccharin. This score suggests that CE is not a likely human carcinogen, especially at low doses. Epidemiologic evidence, while perhaps not yet of sufficient strength to eliminate all concern for the carcinogenic potential of CE, indicates that even for the worst possible case it is a relatively weak human carcinogen. Thus, there is much evidence to suggest the values in the preceding table are adequate for the protection of health in the circumstances of chronic ingestion of CE and would adequately protect human health from all adverse effects. However. cancer data were also considered so as to calculate levels in the event that additional evidence better defining the carcinogenicity of CE is produced.

The values for the estimated safe water concentration, based on carcinogenic potential, are listed below.

Possible water Standards, using non-threshold model:

		10^{-5} Risk	**10^{-6} Risk**	**10^{-7} Risk**
1.	Estimates derived from animal data	24.0 µg/l	2.40 µg/l	0.24 µg/l
2.	Estimates derived from human data	350 µg/l	35 µg/l	35 µg/l

In both of the tables of extrapolated standards, the risk estimates generated from the animal data can be considered reasonably conservative estimates. Gaylor's model has been applied to the second table. which is based on the assumption that CE is carcinogenic; the animal data in that table were considered to give a worst-case estimate of the carcinogenic potential of CE. That estimate was then tested for its relevance to the human situation by extrapolating to, and identifying for comparison, the exposures that would be predicted to produce measurable cancer rates in humans if the base estimates of risk were correct. This test of the data is an upward linear extrapolation from the dose representing the 10^{-5} risk estimate. When this is done. the additional increase in cancer for persons occupationally exposed should be about 17 percent for an exposure, over a 15-yr work interval, to 50 ppm of CE. This incidence is very high and would have been observed in the two actual epidemiologic studies that were made of workers with similar exposure levels and exposure intervals. It can be concluded then that the risk estimates generated here from the animal data *do overestimate* the human risk. A similar test of the extrapolation made from the human data in the two actual epidemiologic studies cannot, of course, be done, but the true risk of CE exposure can be no worse than the estimate derived from the human data in which a cancer incidence was assumed for negative data. Therefore, if it is assumed on the basis of the animal data that CE might carry some carcinogenic potential and risk, a final standard for groundwater could be reasonably chosen as that value corresponding to a previously determined "acceptable risk" level (i.e., 10^{-5}, 10^{-6}, or 10^{-7}) using the extrapolation of the human epidemiologic data.

2.7.9.10. Summary of the Estimates of Risk

It is recognized that risk management is a process in which environmental standards should reflect a variety of important considerations when they are established for contaminant levels in potential human exposure sources such as water. Of primary importance is the protection of human health Once this criterion is satisfied a regulatory standard may reflect other societal concerns as well. For example, while it could be argued that 1 mg/l might adequately protect human health from CE exposure, if subsequent human or animal studies removed concerns for its carcinogenic potential, the value chosen for a standard might be lower and reflect society's desire for a chemical-free drinking water source. For comparative purposes the EPA has established federal primary drinking water standards for a number of chlorinated organic solvents and chlorinated pesticides. *These standards range from the 1-ppb to the 100-ppb level.* Since the 10^{-5}-risk estimates calculated from the animal and human data for our example are reasonably conservative, and generally bracket this range, the state might argue to set the standard somewhere between 100 and 350 ppb. Such a standard should reasonably and safely protect human health within the limits of the uncertainty that are inherent in the database and the models used to calculate the values from it. It would also be in keeping with the intent of the EPA and the guidelines proposed by that agency for other chlorinated aliphatics.
Reiterating that much data are available to greatly lessen concern for the human cancer risk posed by CE, a state Division of Environmental Resources might reasonably select the cancer-risk estimate for a drinking-water standard as high as 350 ppb and at the same time choose to adopt the threshold-derived values in the 1-2-ppm range as a reasonably safe guideline to protect aquatic life and human health under short-term unexpected situations such as spills into rivers acting as drinking-water supplies.
It is hoped that the reader will appreciate the fact that should the state finally pick either level as a standard, various interest groups could argue against one or the other of them by reasoning either that
(1) risk of carcinogenic potential must be assumed until better and more definitive evidence proves there is none, or
(2) since the animal and human data indicate no cancer risk, the higher value is safe because apparently-safe occupational exposures are far higher.

It may interest the reader that currently OSHA allows up to a 500 mg/day exposure to CE-type compounds in the workplace, while the EPA, which has not proposed a formal standard, has recommended about a 60 µg/day exposure. These are clearly very different exposures.
This difference is caused because the EPA criteria are based upon the extrapolation of animal carcinogenicity data, and typically the EPA does not consider modifying factors such as mechanistic and species variations in the carcinogenic response; nor does it use human data as a test or modifier of the animal extrapolation.
Thus, while the OSHA standard is designed to protect workers occupationally exposed for perhaps most of their lifetime, the EPA, by virtue of its risk assessment methodologies, advocates an exposure that is some **60-70,000 times lower**.

Such a disparity between standards or recommendations for federal regulatory agencies is common today, *because each agency assesses and manages risk by a different process.*

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Targets - Bio-transformation

3· Targets - Bio-transformation

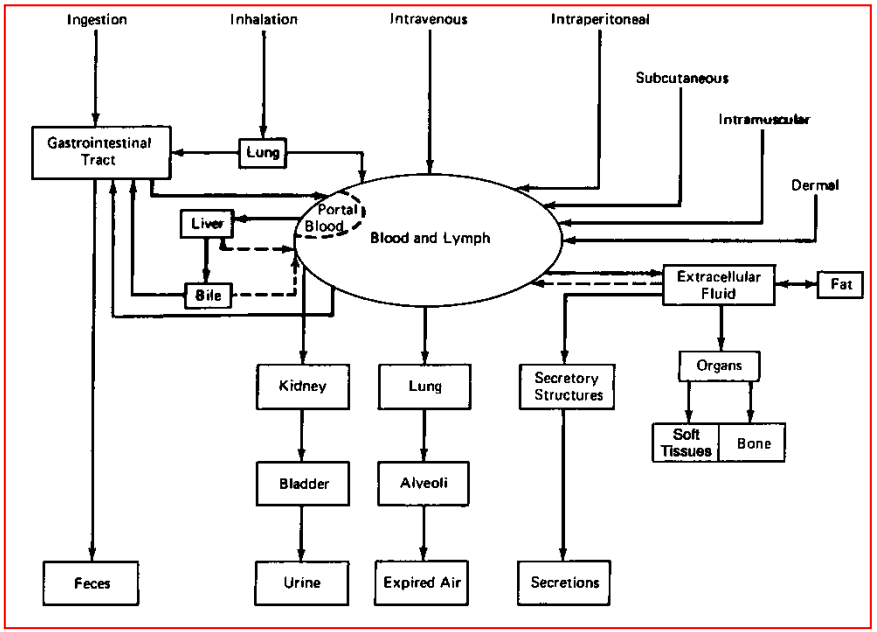
3.1. Routes of Absorption

Toxicants access the body through many absorption routes, spilling into lymphatic and vascular compartments (F3.1). The intravenous route allows complete and immediate penetration, whereas other routes allow delayed and incomplete absorption. The general hierarchy, ordered in decreasing absorption, is:

Injection > Inhalation > Contact on mucosa > Ingestion

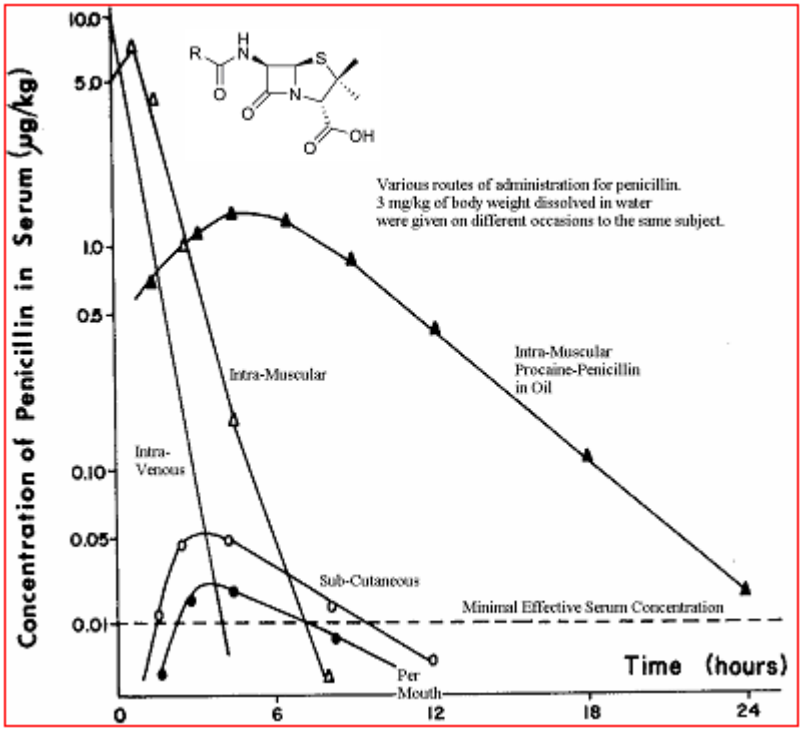
External barriers, such as the skin and the gastrointestinal tract, are quite complex. Internal barriers, as the blood-brain and lung-blood barriers, may have a thickness of only 1 cell, as little as 1 µm. Different routes of exposure (inhalation, cutaneous, ingestion) may result in different amounts of toxicants circulating in the bloodstream, as well as various levels of toxicity. This relates to the toxicant's physical and chemical properties, anatomy (thickness of the membranes, size of internal reservoirs) and biochemistry.

Different routes of administration of drugs result in notably different blood levels over time, as seen in F3.2. In this graph, determining influences are the active excretion of penicillin by the organic acid system of the kidneys, and penicillin's binding to albumin. In some of the curves in the graph, the level in the blood is maintained by oil-phase, sub-cutaneous or enteric compartments.



F3.1. General paths of toxicants through the body.
Casarett & Doull's.

If one views the body as a multitude of membrane layers through which a toxic agent penetrates to reach a site of action, one can anticipate that the variables governing passive diffusion and active transport control the rate and extent of penetration. There may be a large factor (1,000-10,000) between the concentration of a toxicant circulating in the blood and that found at a target site.

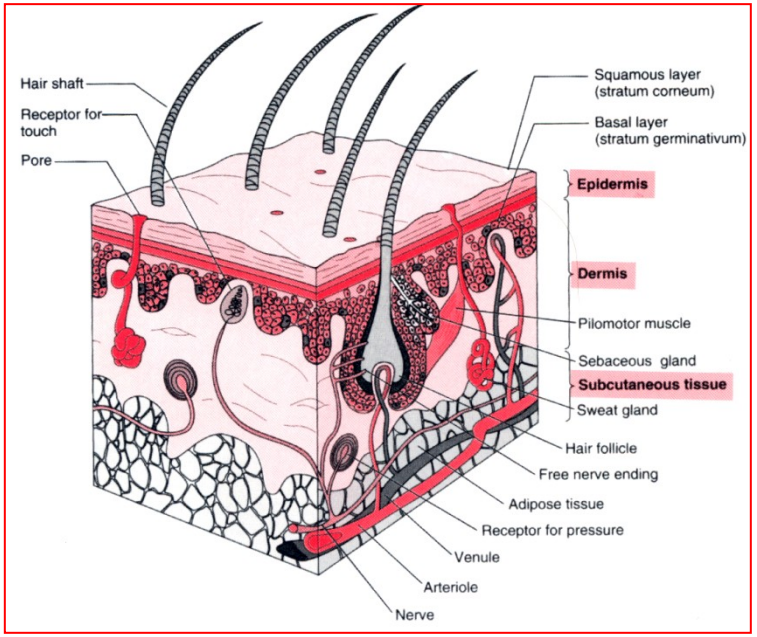


F3.2. Concentration of Penicillin in the blood following various modes of administration. *Pharmacology: Drug Actions and Reactions, 1978.*

3.1.1. Skin

The skin (F3.3) has many roles:

- Keeps out toxicants, UV and micro-organisms.
- Biotransformation and detoxification.
- Elimination of toxicants through sweat.
- Temperature and fluids regulation.
- Sensory reception (temperature, pressure, pain).



F3.3. Skin anatomy. *From Medical Terminology, 1996.*

The *stratum corneum* is only 15-20 dehydrated cells thick. These cells are keratinized[1], extracellular lipids forming a barrier. The thickness of these barrier cells varies tremendously (anticubital fossa vs heel).

Skin penetration is usually through passive diffusion. For example, when swimming or taking a bath or shower, absorption is substantial. Skin is vulnerable to damage, and it's impermeable properties can easily be lost. Toxicants that are small, non-polar and lipid-soluble will diffuse most rapidly. Skin is vulnerable to organic solvents such as chloroform, methanol and dimethyl sulfoxide because of dissolution of skin lipids.

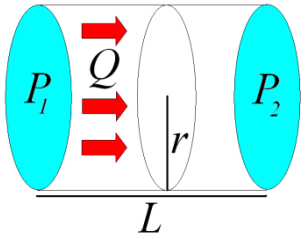
[1] In mammals there are soft epithelial keratins (cytokeratins), and harder hair keratins. As certain skin cells differentiate and become cornified, pre-keratin polypeptides are incorporated into intermediate filaments. Eventually the nucleus and cytoplasmic organelles disappear, metabolism ceases and cells undergo apoptosis as they become fully keratinized.

3.1.2. **Lungs**

Lungs (F3.4) are a very deep, humid cavity that is difficult to clean. Mucus-secreting cells and a ciliated epithelium cooperate to achieve a particle removal half-life of 30-300 min. Coughing can shorten the transit. The movement of the cilia in the lungs is coordinated. One cell may have as many as 200 cilia. The "muco-ciliary escalator" keeps alveolar tissues clean of particles and of many chemicals that absorb readily into the mucus. If clearance of the mucus from the peri-alveolar region is impaired for some reason, the flow of air is impaired into and out of the lungs.

There is a critical physical limit to the flow of air in and out of the lungs (Poiseuille's Law):

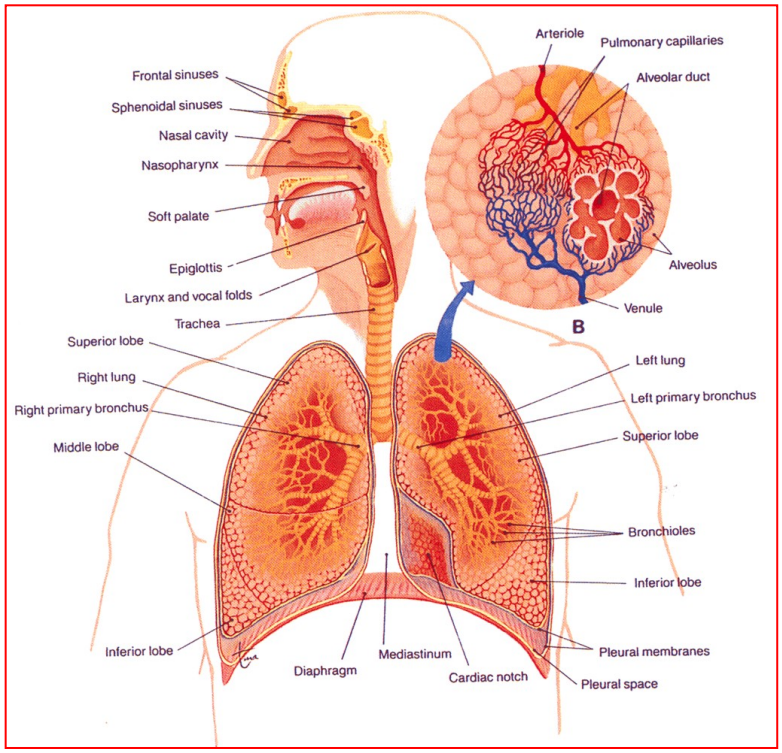
$$Q = \frac{\pi \, \Delta P \, r^4}{8 \, \eta \, L}$$



Q is air flow,
ΔP is pressure difference across the length of bronchiole,
L is length of the bronchiole,
η is viscosity (air 190 μ-poise, water is 10,000 μ-poise),
r is bronchiole radius.

Notice that if bronchiole radius (r) is reduced by a factor of 2, the air flow is reduced by a factor of 16, for a given pressure difference. This explains the importance of micro-airway diameter in asthma attacks.

- Normal respiration volume is about 0.5 liters at 12 respirations/minute = 6 liters/min.
- You can live for a short time on 1.5 liters/minute.
- The spontaneous rate of respiration is controlled by centers detecting pH and CO_2 concentration at the base of the brain. Oxygen concentration is less important.

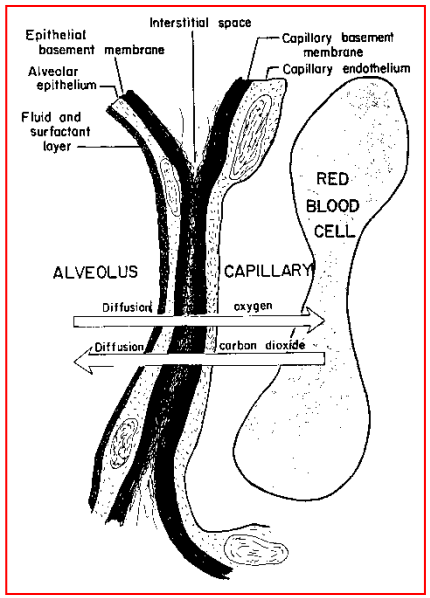


F3.4. General anatomy of the respiratory system.
Essentials of Environmental Toxicology, Taylor and Francis, 1996.

- The rate goes up to 50 liters/min in high level exercise. So, when working strenuously, transfer of toxicants by this route may be considerably enhanced.

There are about one billion alveoli in the lungs, equivalent to a 70 m^2 effective surface, which allow the exchange of gases, primarily oxygen and carbon dioxide. Note that water soluble gases such as HCl or formaldehyde normally do not make it

into the alveoli, as they are dissolved into the mucus while going down the bronchial tree.
Only particles less than 1 µm make it down into alveoli, larger particles being retained at higher levels in the respiratory tract.



F3.5. Epithelial basement membrane of the lung.

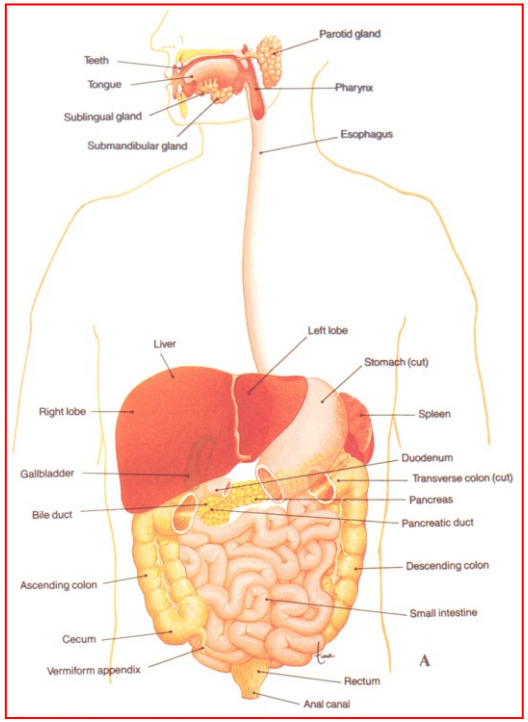
The distance to be covered between alveolus and capillary is only about 0.5 µm. The alveolar epithelial cell is also called a *pneumocyte*.

Q: What happens to a particle of asbestos lodged in the alveolus?
A: White blood cell either from the blood or already in the surface of the alveolus will attempt to phagocytize the particle, and enzymatically digest it.

Blood-soluble toxicants entering the lung will be transported along with oxygen to the heart through the pulmonary vein. The heart then distributes the toxicant to all parts of the body.

3.1.3. Digestive System

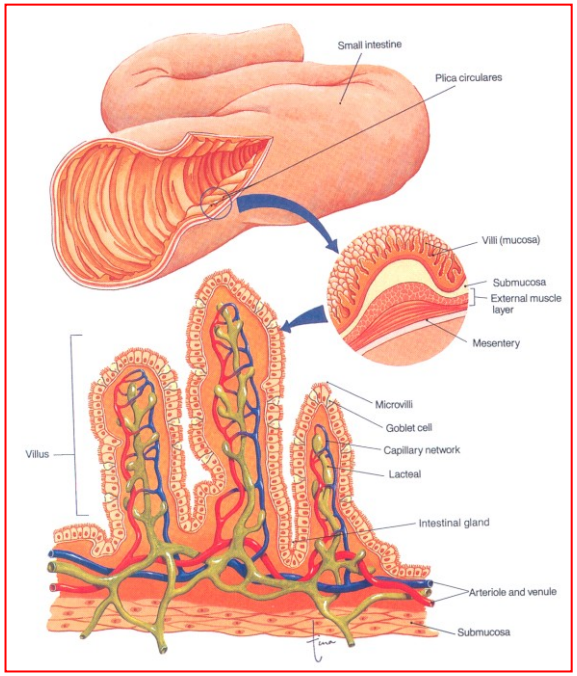
The process of digestion breaks up food mechanically and chemically into useable elements in great part by hydrolysis, which is why you need extra water after eating.



F3.6. Digestive system.
Essentials of Environmental Toxicology, Taylor and Francis, 1996.

The digestive surface is wet and functions well both in absorption and secretion.
Low pH from HCl secretion is observed in the stomach (pH ~2), where bacteria are killed and protein denatured to make them more sensitive to pepsin degradation.

The pancreas has both an exocrine (pancreatic enzymes for digestion) and an endocrine (insulin control over absorption of glucose into cells) function.
Both bile and pancreatic juices are injected into the duodenum, and they need a slightly basic medium (~ pH 8) for optimal function.
Surprisingly, you can live with only a small length of intestine. Although the small intestine usually has few bacterial colonies, the colon is rich with them. This explains why very small amounts of antibiotics can increase the growth rate of many farm animals (and foster antibiotic resistance), as they make nutrients more available to the animal, as opposed to the bacteria.



F3.7 Anatomy of the small intestine.
Essentials of Environmental Toxicology, Taylor and Francis, 1996.

The inner geometry of the small intestine is such that the epithelium is at mechanical risk. It is routinely sloughed off (~every 5 days in the colon), so that regeneration must be continuous. Most absorption of food and toxicants is in the small intestine. A notable exception is alcohol, a small molecule absorbed directly in the stomach. In F3.7, the central duct (green) connects with the lymphatic system, ultimately meeting the blood near the aorta. But a toxin absorbed through the intestinal vein goes to the portal vein and directly into the liver.

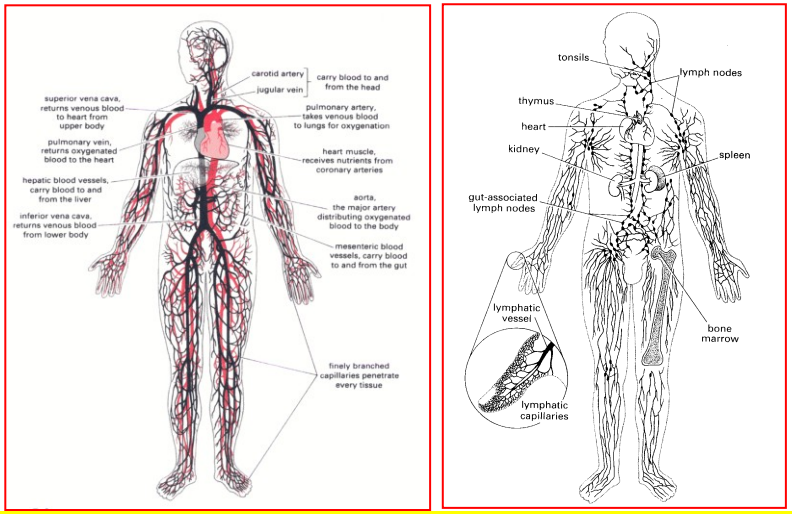
3.2. Target Organs

3.2.1. Perfusion of organs

Toxicants act locally, but are also carried by body fluids. The major fluids of the body, the blood and the lymphatic fluid, (F3.9) have a big influence on where toxicants will act. Very avascular tissues, like the lens of the eye, are less likely to be exposed to fluid-borne toxicants than other tissues. This is because the toxicant is likely to be disposed of before reaching them, or, at least, any crests of exposure are highly attenuated, which implies reduced toxicity.

Region	Mass (kg)	Blood flow (mL/min)	Blood flow (mL/100g/min)	Total cardiac output (% of total)
Liver	2.6	1500	58.0	27.8
Kidneys	0.3	1260	420.0	23.3
Skeletal muscle	31.0	840	2.7	15.6
Brain	1.4	750	54.0	13.9
Skin	3.6	462	12.8	8.6
Heart muscle	0.3	250	84.0	4.7
Other body	23.8	336	1.4	6.2
Total body	63.0	5400	8.6	100.0

T3.8. Blood perfusion of various body regions.
Medical Physiology, 1961.



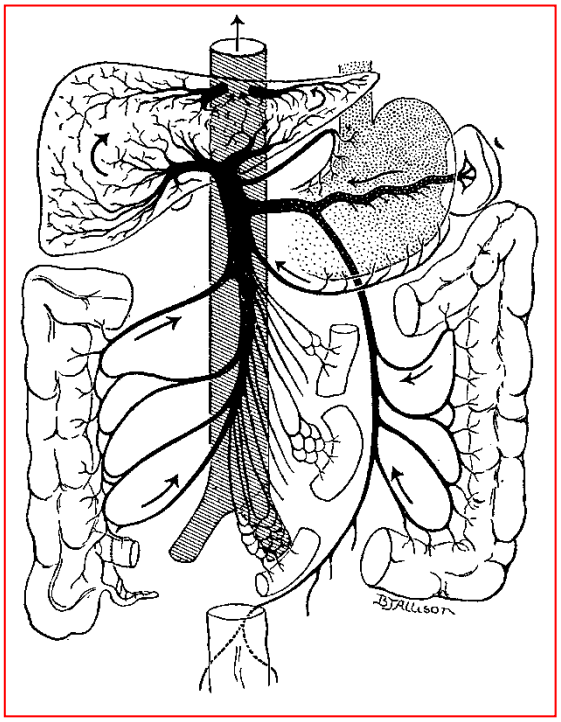
F3.9. Blood and lymphatic circulations.
Essentials of Environmental Toxicology, Taylor and Francis, 1996.

3.2.2. First Pass Effect

For any drug or toxicant reaching the blood, we should be concerned with what is known as the "first pass effect". Because 28 % of the blood circulates through the liver in dense capillary beds at low pressure and flow, and because the liver

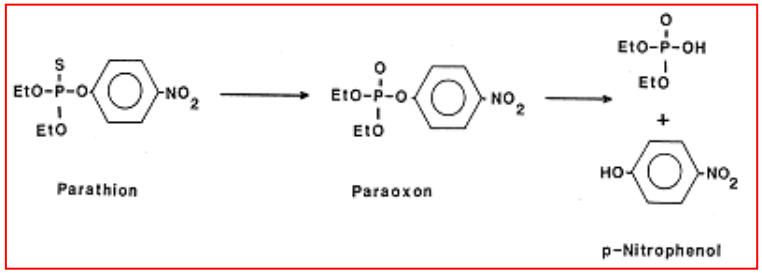
has such good extractive capabilities, the agent may be metabolized as it passes through the liver for the first time (F3.10). Approximately 70 % of the blood supply to the liver comes from the portal vein and 30 % via the hepatic artery. In many cases, if the level of the agent in the blood (acquired by ingestion, inhalation or dermal exposure) is “low”, little or none will escape into the systemic blood (vena cava) on the other side of the liver. It will be retained and metabolized locally, even up to “moderate” concentrations of the agent, because of the liver’s high filtration capacity. This may result in *hepatotoxicity*.

At high levels of exposure and elevated concentrations in the bloodstream, some agent will escape entrapment in the liver and will be forwarded through the *vena cava* to the heart and the systemic circulation. Toxicity may result from the "escaped" fraction reaching other organs.



F.3.10. Incoming arrows show circulation from the digestive system into the portal vein, while the vertical arrow at top shows exit to the *vena cava*.

As an example, the insecticide parathion must undergo oxidative desulfuration (using the non-specific mono-oxygenase aryl hydrocarbon hydroxylase, or AHH, also known as CYP1A1) to the much more reactive anticholinesterase (acetylcholinesterase-inhibiting) agent, paraoxon, before it can be degraded further (F3.11). At low-to-moderate blood levels, no parathion will escape the liver, all being destroyed *in situ*.



F3.11. Degradation of parathion. Et = Ethane

However, following excessive exposure (suicide attempts, sprayers, field workers, etc.), not all of the parathion is extracted by the liver, some escapes into the general circulation to be biotransformed in tissues. In nerves (parathion is highly lipid soluble), the intermediate, paraoxon, can inhibit acetylcholinesterase, resulting in the accumulation of the neurotransmitter acetylcholine.

Muscarinic, nicotinic and central nervous system toxic signs and symptoms appear (T1.17). It is thought that the systemic toxicity of parathion in poisoning cases arises from the extra-hepatic biotransformation of the parathion not destroyed by the liver.

3.3. Penetration of Barriers

Indiscriminate absorption of foodstuffs and toxicants into the body is prevented by *tight junctions* between cells. In a lot of the epithelium (ie, skin, inside of vessels, respiratory and digestive tracts), *tight junctions* prevent passage of molecules

between cells by simple diffusion (there are no gaps between cells).
The *Blood-Brain barrier* is an example of such an anatomical barrier that is particularly effective, making it difficult for physicians to obtain therapeutic doses of some medications in the Central Nervous System.

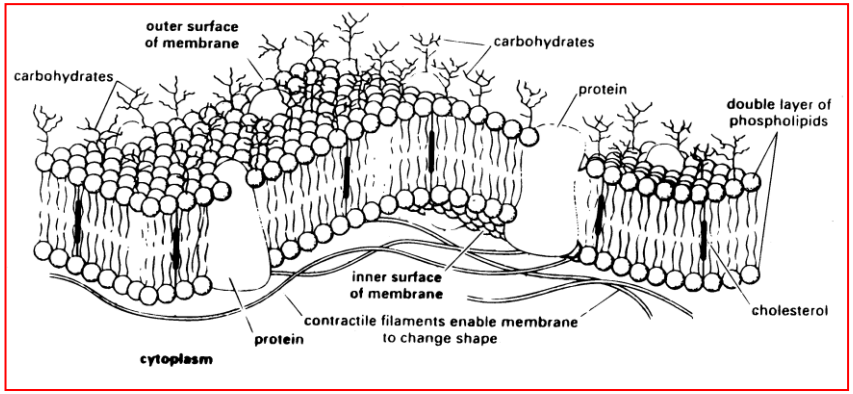
> For example, manganese, even if absorbed into the blood, is limited in its effects on the nervous system by the blood-brain barrier. Unfortunately, particles can dissolve on the nasal olfactory mucous membrane, then proceed to nerve cells that lead to the olfactory bulb in the brain, where it is redistributed to do neurological damage…

"Pinocytosis" is a mechanism of volume bolus absorption that is active in the gut, but is relatively rare. Down the line, pinocytic particles are expected to meet peroxysomes for molecular digestion. In spite of such marginal absorption mechanisms, however, most toxicants have to enter the body by going through cell membranes…

Proteins have a notoriously difficult time traversing the hydrophobic layers of the plasma membrane.
But *Bacillus anthracis* has a clever ways of pushing its proteins through. Anthrax kills with a toxin, a compound composed of three proteins—protective antigen, lethal factor, and edema factor—that somehow penetrate the plasma membrane of the host cell and enter the cytosol, where they make their kill[5].

Sometimes, a tough cell membrane can be a problem. An organism that infects one-third of the world's population, *Mycobacterium turberculosis,* has evolved expert multi-drug resistance primarily by resisting toxicant penetration. The membrane recipe is a wall of peptidoglycans followed by galactans and arabinans and further by mycolic acids. Most substances cannot diffuse across this boundary. The survival of the bacterium is enhanced by a slow division rate and de-toxifying metabolism.

3.3.1. **Phospholipid Bilayer**



F3.12. PhosphoLipid bi-layer.

TRANSPORT MECHANISMS THROUGH THE BILAYER

1. Passive diffusion (from a concentration gradient across the bilayer): molecular size is paramount only in the case of large molecules (fibers, dusts, peptides, proteins). Since most of the chemicals of concern are of rather small molecular weight, *solubility* is more important. Penetrating substances are lipid-soluble enough to penetrate cell membranes, but not lipid-soluble enough to remain trapped there, such as is the case for the volatile organics that exert an effect on the CNS.
The rate of transport (dD/dt) across a membrane is governed by Fick's Law:

$$\frac{dD}{dt} = \frac{K \times A \times (C_0 - C_i)}{T}$$

A is the cross section exposed to the compound, C_x are the concentrations on either side of the membrane, T is the thickness of the membrane and K is the diffusion constant.

Protein binding to blood and tissue proteins can enhance or retard transport by passive diffusion. Two features, the extent of binding (ie, 30 % or 85 % bound) and the tenacity of binding (loosely or tightly bound) are important. Unbound (free) agents can be transported by passive diffusion, bound agents cannot. To use passive diffusion effectively, a toxicant must be of small molecular size (less than ~600 Dalton), non-polar, and lipid-soluble (Lipinsky Rules).

2. Facilitated transport: mechanisms for sugars (glucose) and amino-acids (vitamins), whose details are not entirely understood. Involves membrane proteins which encourage transfer.

3. Active transport for inorganic ions and for xenobiotics: this process needs ATP. These systems maintain membrane potentials and allows cell-level excretion of xenobiotics (drug resistance).

Most of the chemicals of concern to the toxicologist are relatively small molecules that enter the body and reach critical sites by passive diffusion. A few exceptions include heavy metals (Pb, Cd, Zn, Co), which are acquired by active transport, using carrier systems designed to transport calcium or iron. These transport systems are not sufficiently selective to exclude other divalent cations of roughly the same size and charge (see example, F7.4).

Although passive diffusion always *follows* a concentration gradient, active transport can concentrate agents *against* such a gradient.

- The CNS has 2 active transport systems: one for organic acids, the other for organic bases.
- The kidney has 2 active transport systems: one primarily for sodium, the other for hydrogen.
- The liver has 4 active transport systems: 2 for organic acids, one for organic bases and one for neutral compounds.

The transport processes involved may be carrier proteins (transferrins for iron, etc.) or enzyme systems in which energy is expended. Specificity (sufficient or not) and binding capacity limits are important, as these systems can be saturated. According to the concentration of agent available, up to a certain maximum, the agent will be transported effectively.

Lead, for example, is transported by the same carrier system as calcium and, indeed, competes with calcium. They are both divalent cations and, in calcium deficient states, lead will be absorbed more efficiently. The final "resting place" for lead is in the long bones where one finds much higher levels than are circulating in the bloodstream (up-gradient transport).

3.3.2. Partition Coefficient

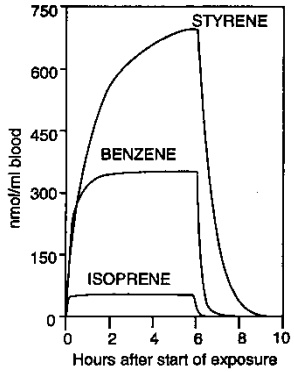
Since membranes must be crossed, the solubility of a drug or toxicant from one medium into the next is a critical determinant of its migration into tissues.
The determination of migrating ability between two media can be specified between any two liquid or solid media, but frequently, reference media are used such as from water to chloroform, hexane or octanol (see below).

$$P_{OW} = \log_{10} \frac{[in\ Octanol]}{[in\ Water]}$$

In the example of F3.13, one can see that as rats are exposed to breathing various Volatile Organic Compounds, the ones with the larger Partition Coefficient between Blood and Air tend to be draw out of the inspired air more rapidly and in greater amounts (less isoprene than benzene than styrene in the blood). As well, upon cessation of exposure, some substances tend to remain in the blood for longer periods. However, the decay in a complete animal can also be influenced by fat stores, since the three compounds shown migrate to fatty tissues from the blood (and vice-versa).

Partition Coefficients for Four Volatile Organic Chemicals

CHEMICAL	BLOOD/AIR	MUSCLE/BLOOD	FAT/BLOOD
Isoprene	3	0.67	24
Benzene	18	0.61	28
Styrene	40	1	50
Methanol	1350	1.3	1.1



F3.13. Effect of blood-air partition coefficient on venous blood concentrations in rats for a 6-h exposure to 2 g/m^3 (model).

The partition coefficient (P_{OW}) of a substance between a lipophilic solvent, usually *n-octanol*, and *water* is commonly and conventionally used to quantify substance transfer not only between body compartments but also between compartments in the environment (biota vs air, water or soil).

T3.13a. Hydrophilic - Lipophilic Molecules	**P_{ow}**
Acetamide	-1.16
Methanol	-0.82
Formic acid	-0.41
Diethyl ether	0.83
p-Dichlorobenzene	3.37
Hexamethylbenzene	4.61
2,2',4,4',5-Pentachlorobiphenyl	6.41

Significant relationships have been demonstrated between P_{OW} and the potential for a chemical to bio-accumulate in the aquatic environment, to concentrate in fish and adsorb on soil and sediments. A low P_{OW} (less than 3) can be used to convince regulatory authorities that an agent is unlikely to bio-accumulate or cause aquatic toxicity.

3.3.3. Dissociation Constant

Since biological membranes tend to give passage preferentially to uncharged molecules, it is important to know the degree of ionization of molecules when dissolved in body fluids.
The degree of ionization can be calculated from K_a, the Dissociation Constant of the molecule, and the Henderson-Hasselbalch equation, shown below.

$$K_a = \frac{[H^+][A^-]}{[HA]}$$

K_a is so named because it refers to dissociation of the acid (H^+) part of the molecule, as opposed to the basic part (OH^-). The Dissociation Constants (K_a) of various substances can be found from tables.

As an application of this equation, let us look at the absorption of acetylsalicylic acid (aspirin) from the human stomach…
Remember that pH = -log [H^+] and pK_a = - log [K_a].

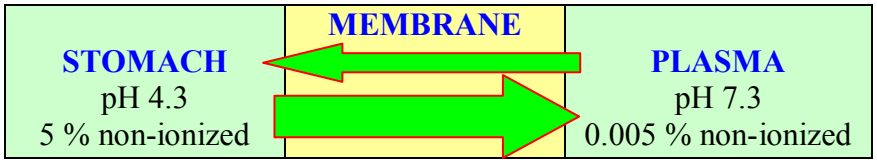
The pK_a for salicylic acid is 3.
The pH of blood is 7.3.
The pH in a full human stomach could be 4.3.

We use a modified Henderson-Hasselbalch equation:

$$\log \frac{[non-ionized\ \ form]}{[ionized\ \ form]} = \log \frac{[HA]}{[A^-]} = pK_a - pH$$

According to the equation, if pK_a = pH, then pK_a - pH = 0, and half of molecules are ionized, the other half not; that is, [non-ionized] = [ionized].
If we put aspirin in the stomach at pH 4.3, then
pK_a - pH = 3 – 4.3 = -1.3, which means that about 5 % of the aspirin is non-ionized ($10^{-1.3}$).
This 5 % of molecules of aspirin is able to cross into the hydrophobic membranes of the stomach wall, and ultimately into the circulation.

When these molecules cross into the intracellular fluid and into the blood, where the pH is 7.3, the ratio of ionized to non-ionized is altered: pK_a – pH is now 3 – 7.3 or - 4.3,
which means that only 0.005 % of the molecules are now non-ionized, and able to cross cell membranes.



This implies that most aspirin molecules that get into the stomach wall (plasma) are unable to diffuse back into the stomach, while there is a continuous 5 % of aspirin molecules able to cross into the stomach wall from the stomach.
Aspirin concentration rises in the stomach wall as a result of that trap, and aspirin is steadily pumped by diffusion into the blood and absorbed effectively.

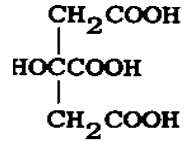
The pH difference between compartments has created a gradient of aspirin concentration.
Since pH is so important to agent penetration, the body has many systems to control it. You can live only for a little while with deviations in pH of about 0.5 units around 7.3.

pH is stabilized by :

- Buffer systems (bicarbonate, phosphate, protein systems),
- Breathing: CO_2 elimination helps regulate pH,
- Kidneys.

In case you are wondering, the mechanism of aspirin action has been at least partly elucidated: the acetyl group of aspirin seals access to a cavity of the enzyme prostaglandin H_2 synthase ("PGHS"), preventing transformation of arachidonic acid into prostaglandin H_2 (see F3.14).

More complex molecules, such as citric acid (shown), may have many dissociation steps. In the case of citric acid, they occur at pK_as of 3.14, 4.77 and 6.39.



HENDERSON-HASSELBALCH EQUATION

$$\log\frac{[non-ionized\ form]}{[ionized\ form]}=\log\frac{[HA]}{[A^-]}=pK_a-pH$$

For Aspirin (pK_a =3):

$$\log\frac{[non-ionized\ form]}{[ionized\ form]}=\log\frac{[HA]}{[A^-]}=3-7.3=-4.3$$

Aspirin in Stomach (pH 4.3):

$$\log\frac{[non-ionized\ form]}{[ionized\ form]}=\log\frac{[HA]}{[A^-]}=3-4.3=-1.3$$

Aspirin in Pancreatic Secretion (pH 8):

$$\log\frac{[non-ionized\ form]}{[ionized\ form]}=\log\frac{[HA]}{[A^-]}=3-8=-5$$

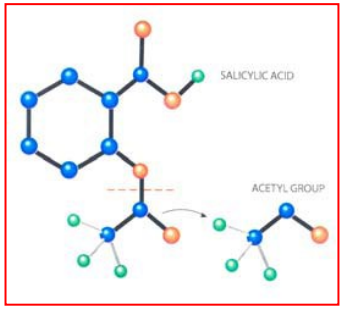
Aspirin in Plasma (pH 7.3):

$$\log\frac{[non-ionized\ form]}{[ionized\ form]}=\log\frac{[HA]}{[A^-]}=3-7.3=-4.3$$

Computation example (Stomach): $10^{-1.3} = 0.05 = 5\%$ Non-Ionized

LOCATION	% Non-Ionized	% Ionized
Stomach	5	95
Pancreatic Secretion	0.001	99.999
Plasma	0.005	99.995

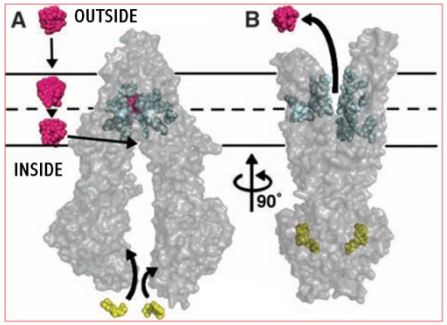
Result:
Aspirin is drawn from stomach to plasma and from plasma into pancreatic secretion.



F3.14. Mechanism of action of aspirin. Aspirin stops an inflammatory cascade which converts arachidonic acid, a fatty acid of cell membranes, into prostaglandins, by inhibition of the enzyme *cyclo-oxygenase*.

At the cellular level, physical and chemical properties may prevent entrance of toxicants inside cells. If toxicants do penetrate, they can be pumped out by efflux pumps or multi-drug resistance transporters, the same types of pumps responsible for drug resistance in bacteria:

- Multi-Drug Resistance associated P-glycoprotein (MDR1 gene) is weakly expressed in the liver, but more strongly in the intestines as well as T cells. It is a key factor in the blood-brain barrier, and has been likened to a "hydrophobic molecule vacuum-cleaner". The diagram at right shows (A) the transport of a red substrate from the membrane to a pocket, and (B) exclusion of the substrate using an ATP(yellow)-dependant conformational change. P-glycoprotein can export hundreds of chemically unrelated toxins, using binding sites in an internal cavity capable of stereoselectivity based on hydrophobic and aromatic interactions[15],



- the phospholipid export pump (MDR3 gene) translocates phospholipids (from bile, for example) from inner to outer aspect of membranes,

- the conjugate export pump (MRP2 gene) transfers organic anions into bile (conjugates of bilirubin, glutathione, glucoronide and sulfates).

Chewing with metal dental fillings releases mercury and activates these pumps, reducing the exposure of cells. Some chemicals, such as synthetic musks, are capable of specifically disabling these cellular pumps, leaving the cells vulnerable to other exposures[9].
Once in the bloodstream, toxicants can be taken up by organs specialized to deal with them, or damage organs that are not.

3.4. Distribution

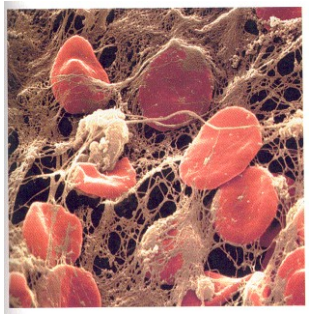
The two Circulatory Systems are by far the most powerful distributors of toxicants throughout the body.

3.4.1. Lymphatic Circulation

The lymphatic fluid can be seen as a yellowish crystalline-like fluid exuding from a cut. It carries away extra-cellular space proteins and particulate matter. It is driven mostly by muscle contraction, but also by a specialized system of valves throughout the system of lymph capillaries (F3.9), using only partly blood circulation as a propellant. Without lymph flow, you could only live for 24-hours.

3.4.2. Blood circulation

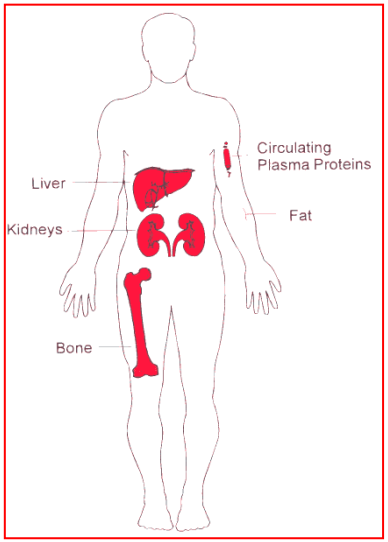
F3.15. Red Blood Cells trapped in fibrin clot.



The delivery of toxicants to target organs is primarily from the blood, because of the speed of its transit. Blood is made of *erythrocytes, leucocytes, platelets* and *plasma*. If fibrin is removed from *plasma* (stirring *plasma* with a glass rod will polymerize fibrinogen to it), one gets *serum*.
Keep in mind that plasma protein such as **albumin** (normal levels of albumin are 3.5-5 grams/dl) can keep toxicants in the blood for a period of time (binding 0 to 100 %), attenuating the release of toxicants to the organs.
Total plasma volume in the average human adult is **3 liters**, so any soluble toxicant injected into a vein would quickly be diluted into that volume.

Considerations of molecular size and lipid solubility are again of importance in gaining access from the blood into organs. Some chemicals present in the blood find more affinity for specific tissues such as **fat** (DDT) and **bone** (lead), and may concentrate there over time.

F3.16. Typical toxicant storage sites in the body. *Essentials of Environmental Toxicology, Taylor and Francis, 1996.*



Where toxicants end up is of great importance in final determination of toxicity. But controlling drug deposition in the body is difficult. In the pharmaceutical industry, unfavorable "ADMET" (absorption, distribution, metabolism, excretion and toxicity) is responsible for more than half of compound rejections at the level of clinical trials.

3.5. Storage

Storage is a type of *bioaccumulation*. Tissues and animals may bio-accumulate toxic halogenated defenses from compounds

they synthesize (Sea Slug, left) or from external sources such as their diet (*Aplysia,* right).

These chemicals make them unappetizing to predators, including sharks. Some of these compounds have been shown to move up the food chain into the milk of Faroe Island women (from whale blubber), just like PCBs[8].

Yet other animals get toxins from their prey, and then metabolically upgrade their toxicity (x 5) to protect themselves. This small tropical frog, *Dendrobates*, sports a very toxic skin *(pumiliotoxin)*.

3.5.1. Bone

Bone is actually in a state of perpetual regeneration every 7 years. One major constituent of bone is hydroxy-apatite $Ca_{10}(PO_4)_6(OH)_2$. In intoxication, fluoride may substitute for OH (making bones brittle), and radio-active strontium or lead for calcium.

3.5.2. Fat

Body fat is a storage compartment that varies in size, depending on the individual. In dieting, autointoxication can occur as toxic substances are released into the blood. Volunteers who lost 10 kg saw an increase of 23 % in blood levels of 14 PCBs and 11 pesticides. These increased concentrations in turn reduced the 15 subjects' metabolic rates either through effects on mitochondria or on the thyroid gland[7]. Since fat is so adept at absorbing a large category of liposoluble compounds, can it be viewed simply as a compartment designed to attenuate the exposures of the remaining body compartments to toxicants?

3.5.3. Resulting Distributions for Lead and Dioxin

As can be seen in T3.17, body tissue distributions may vary substantially between toxicants. Hair and nails have very high concentrations of lead, and are often used to document lead intoxication.

T3.17. Distribution of Pb and Dioxin

LEAD	%	DIOXIN	%
Bone	95.0	Fat	95.0
Liver	4.4	Liver	1.1
Lung	0.1	Muscle & Skin	2.5
Kidney	0.3	Gut	1.3
Brain	0.2	Other	0.2

3.6. Biotransformation

It has been estimated that 80 % of drugs taken by people emerge from the body intact. But what happens to the remaining 20 % is interesting…
The major function of the various biotransformation pathways in body tissues is to convert relatively lipid-soluble exogenous (and endogenous) agents into water-soluble, nontoxic and readily excreted compounds. The strategy is: make toxicants water-soluble so they will not diffuse back across cell membranes, and will be more easily controllable for excretion.

GENERAL MECHANISM OF BIO-DETOXIFICATION

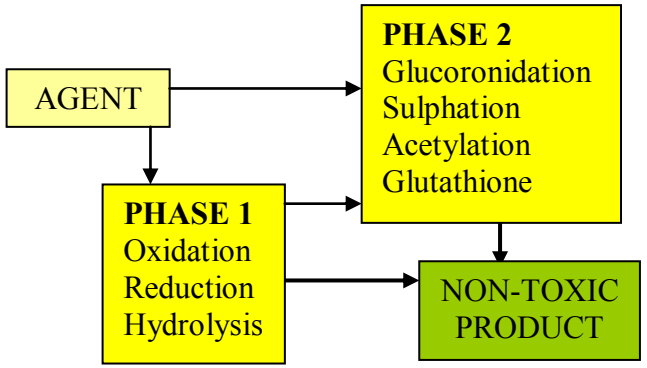
- **hydrophilic toxicants** are generally eliminated from the body in their original chemical form,
- **lipophilic toxicants** must be biotransformed for elimination.

Many of the detoxifications reactions occur within the endoplasmic reticulum of individual cells.
Unfortunately, biotransformation can also result in *bio-activation* or *toxication*, which involves the production of a more powerful toxicant derivative from the original toxicant.

The major organ for detoxification is the **liver**. Detoxification is conventionally segmented into **Phase I** and **Phase II** reactions. These reactions are also used by the body to eliminate normal by-products of metabolism. For example, hemoglobin is metabolized to bilirubin, which goes through the biliary system into the feces. Molecular weights smaller than 250 Dalton are excreted in urine, molecular weights larger than 350 Dalton are excreted in bile and feces.
Not all chemicals are processed through both Phases (F3.18). Generally, Phase I puts a molecular *handle* on molecules, while Phase II makes them more hydrophilic.
Heroine and codeine metabolism involve Phase I, acetaminophen (Tylenol) and morphine go directly to Phase II.[1]

3.6.1. Variations in Biotransformation

The importance of bio-transformation in biological organisms is underlined by the fact that most proteins are actually *enzymes*[▽] (as opposed to structural, messenger or transport proteins), agents that facilitate the transformation of one chemical into another.



F3.18. Biotransformation pathways.

When a single chemical agent is biotransformed, it is common that multiple forms of the agent result from the biotransformation process. As these reactions are often dependant on enzyme kinetics, exposure concentration may influence the variety of molecular species produced. These multiple forms of the original agent make toxicity more complex.
However, most of the differences in biotransformation are **quantitative** (as opposed to qualitative), resulting from changes in the rates at which toxicants are converted to intermediates and to water soluble, readily excreted products.
Biotransformation varies according to many factors such as species, strain, age, weight, emotional state, gut bacteria and sex.

▽ The molecular specificity of enzymes is determined by mechanical fitting (shape), electrostatic and dipolar interactions, as well as electron and proton tunneling.

3.6.1.1. With Metabolic Rate

For example, **warfarin** is used both as a rat poison and as an anticoagulant, and is primarily broken down by CYP2C9 (F3.23). 18 % of people have a variant of CYP2C9 that slows their metabolism of the drug. They are more susceptible to side-effects, such as severe internal bleeding. In this case, slower metabolism leads to increased effects because the AUC is larger.

Rodents metabolize drugs more rapidly than people do. To reach the same blood level in a rodent model, administered doses are increased. Although the blood levels are then comparable, gastrointestinal tract and liver levels become much higher in the rat, which increases tumors in the liver and digestive system. In this case, faster metabolism leads to increased effects.

3.6.1.2. With Sex

In rodents, males have a capacity for some bio-transformations about 5 times that of females. This often corresponds to major response variations in animal experiments.

An example of a sex-based variation is the effect of the phyto-estrogen diadzein in rats. Injections increase growth hormone, testosterone and muscle mass in males, while the opposite happens in females[10].

T3.19. Genes Expressed differently between the Sexes

Mouse Tissue	0 to 20% difference	300% difference
Liver	72 %	0.5 %
Fat	68 %	0.3 %
Muscle	55 %	0.1 %
Brain	14 %	0.1 %

Many of the sex differences in gene expression in the table above are hormone dependant: the differences are considerably attenuated if testes and ovaries are removed[12].

3.6.1.3. With Route of Administration

The toxic reaction is also influenced by the route of administration, as this determines the order in which the toxicant will encounter various organs, and the toxicant flow.

3.6.1.4. With previous Exposures

The biotransformation activity can be enhanced (*up-regulated)* by exposure to compounds such as phenobarbital, a technique used to harvest large quantities of the enzymes.

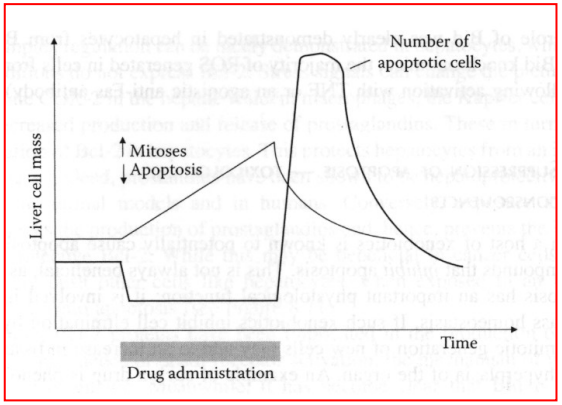
Not only will individual liver cells have more enzymes under phenobarbital induction, but the overall liver mass will increase with chronic exposure (F3.20).

Receptors such as Aryl Hydrocarbon Receptor (AHR), Pregnane X Receptor (PXR) and Constitutive Androstane Receptor (CAR) mediate the transcriptional responses that amplify the responses to xenobiotic exposures.

3.6.1.5. With and Within Species used

Rodents are frequently used as test animals, not because they are similar to humans, but because they are inexpensive, easy to house and breed, require minimal care and are easy to handle.

F3.20. Phenobarbital induces liver weight gain and enzyme production, and inhibits apoptosis in the liver. As the drug is removed, a sharp increase in apoptosis restores balance.
Boelsterli, 2007.



Commonly used test species *share* some of the metabolic capabilities of humans, but *lack* many others. These metabolic variations occur not only between species and strains, but between different individuals of the same species.

Even within a group of 75 inbred rats, the same high dose of acetaminophen (Tylenol) produces various degrees of liver damage. But pre-exposure analysis of urine makes it possible to predict with 85 % accuracy the level of liver damage that will be produced by the drug in individual rats[11].

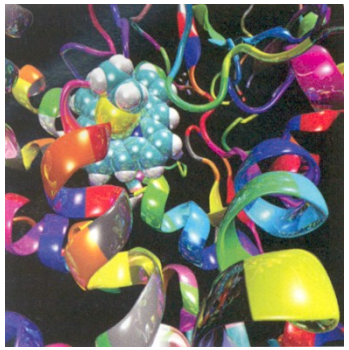
Some differences can be traced to minor genetic variations among individuals, called single-nucleotide polymorphisms, or SNPs. Genome investigators at the National Institutes of Health (US) have already compiled more than 800,000 examples of such variations in humans.

3.6.2. Phase-I Biotransformation

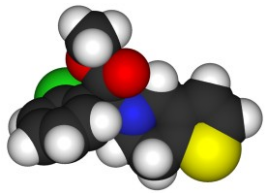
These **catabolic** (breakdown) enzymatic reactions deal with the insertion, addition or exposure of reactive groups, initiating the conversion of the molecule: a polar group is either unmasked or added, to enhance water solubility. The typical mechanisms used are **oxidation, reduction and hydrolysis**. The reactions are carried out by enzymes in microsomes and in the endoplasmic reticulum.

The major system is **cytochrome P-450** (F3.21, F3.23), an array of (7700 known distinct sequences) mono-oxygenase enzymes in the smooth endoplasmic reticulum. Fe^{++} acts as a source of electrons and oxygen. The "450" (nm) refers to the bluish color of the enzymes.

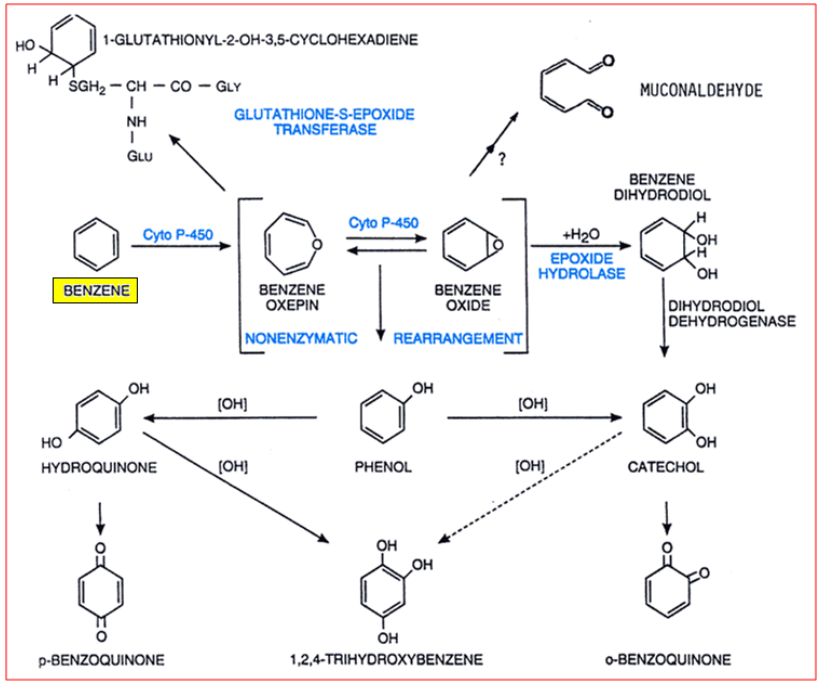
The role of the **mono-oxygenases** is the oxidation or reduction of existing groups present on the molecule or the insertion of molecular oxygen into aromatic rings to form oxepin or benzene oxide (F3.22, center).



F3.21. Part of a CYP450 (multicolored ribbon) enzyme binding to putidaredoxin (light blue and white).
Center for Computational Research, U. of Buffalo.



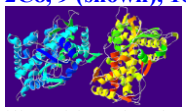
Clopidogrel inhibits blood clots and is prescribed after a heart attack to reduce subsequent coronary events. Clopidogrel is inactive, and must be metabolized in the liver by cytochrome P450 enzymes, including CYP2C19. Clopidogrel-treated patients who carry one or two ineffective variant alleles of CYP2C19 are 1.5 to 3.5 times more likely to die or experience cardiovascular-related complications than patients who carry high-functioning alleles. Genotyping would allow patients with these mutations to use alternate drugs[13,14].



F3.22. Metabolic pathways for benzene. *Casarett & Doull.*

NNK (nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone) is a tobacco-specific nitrosamine that requires metabolic activation by cytochrome P450 enzymes. Smokers who have two fully functional copies of the CYP2A6 gene smoke 7-10 more cigarettes per day than smokers who have only one.
The enzyme CYP1A2 activates many cancer-causing chemicals in tobacco smoke. The enzyme is inhibited by drinking 500 ml of grapefruit juice every day, a tactic to reduce the chances of cancer from smoking.

CYP1A1 (AHA)
Benzo[a]pyrene and other polycyclic aromatic hydrocarbons
CYP1A2
Acetaminophen, 2- Acety laminofluorene, 4-Aminobiphenyl, 2-Aminofluorene, 2-Naphthylamine, NNK, Amino acid pyrrolysis products (DiMeQx, MeIQ, MelQx, Glu P-I, Glu P-2, IQ, PhIP, Trp P-I, Trp P-2), Tacrine
CYP2A6
N-Nitrosodiethylamine, NNK, nicotine.
CYP2B6
6- Aminochrysene, Cyclophosphamide, Ifosphamide
CYP2C8, 9 (shown), 18, 19



Tienilic acid, Valproic acid
CYP2D6
NNK*
CYP2E1
Acetaminophen, Acrylonitrile, Alcohol, Benzene, Carbon tetrachloride, Chloroform, Dichloromethane, 1,2-Dichloropropane, Ethylene dibromide, Ethylene dichloride, Ethyl carbamate, Halothane, N-Nitrosodimethylamine, Styrene, Trichlorothylene, Vinyl chloride
CYP3A4
CYP3As metabolize 50 % of drugs and are major determinants of first pass and systemic clearances. Grapefruit juice elevates blood levels of many drugs primarily by inhibiting intestinal CYP3A4 during absorption.
Acetaminophen, Aflatoxin B, and G, 6-Aminochrysene, Benzo[a]pyrene 7,8-dihydrodiol, Cyclophosphamide, Ifosphamide, 1- Nitropyrene, Sterigmatocystin, Senecionine,
Tris(2,3-dibromopropyl) phosphate
CYP4A9/11
None known

F3.23. Xenobiotics activated to more toxic forms by individual CYtochrome P450 enzymes. The identification following "CYP" refers to enzymatic activity traceable to specific genes: class-subclass-gene. Growth hormone pulse profile♣ is a major determinant of which P450 enzymes are expressed. *Casarett & Doull.*

♣ Hormones are often most effective when released in **pulses** as opposed to continuous releases.

Another group of Phase I enzymes includes the **hydrolases** (esterases, proteases, lipases), their function being to hydrolyze peptide and ester bonds of a wide variety of chemicals to expose polar carboxyl, and hydroxyl or amino groups.
These enzymes sometimes transform aromatic compounds into epoxides (R-O-R), compounds significantly more toxic (mutagenic) than their parents. There are many examples of xenobiotics activated by individual P450 enzymes to more toxic species, as shown in F3.23.
On the other hand, CYP2B6, for example, also disables more than a dozen known herbicides, pesticides and industrial chemicals.

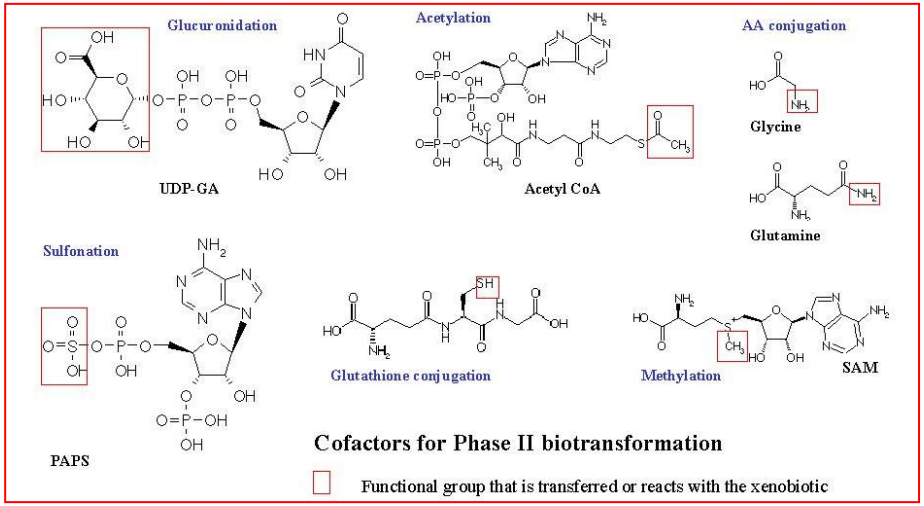
Many drug companies now conduct lab tests to determine the influence of potential drugs on common metabolic pathways, such as cytochrome P450 enzymes. If the drug affects them, it is pulled from further development, so that no interference with other drugs can occur.

3.6.3. Phase-II Biotransformation

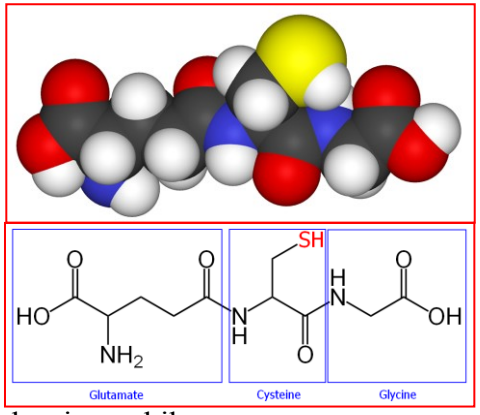
The introduction or exposure of reactive groups by Phase I prepares the way for Phase II. Although the enzymatic reactions also occur in microsomes and mitochondria, it is mostly cytosolic enzymes (F3.24) that **transfer covalently** normal body constituents (sulfate, glutathione, acetyl/glycine, methyl, glucuronyl, amino acids as well as ornithine) to reactive groups.
Note that a molecule must be *provided by the body* to the toxicant to further enhance hydrophilicity in these conjugation reactions.
The conjugation converts the intermediates into water soluble products amenable to excretion via the urine and feces.

F3.24. PHASE II ENZYMES ("transferases")

DeTox Group	Enzyme Family	Enzymes	Substrates
Sulfate	SulfoTransferases	**SULT** 1A1 to 2B1	Phenol, Toluene, Acetaminophen
Glutathione	Glutathione S-Transferases	**GST** A1 to Z1	Peroxide, Acetaminophen, Heavy Metals
Acetyl	Arylamine, Aralkylamine or Glycine N-Acetyl Transferases	**NAT** 1 to 2 AANAT, GLYAT	Arylamine and Hydrazine drugs
Methyl	Methyl Transferases	COMT	Cathecols, Indolamines, Thiouracil
Glucoronyl	UDP-Glucuronosyl Transferases	**UGT** 1A1-2B28	Phenol, Trichloroethanol, Nicotinic acid
Amino Acid	Amino acid N-acyl Transferases	BACATs	CoA-activated Bile Acid
Ornithine	Ornithine Carbamoyl Transferases	OCT	Choline



3.6.3.1. Glutathione

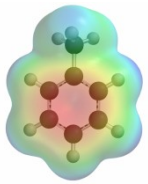


Glutathione (shown) binds to heavy metals with its sufhydryl (-SH) extremity. The transport proteins Multi-Drug Resistance Protein (MRP) and P-glycoprotein (Pgp) ship these adducts out of cells, and the body then eliminates the molecular complex through urine or bile.

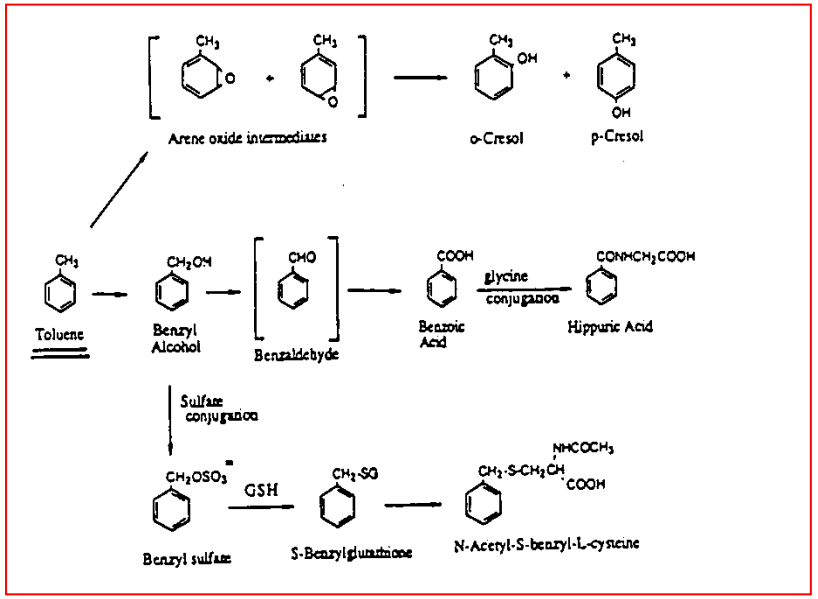
The conjugation of toluene derivatives with glutathione (F3.25) may result in the formation of nephrotoxic cysteinyl thioether derivatives (F3.25 - toluene conversion into benzyl sulfate, then into an N-acetyl-S-benzyl-L-cysteine derivative).

Free hydroxy groups, as in o-cresol or p-cresol formed from toluene, are susceptible to glucuronidation and sulfation. Many chemicals already possess electrophilic groups (as in cresol or phenol, which have hydroxy groups) and do not need to undergo Phase I biotransformation, proceeding immediately to Phase II reactions.

3.6.3.2. Toluene



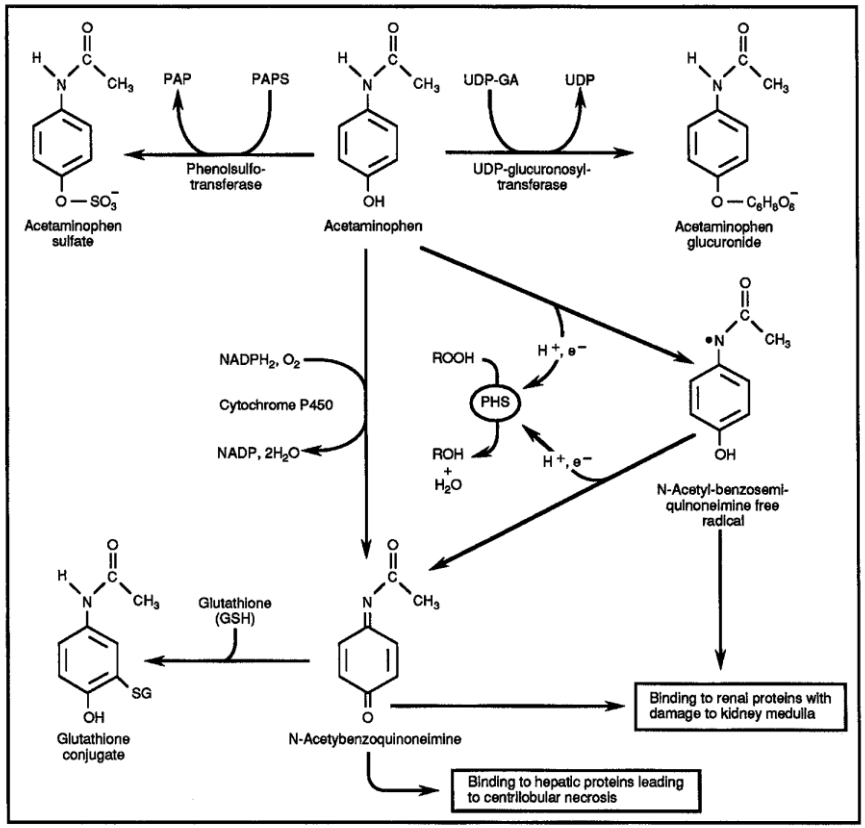
Toluene can be biotransformed into a number of products (F3.25), but not all of these may be produced by all species. Certain pathways may occur at low levels of exposure while, at high levels, a second or even a third pathway may become active. In any study of a chemical's excretion, it is advisable to know which excretory products are found at which dose, and have a method by which all can be quantitated. The rodents are notorious for introducing a new pathway when a threshold dose is attained.



F3.25. Metabolic pathways for toluene.
Low et al. Toxicol. Indust. Health 4, 49-75, 1988.

3.6.3.3. Acetaminophen

Humans may do the same, as is shown in F3.26 for the drug acetaminophen. Acetaminophen ("*Tylenol*") is both hepatotoxic and nephrotoxic at high doses. The drug (F3.26, top-center) already has a handy hydroxyl group, useable to tag glucuronide or sulfate conjugates. However, these pathways can saturate at high exposures, because of enzyme limitations.



F3.26. Pathways of acetaminophen disposition.
(PHS = Prostagladin H synthase).

With higher dosage, the cytochrome P-450 mono-oxygenases begin to react with the unconjugated drug, producing a quinoneimine derivative which can be utilized by the glutathione conjugating enzyme to form a mercapturic acid derivative. At excessively high dosage, this pathway can be saturated and even halted.

This leaves the reactive, electrophilic and unstable intermediate free to seek a "neutral" environment by reacting with any nucleophilic groups in the region - DNA, membrane proteins, cellular proteins, etc.
The result is cellular damage with leakage through damaged membranes and, if severe enough, necrosis and cell death.

3.6.3.4. Phase I and II as Rate-Limiting

In most tissues of the body, there are excess amounts of mono-oxygenases for the cells' needs, but the various conjugating enzymes are limited. Although sufficient for low level exposure, the rate-limiting step at high exposures is the activity of these enzymes and their dependency on

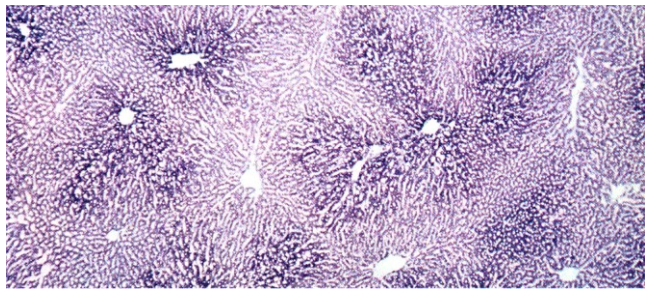
(1) a sufficient cellular store of the conjugating substance, and
(2) the rates of replacement or re-synthesis of these substances (glutathione, glucoronic acid, sulfate, glycine, etc.).

A dramatic decrease in the activity of the enzymes occurs until the stores are replaced, a period of 18 to 48 hr in many species. Repeated exposures result in repeated insults to the body's tissues and considerable damage if the reactive intermediates formed cannot be conjugated.
The formation of certain conjugates is quite different between species. For example, the human glucuronidates much of the acetaminophen ingested, with a small amount of sulfate formed unless the level of exposure is high, and then glutathione derivatives (leading to mercapturic acids) will be found. The guinea pig does the same. The rodents (mice, rats) produce quantities of both glucuronide, sulfate and glutathione derivatives.
The conjugation of a simple molecule such as **phenol** is quite species-specific, as is shown in F3.28, showing the pathways of phenol and the oxidative hydroxylation product, quinol.

While Phase I and II enzyme complexes are ubiquitously distributed through the body's tissues, they are not *uniformly* distributed, but bear some relationship to the overall metabolic activity of the particular organ. Liver has the highest activity, the kidney has approximately 20 % of the liver's activity, while brain, muscle, etc. may have 1 to 10 %. This concentration of enzyme activity is responsible for chemical-induced cellular damage in the liver.
The detoxification properties of Phase II enzymes can be manipulated, even by simple dietary changes. Broccoli sprouts contain a substance, sulforaphane, which stimulates the production of Phase II enzymes, causing carcinogens to be more efficiently detoxified.

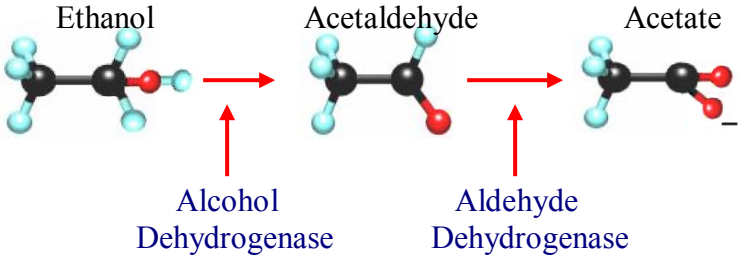
3.6.4. Toxicant Interactions

If more than one agent solicits the liver enzymes, interference or competition between two chemicals for biotransformation by the same or similar mono-oxygenases may result. The chemicals might be two components in a mixture or an "occupational" chemical plus an over-the-counter or prescription drug. Unfortunately, there are too few good, well studied, examples of toxicant interactions in the literature. However, three such studies are listed in the references[2-4].



F3.27. Distribution of glucose-6-phosphatase enzyme in the liver. X 25. *Biological structures, 1979.*

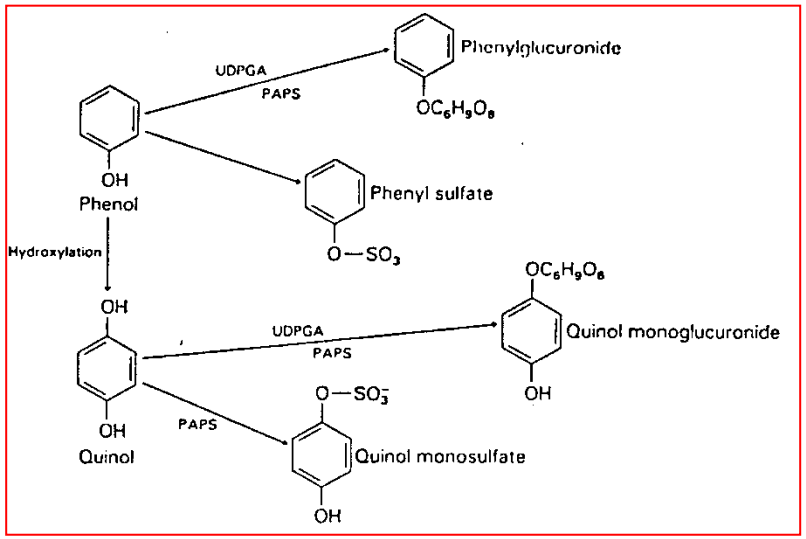
In the metabolism of **ethanol**, shown below, the enzyme alcohol dehydrogenase has an affinity for ethanol 100 times greater than to ethylene glycol.
In case of ethylene glycol (car coolant) intoxication, intravenous alcohol is recommended. This allows ethanol to monopolize the enzyme, and for the renal excretion of unchanged ethylene glycol to occur slowly, avoiding its conversion to more toxic metabolites.



You might have noticed a slight trembling the day after alcohol intake. The enzymes monopolized for alcohol digestion would normally provide nervous system nutrients, but are not available in sufficient quantity to fill the needs of the nervous system.

F3.28. Species variation in the metabolic conversion of Phenol *in vivo*. Percent of 24-hour excretion as…

	Glucuronide		Sulfate	
Species	**Phenol**	**Quinol**	**Phenol**	**Quinol**
Pig	100	0	0	0
Indian fruit bat	90	0	10	0
Rhesus monkey	35	0	65	0
Cat	0	0	87	13
Human	23	7	71	0
Squirrel monkey	70	19	10	0
Rat tail monkey	65	21	14	0
Guinea pig	78	5	17	0
Hamster	50	25	25	0
Rat	25	7	68	0
Ferret	41	0	32	28
Rabbit	46	0	45	9
Gerbil	15	0	69	15



3.7. Excretion

3.7.1. Lungs

Lungs can breathe OUT toxicants as well as they can breathe them IN. For example, 90 % of alcohol is transformed into acetaldehyde in the liver, but 10 % is left unchanged, and can be exhaled or urinated (thus, the roadside test).

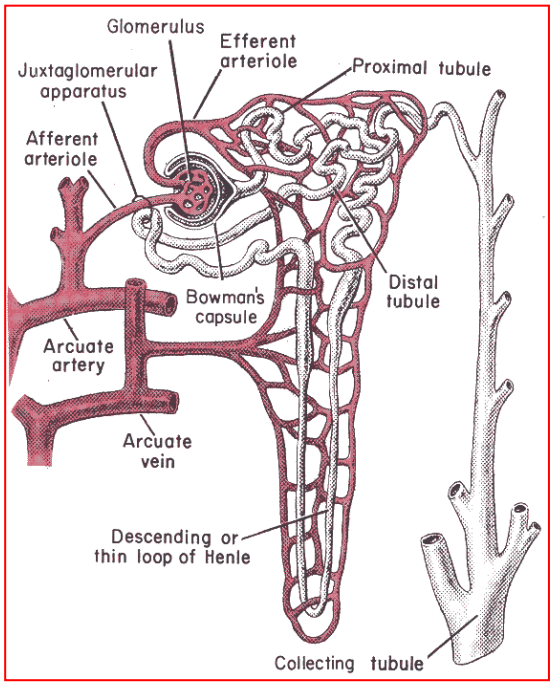
3.7.2. Liver

Feces can contain ingested toxicants which were not absorbed by the body at all, or toxicants that were secreted out either by the intestine or the bile (DDT and lead).
Toxicants that are being expelled through bile can be reabsorbed into the body, possibly to produce more damage to the liver. This re-absorption is called the **entero-hepatic loop**.
Techniques have been developed to interrupt the loop by ingesting an adsorbent such as carbon black, that will bind tightly and carry toxicants into the feces.
Bile secretions contain detergent-like acids for digestion of fats in the intestine. Most of these acids are reabsorbed in the intestine. Lithocholic acid is not reabsorbed, and is one of the most toxic natural substances in the body, acting as a mutagen that also inhibits DNA repair, which has led in animal models to colon cancer[6].

3.7.3. Urine and Feces

There are about 1 million nephrons in a kidney. The incoming blood loses fluids through the glomerulus by mechanical filtration (sieve) and proceed to recuperate needed components at the proximal tubule, the loop of Henle, and the distal tubule. The kidney is therefore an organ of RECUPERATION,

throwing out everything, and selectively reabsorbing precious components.



F3.29. Diagram of a nephron. *Medical Physiology, Guyton, 1981.*

The strategy of making toxicants *hydrophilic* then becomes clearer. At the glomerulus, hydrostatic pressure forces small molecules smaller than 60,000 Dalton out into Bowman's capsule, thus conserving blood cells and large proteins. 20 % of all dissolved compounds less than protein size are initially lost in the kidney. From the expelled fluid, 99 % of the needed components (for example, glucose) are recuperated in the loop of Henle. We would die rapidly without this constant recuperation. Some *active secretion into the urine* occurs in the distal tubule, such that compounds that are actively secreted (PAH, Diodrast) can have a clearance greater than the glomerular filtration rate. The pH of the urine can vary between 4.5 to 8. With this delicate anatomy, is it surprising that with a little hypertension, some protein or blood could leak into the urine?

The rate of elimination of many fat-soluble compounds can be enhanced by increasing the lipophilicity of the gastro-intestinal tract content, for example by adding mineral oil to the diet.

Older people are know to be more sensitive to drugs. For example, they have increased sensitivity to central nervous system depressants, to side effects such as high blood pressure from psychotropic medication, and are more likely to experience hemorrhage from anti-coagulants. These changes correspond to alterations in the distribution and transport of drugs as a result of reduction in lean body mass (vs fat), serum albumin in the plasma, and total body water, as well as reductions in the function of organs which assist in the metabolism (enzymes) and elimination of substances.

3.7.4. Species Variations

Using benzene as an example, most animals appear to excrete low internal doses as metabolites in the urine. The urinary profiles in benzene-exposed mice, rats, and primates are shown in T3.30. For the low-dose treatments, a higher fraction of benzene is converted to hydroquinone and its conjugates in the mice compared to the rat. The urine of the monkeys, on the other hand, contains approximately the same fraction of hydroquinone as found in the urine of mice. One must keep in mind, however, that a smaller fraction of inhaled benzene is metabolized in the monkey compared to the mouse. The urinary profile of the chimpanzee is similar to that of the rat in regard to the hydroquinone fraction.

Urinary metabolites (% of total)	6 hours at 5 ppm			6 hours at 50 ppm			6 hours at 600 ppm		
	Mouse	Rat	Monkey	Mouse	Rat	Monkey	Mouse	Rat	Monkey
Phenyl Conjugates	37	58	61	37	72	73	67	74	78
Hydroquinone Conjugates	33	12	27	40	3	15	11	2	9
Cathecol Conjugates	na	na	8	na	na	6	na	na	9.9
Pre-PhenylMercapturic Acid	6	10	na	1	11	na	15	17	na
Muconic Acid	23	19	4.4	21	14	3.1	5	4	1.3

F3.30. Urinary Excretion of (^{14}C) Benzene. *from Sabourin*[16 17,18].

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Toxicokinetics

4· Toxicokinetics

Toxicological science is not explicitly concerned with the difference between Toxication and Intoxication.
Toxication is the instantaneous toxic action resulting from the action of a toxic agent at a given moment in time. Whereas *Intoxication* refers to the effects of having been exposed to a toxic substance. So,

$$In-toxication\ (t) = \int_0^t Toxication\ (Agent\ Concentration)\ dt$$

This emphasis on Intoxication results from difficulties in the practical measurement of Toxication.
Usually, time effects in Toxicology are dealt with using indirect concepts such as categories of acute, sub-chronic and chronic effects.
Further, the evolution over time of the agent (primarily, agent concentration) is separated from the evolution of the organism itself.

Toxico*kinetics* is a quantitative description of the tissue levels of toxicants as a function of time in body compartments. It is concerned with peak blood levels, half-lives, body burdens and persistence of toxicants in organisms.

Toxico*dynamics*, a closely related field, investigates the changes in an organism as a result of toxicant exposure.
Note that toxicodynamics also happens at the level of species, through evolution. Species change their design as a result of toxicant exposure.
2.5 billions years ago, there was no oxygen in Earth's atmosphere, and there was little in the oceans until 1 billion years ago. It arrived after the proliferation of cyanobacteria. People did not invent pollution...it has been with us forever. Living systems adapted to a point by, for example, integrating mitochondria into their design. A shorter-term example is the prevalence of hemoglobin S in Africa to counter malaria. Extremely fast toxicodynamics is the development of drug resistance in bacteria. Humans are adapted to their toxic environment, but there is no doubt that the "design" can be improved[6].

In an animal model with null toxicodynamics, the animal would not change as a result of intoxication. In this (unlikely) case, it would be possible for a single animal to be exposed to successively higher doses of a toxicant, and the experiments would yield the same results as if many animals were exposed to a range of single doses. In fact, the various manifestations of a toxicant could be uncovered by monotonously increasing the dose on a single animal.

Toxicokinetics quantifies absorption, distribution and elimination of an agent in an organism. It has the same basis as clinical pharmacokinetics[2], which monitors body burdens (blood and tissue levels) and therapeutic benefits.
The ideal Toxicokinetics for a drug (achieving the ideal plasma concentration) are not always easy to achieve. In the last 10 years, many oncology drugs have been approved which are in fact only innovations in drug delivery: getting the proper amount of medication into the proper compartment for the proper time interval.

Toxicokinetics is concerned with the duration and intensity of toxic action by taking into account:

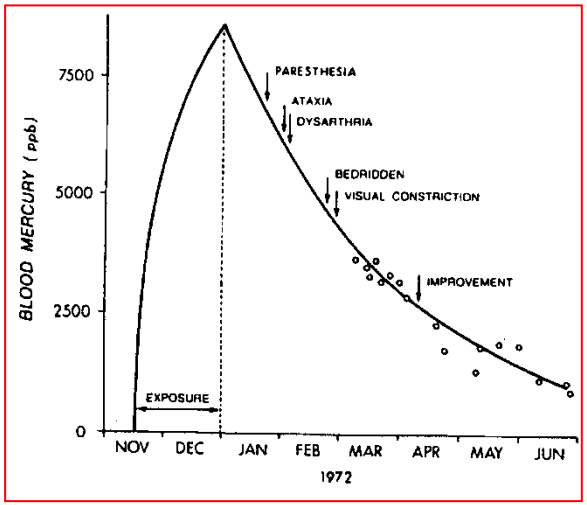
- the persistence of the toxicant and its metabolites in target organs,
- the accumulation of the agent in storage sites (body burden),
- the rate of elimination of the toxicant.

The *first step* in toxicokinetics is to divide the human body into

1. a central compartment (usually, the blood together with highly vascularized tissues), and

2. a certain number of peripheral **compartments** representing body segments with common properties (F4.2). The simplest model assigns to a single peripheral compartment the organs that are more poorly vascularized.

The *second step* of toxicokinetics is to make assumptions about the **rate of elimination** of the toxicant from the central compartment, specifically **zero order** or **first order** elimination (the models most commonly used).

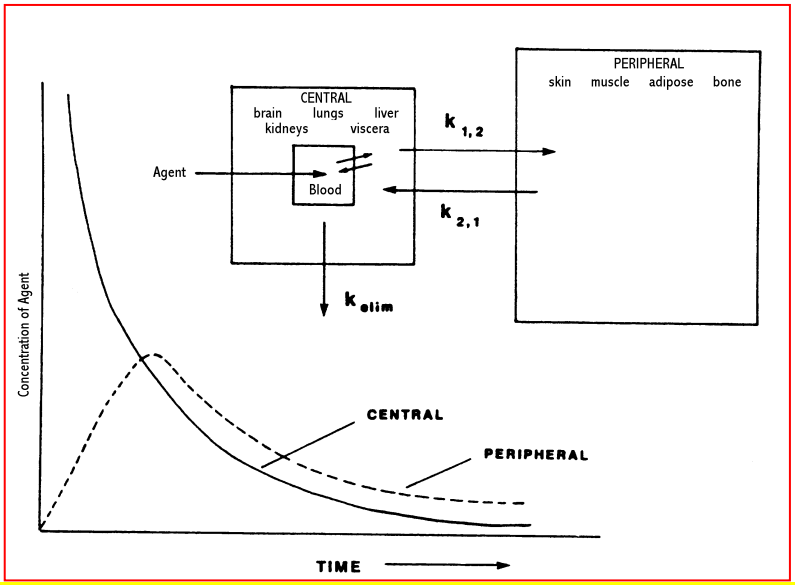


F4.1. Clinical symptoms in victims of methyl-mercury poisoning in the Iraq outbreak of 1971-72. The solid line represents blood levels based on intake and the pharmacokinetics of methyl-mercury. Dots are actual observed blood concentrations.
Basis of Toxicity Testing, 1997.

Toxicokinetics is of great clinical usefulness in predicting the evolution of symptoms in victims of intoxication. Optimal patient care needs quantification of toxicant concentration for many agents: acetaminophen, aspirin, ethylene glycol, methanol, ethanol, iron and other metals, lithium, carboxyhemoglobin and digoxin.

4.1. Notion of Compartment

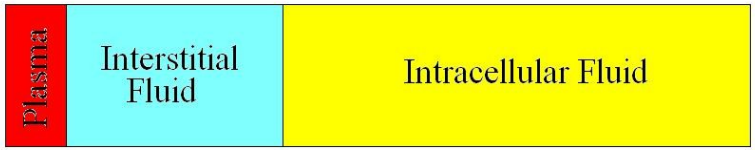
A *compartment* is a part of the body with a uniform toxicant concentration. Examples of compartments: blood, plasma,



F4.2. Concentration of an agent in body compartments after injection into the blood (central square). Rapid equilibration between blood and Central compartment (short arrows) is already achieved before the beginning of the graph. The curves show equilibration between Central and Peripheral compartments, as well as elimination from the Central compartment. There is a route of escape from the central compartment only (k_{elim}, due to bladder, gastro-intestinal tract, lungs). There are rate constants to and from the peripheral compartment but no escape route, indicating that what goes out to the peripheral compartment (adipose tissue, bones) must be released back into the bloodstream for transfer to the central compartment.
Basis of Toxicity Testing, 1997.

stomach, bone, liver, extracellular fluid, mitochondrial volume, bound with albumin, etc. Almost anything can be a compartment, but the choice of modeled compartments depends on the properties of the toxicant.
Early studies used one or two "abstract" compartments (even if they had no physical reality) to describe the evolution of molecular concentrations over time. Now, the fashion is to use physiologically meaningful compartments of individual organs (intracellular, extracellular, alveolar space). Unfortunately, the basic data needed to set-up such models is often missing, partial or approximate.

BODY FLUID VOLUMES (8, 29 AND 63%)

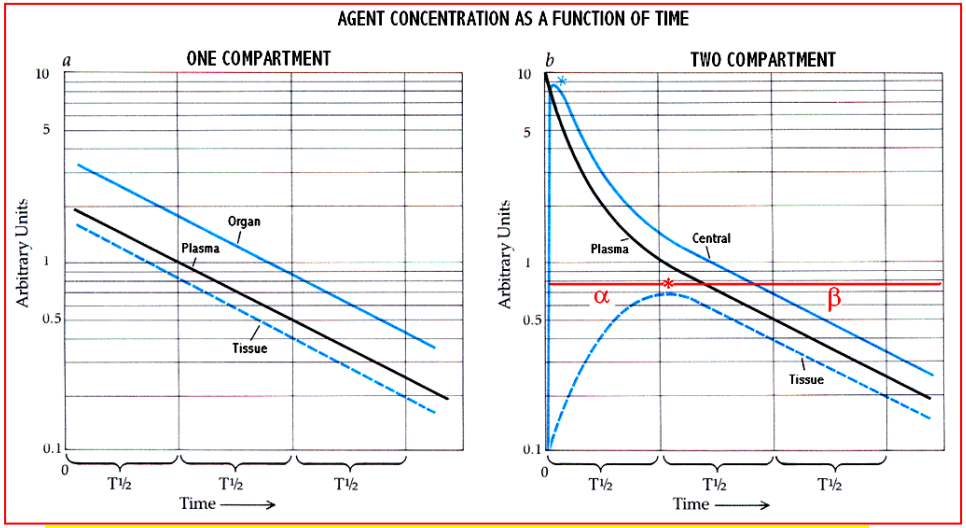


Although a compartment has a single concentration of a toxicant, the value usually drifts over time. An exposure generally results in the appearance of the agent in the bloodstream, but it usually does not remain there exclusively, as it is drawn out of the blood into other compartments.
In F4.2, an agent injected into the blood is rapidly equalized in the **central** compartment made up of highly perfused tissues (this process is too fast to be shown in the graph). The blood level in the central compartment thereafter declines, indicating distribution into the **peripheral** compartment made up of the less vascularized tissues such as skin, muscle, adipose tissue and bone.

4.2. Theoretical Toxicokinetic Curves

Three important phenomena can be displayed in toxicokinetic curves (F4.3b): Absorption, Distribution ("α phase") and Elimination ("β phase").

When a toxicant is eliminated from a single compartment model (F4.3a), one mostly *observes agent elimination* ("β phase") in the data. The absorption phase is vanishingly short, since the agent was administered directly into the blood, and there is no distribution, since there is only one



F4.3. Intravenous administration of a drug in a One- (a) and Two- (b) compartment model.
Modified from Scientific American Medicine.

compartment. The concentration differences between tissue, plasma and organ are due to the *partition coefficient* between the regions. Toxicokinetics based on blood analysis gives the investigator a way to predict future levels of the agent in the blood of the victim, but not in the target organs, unless the *partition coefficient* is known for all metabolites.

When elimination is from a two-compartment model (F4.3b), the toxicant rises instantly to its maximum value in plasma,

since it is injected. After reaching its peak in the blood (time 0), it is rapidly equilibrated between plasma and central compartment (blue asterisk).
After the peak concentration in the central compartment, the central and plasma variables are essentially the same, except for the influence of the *partition coefficient*.
Tissues, however, equilibrate more slowly, reaching a maximum later (red asterisk). After the tissue peak (red asterisk), all rates of decay are parallel. Before the red asterisk is the absorption and distribution phase, or α phase, and after the red asterisk is the elimination phase, or β phase.
The half-life of the drug changes during the α phase and is constant during the β phase.
The α phase may last for a few minutes, or for days or longer, depending on the speed of exchange of toxicant between plasma-central compartments and tissues.
Of interest to pharmacologists is whether the central concentration of a drug reaches a high enough value for a long enough period so that it is *efficant*.

Elimination and some aspects of **biotransformation** can readily be measured by sampling urine, feces, exhaled air and carrying out chemical analyses for the agent and its biotransformation products. **Storage** cannot be measured conveniently, requiring invasive and frequently unpleasant techniques to secure appropriate samples.
In the case of heavy metals or halogenated hydrocarbons such a polychlorinated biphenyls (PCBs), the amount circulating in the bloodstream at any one time may represent only a small fraction of the body burden. 95% of lead body burden is stored in bone.

4.3. Elimination

Some biological fluids are available for sampling without using invasive techniques: exhaled air, urine, feces[5]. The data points on the right of curve of F4.3b correspond to pure elimination of the agent from the body.
The long-term excretion of a compound localized in a body storage site (adipose tissue, bone) is generally a slow process of equilibrium between the depot and the blood. Once the agent is in the blood (central compartment), metabolism and excretion (urinary, fecal, exhaled air) often occur, no elimination generally occurring from the peripheral compartment.
Examples of slowly eliminated compounds are PCBs, DDT and lead. When elimination of toxicants is very slow, elimination (particularly of heavy metals) can sometimes be improved by the administration of chelators: for example. the slow elimination of lead can be accelerated by EDTA chelation (T4.4).

T4.4. Chelators of Heavy Metals

Metal	Chelator
Arsenic	Meso, 2,3-dimercapto-succinic acid (DMSA) 2,3-dimercapto-1-propanesulfonic acid (DMPS) Dimercaprol (BAL) D-penicillamine (PCN) N-acetly-L-cysteine (NAC)
Cobalt	Ethylenediamine-tetra-acetic acid (EDTA)
Copper	DMSA, DMPS, PCN, Trientine dihydrochloride (Trien)
Iron	Deferoxamine (DFO), Deferiprone (Li)
Lead	DMSA, EDTA, BAL, PCN
Mercury	DMPS, DMSA, BAL, PCN

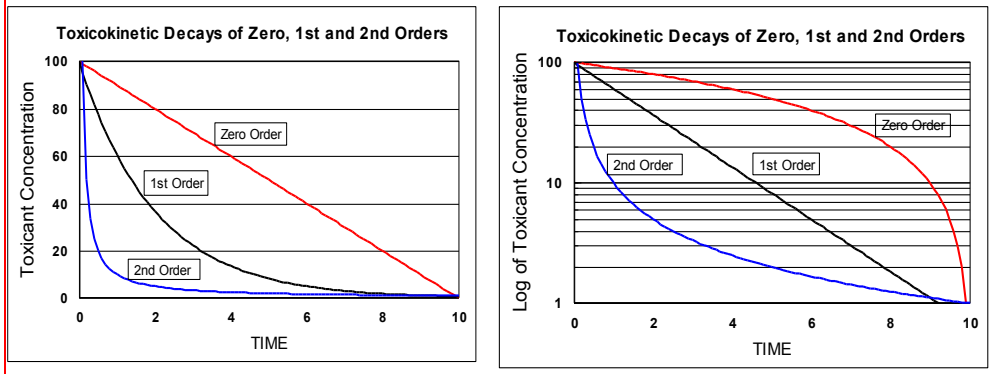
Another prominent chelator is DMSA. 20% of orally administered DMSA (10 mg per kg every eight hours for five

days) is absorbed from the gastrointestinal tract. One of the sulfhydryls in DMSA binds to a cysteine molecule on albumin, leaving the other S-H to chelate metals. DMSA produces the best urinary excretion of mercury, greatest in the first eight to 24 hours after ingestion, and is effective at removing mercury from the blood, liver, brain, spleen, lungs, large intestine, skeletal muscle and bone. DMSA has removed two-thirds of the brain mercury deposits in animal studies. Quantitatively, elimination of the toxicant may proceed by various methods:

(0) a fixed weight of the toxicant per unit time. This is "zero-order" or *saturation kinetics,* because the exponent of Toxicant concentration = 0,

(1) a fixed percentage of the central compartment content per unit time, or so-called "first order",

(2) or the elimination can be even higher order (concentration squared is pictured in F4.5), reflecting facilitated elimination at higher toxicant concentrations.



F4.5. Zero-order and First-order decays are most frequent in toxicity studies, First-order usually succeeding Zero-order. By converting concentrations (µg/ml) into logarithmic values, the curves at right are obtained from the curves at left. In the log graph at right, Zero-order decay is curvilinear, and First-order decay linear.

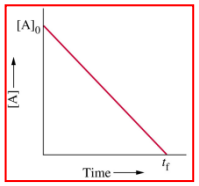
4.3.1. Mathematical forms of Elimination

(k_x are the Elimination Rate constants; [ ] denotes a concentration)

Elimin. rate = k_0 [Toxicant]^0 + k_1 [Toxicant]^1 + k_2 [Toxicant]^2+... (mg/hour)

Let us consider 3 particular cases of elimination…

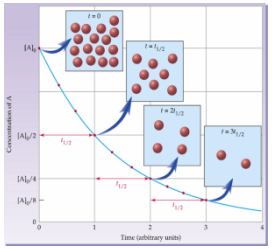
ZERO-ORDER: If k_1 and k_2 = 0,



Elimination rate = k_0 (mg/hour)

The elimination per unit time is then simply a constant equal to k_0, that is a fixed amount of compound is eliminated for each unit of time. Elimination is independent of toxicant concentration, since the exponent of [Tox] is 0.

FIRST-ORDER: If k_0 and k_2 = 0,

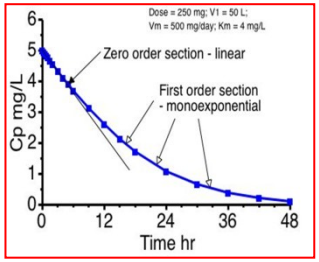


Elimination rate = k_1 [Toxicant] (mg/hour)

The elimination rate is proportional to the first power of toxicant concentration, ie [Tox]^1. The concentration of toxicant is proportional to the elimination rate.

ZERO and FIRST-ORDER: If k_2 and higher terms = 0,

Elimination rate = k_0 + k_1 [Toxicant] (mg/hour)



Many processes are first detected as "zero-order", and then morph into "first-order" as time passes and toxicant concentration decreases (F4.8).

4.3.2. The Mathematics of First-Order Elimination

If the rate of elimination of a toxicant from the body is directly proportional to its concentration, this is expressed mathematically as the "linear first-order homogenous differential equation" shown below.
The derivative (d) of [Tox] with respect to time (dt) is equal to a constant (*k)* times the concentration itself [*Tox*].

$$\frac{d[Tox]}{dt} = -k\ [Tox].$$

"k" determines the speed of elimination (relative change in toxicant concentration per unit time and concentration), the minus sign in front of it signals a decrease in concentration with time. By moving factors in the equation above and integrating, we obtain

$$\int \frac{d[Tox]}{[Tox]} = \int -k\ dt, \text{ or } \quad \ln [Tox_t] - \ln [Tox_0] = - k\ t.$$

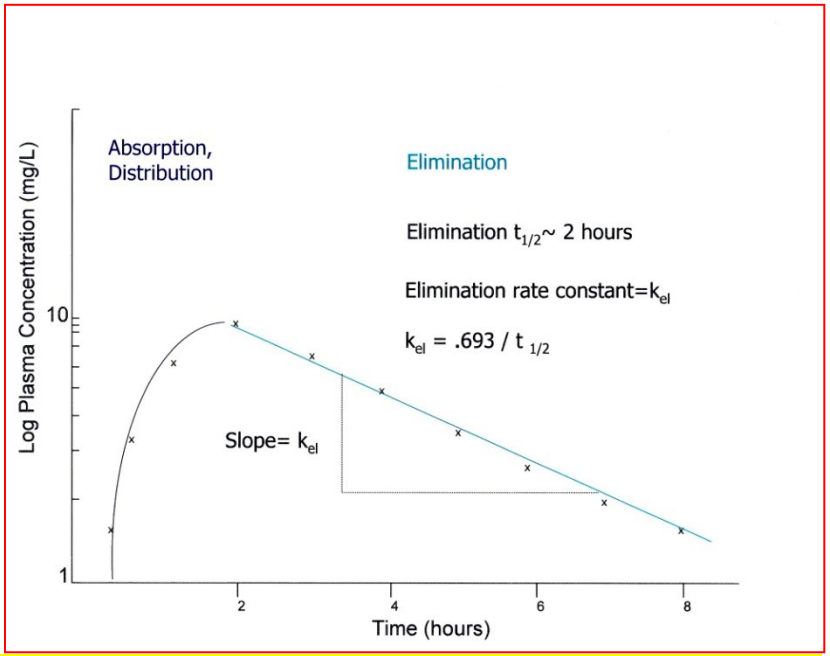
The equation above allows us to find the concentration of the toxicant at any time, [Tox $_t$], if we know what the concentration was at t = 0, [Tox $_0$].

If we prefer to use *common* (base 10, log), rather than *natural* (base e, ln) logarithms, the equation transforms as

$$\log_{10} [Tox_t] - \log_{10} [Tox_0] = - k\ t / 2.303$$

$$\frac{\log_{10}[Tox_t] - \log_{10}[Tox_0]}{t} = -\frac{k}{2.303}$$

The expression above indicates that if we plot the <u>log</u> of toxicant concentration, then the fall in concentration between time 0 and time t divided by the elapsed time t is equal to a <u>constant</u>.



F4.6. Half-life [$t_{1/2}$ or $\beta_{1/2}$] determination in the β-phase.

Graphically, this means that the slope of the curve (term at left) is negative (decreasing values of concentration) and is a constant (k/2.303), yielding a straight line, such as at the right of F4.6.

4.3.3. Half-Life Determination

Half-Life can be evaluated *only* during First-order elimination or beta (β)-phase, as shown in F4.6.
A value, known as the ß-*half-life* ($ß_{t/2}$) can be found from the slope of the straight blue segment. The $ß_{t/2}$ is the *time required to deplete the compartment (usually the blood) of 50 percent of the agent*.

If we assume that the concentration [Tox] is reduced in half at the half-life, the following equation holds:

$$\frac{\log_{10}[\frac{Tox_0}{2}] - \log_{10}[Tox_0]}{\beta_{½}} = -\frac{k}{2.303}, \text{ or}$$

$$\beta_{½} = -\frac{2.303\ \left(\log_{10}[\frac{Tox_0}{2}] - \log_{10}[Tox_0]\right)}{k}, \text{ or}$$

$$\beta_{½} = -\frac{2.303\ (-\log_{10} 2)}{k}, \text{ or}$$

$$\beta_{½} = \frac{0.693}{k}.$$

This equation relates the observed half-life to the elimination constant, k.
In practice, using experimental data, the half-life of a compound can be found from any two points ("1" and "2") which are part of a straight line on a log-linear curve.

$$Slope\ on\ \log-linear\ graph = \frac{\log[Tox_2] - \log[Tox_1]}{t_2 - t_1} \quad (1)$$

$[Tox_1]$ and $[Tox_2]$ represent blood concentrations and t_1, t_2 the times at which these concentrations were observed.

Applying the above formula to the specific time interval t_2 which follows t_1 by one half life $[\beta_{½}]$ and reduces $[Tox_1]$ to half its value:

$$Slope\ over\ one\ half-life = \frac{\log\left[\frac{Tox_1}{2}\right] - \log[Tox_1]}{t_1 + \beta_{½} - t_1} = \frac{-\log\ 2}{\beta_{½}} \quad (2)$$

To find the numerical value of $\beta_{½}$, we need to equal expression (1) (with points 1 and 2 chosen on the straight segment on the curve) to the rightmost part of expression (2), as shown below:

$$\beta_{½} = \frac{-\log\ 2\ \times(t_2 - t_1)}{\log[Tox_2] - \log[Tox_1]} \quad \text{or}$$

$$\beta_{½} = \frac{-0.301\ \times(t_2 - t_1)}{\log[Tox_2] - \log[Tox_1]}.$$

Log-linear tracings are indispensable for easy visualization of elimination rates. Such tracings are obtained through plotting software or, if manual plotting is preferred, log-linear paper. Such paper can be generated through a printer by software (Graph Paper Printer 4.21 for Windows, DVD Drive:\Graph Paper Program) or by using the sheets supplied at the end of this text.

The formalism above should not obscure the fact that half-lives can usually be found easily by simple curve inspection on a log-linear graph, or by simply inspecting the values of a table...

Follow these steps:

1. Delimit a straight segment on the log-linear curve.
2. On this segment, find two concentrations that are a factor of 2 apart (ie, 10 and 5).
3. The time interval between these two concentrations is the half-life.

This simple method should be used to verify calculations...
Since many compounds can be stored effectively in relatively inaccessible body compartments with an exceedingly slow

release of the agent, half-lives in humans can be years.
For example the PCBs stored in our fat have elimination times that increase as the PCB concentrations decreases. Even if our exposure stopped tomorrow, we would all still die with a measurable body burden of PCBs.

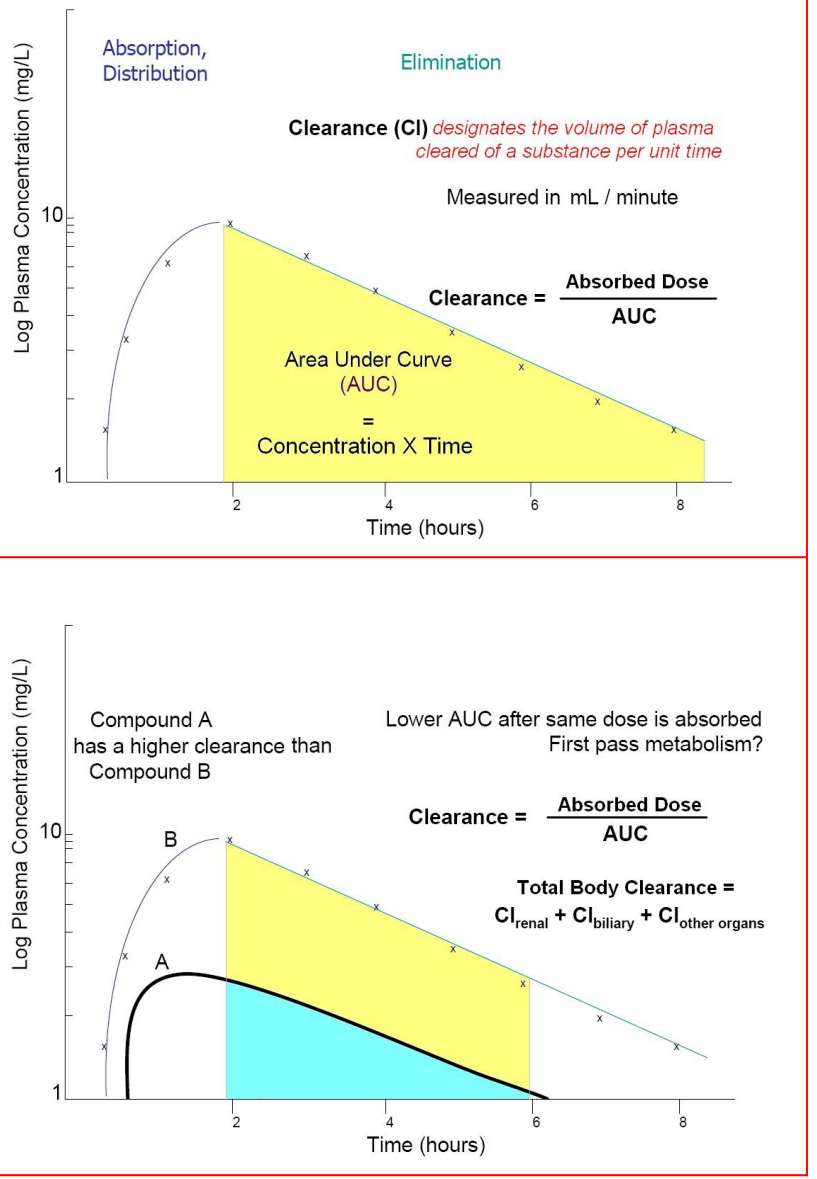
4.3.4. Clearance

A good example of a first-order elimination concept often used in medicine is **clearance**. Clearance in ml/min is the volume of fluid that is totally cleared of the toxicant per minute.
If a chemical is not reabsorbed at all by the kidney, the **clearance is equal to the glomerular filtration rate**.
This is true of inulin and mannitol, used as test substances for glomerular filtration rate determinations.
If the substance is actively secreted, the clearance can be greater than the glomerular filtration rate.
A convenience of *clearance* is that it is additive between organs such as kidneys, liver, intestine, etc…
Clearance is of critical importance in recognizing kidney diseases in the clinic.
F4.7 (lower) shows how two compounds administered in the same amount can have their clearance determined by continuous measurement of the plasma concentration of the substance.

4.4. Analysis of Toxicokinetic Curves

4.4.1. Summary Characteristics of Zero-Order Elimination

1. Rate of elimination is a constant, independent of the amount of toxicant present in the body.
2. Quantity of Toxicant in the tissues decreases by a constant amount per unit time (mg/hour).
3. *Linear plot* of Toxicant concentration yields a straight line.



F4.7. Practical determination of clearance.

4. Toxicant half-life concept is inapplicable.

4.4.2. Summary Characteristics of First-Order Elimination

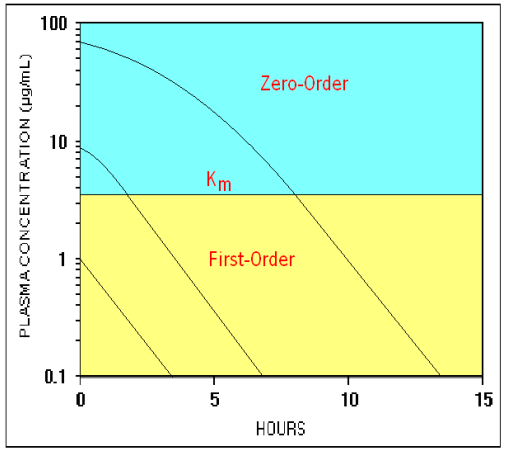
1. Rate of elimination is proportional to the amount of toxicant present in the body.
2. Quantity of Toxicant in the tissues decreases by a constant fraction per unit time (% / hour).
3. *Semi-log plot* of Toxicant concentration yields a straight line.
4. Toxicant half-life is independent of the dose.

An elimination process limited by bio-synthesis (such as Phase II biotransformation), may be **0-order** if the conjugation product is produced by the body at a fixed rate. The liver has a limited ability to synthesize the needed molecules, and excretion can only proceed after conjugation.
Often, a *zero-order elimination reflects a limit in liver bio-synthesis or in the kidney's active secretion capability.*

However, if a process is limited by *diffusion*, one might expect **first-order** kinetics, since a higher concentration gradient of the agent will result in more toxicant transfer and in more toxicant being processed, assuming no other limits.

Elimination in the kidney is typical of such a process. The more toxicant enters the glomerulus, the more will be carried away into the urine, assuming no further transport of the agent across membranes.
Therefore, *a first order elimination often reflects a process of elimination governed by passive elimination by the kidneys.*

Many elimination curves are first "zero-order" and then morph into "first-order", as time passes and toxicant concentrations decrease.
This is explained as follows. In the initial stages of high level intoxications, stored reserves of conjugation products are used very rapidly, thereafter leaving the elimination process to work only with conjugation products that must be synthesized *de novo*. This limits the speed of elimination to a zero-order process. As concentrations diminish, conjugation products again become plentiful and diffusion, rather than conjugation product availability, becomes limiting.



F4.8. Zero to first order kinetics.

The transition from zero-order to first-order occurs at the *Michaelis-Menten constant concentration (K_M)*, which is defined as *the toxicant concentration at which the velocity of an enzyme reaction is half the maximal velocity*.

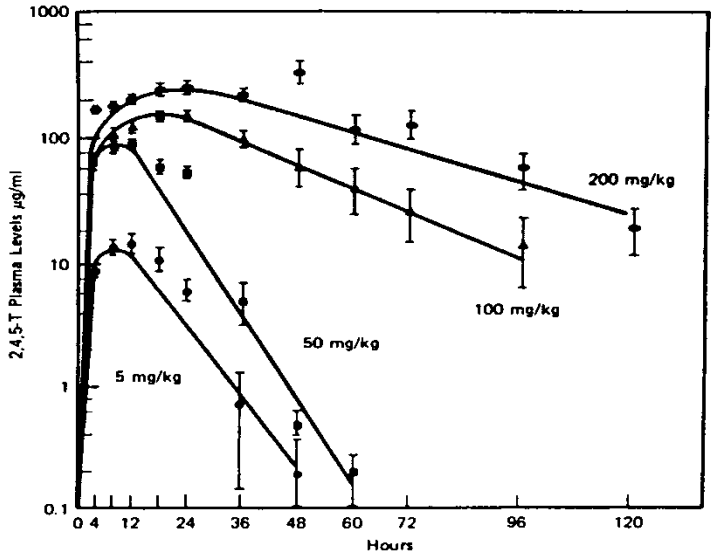
- When the concentration of a chemical in the body is **higher than K_M**, the rate of elimination is no longer proportional to concentration (**Zero-Order**).
- When the concentration of a chemical in the body is **smaller than K_M**, the rate of elimination is proportional to concentration (**First-Order**).

4.5. Toxicodynamics: drifting Elimination with Dose

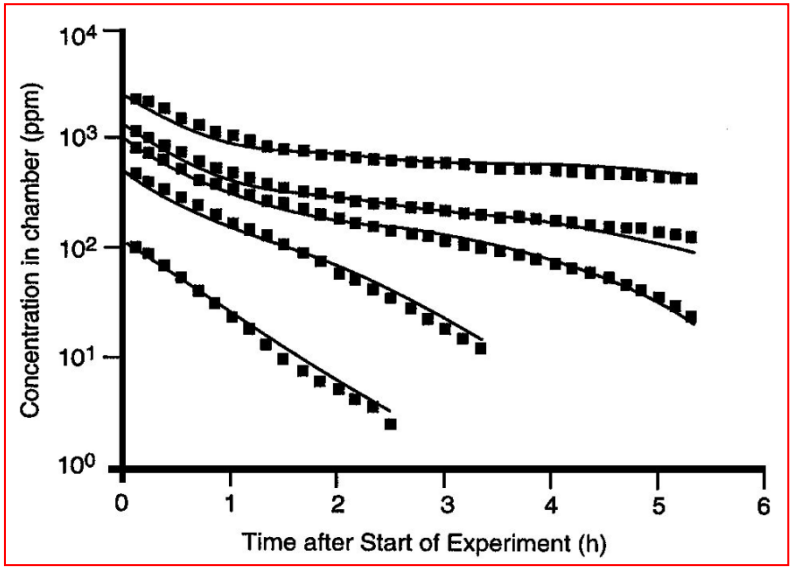
A further complexity is introduced in the process of elimination by the fact that the *elimination rate* in the beta phase can change with the dose administered.

The same agent can display different half-lives at various doses, as shown below for the herbicide, 2,4,5-T.
2,4,5-T is a highly polar organic anion that does not undergo extensive storage, is not bio-transformed in the body, but is eliminated via the kidney by both glomerular filtration (the major route) and active secretion through the cells in the proximal tubule of the nephron.
Essentially, the straight curve segments show urine excretion, with only one parameter of the ß-phase (elimination) in operation.
The fact that at a given concentration in the blood, the half-life of the chemical can show different values (slopes in F4.9) indicates that the elimination process is altered by the dose administered (*Toxicodynamics*). Therefore, the *elimination performance of the organism was altered by the drug*.

A particularly ingenious experiments is shown in F4.10 below, where 3 rats are left to metabolize chloroform ($CHCl_3$) inside a closed cavity. The "biomonitoring" is achieved by simply measuring the concentration of chloroform in the atmosphere of the cavity. As the dose of chloroform increases in various separate experiments, we can see the transition from First-Order to Zero order elimination, reflecting a likely saturation of Phase II synthesis.



F4.9. Plasma 2,4,5-T of rats following various single oral doses.
Basis of Toxicity Testing, 1997.



F4.10. Disappearance of chloroform from a chamber containing three rats.

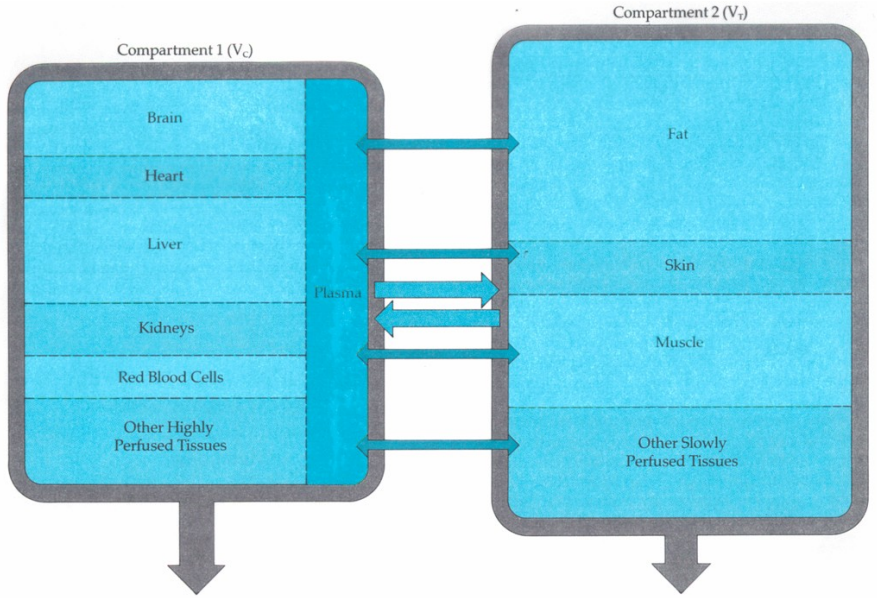
4.6. Multiple Compartments

A **one** compartment model is typical of the case when blood and body organs reach equilibrium rapidly between them for toxicant concentration: a single compartment is therefore representative of the whole body, and excretion proceeds from this single compartment.
In many cases (such a lead, or any lipophilic chemical), a single compartment is not enough, and one must resort to two or multiple compartments, with elimination proceeding (in most implementations) from a central compartment that is dynamically connected for toxicant exchange with other compartments, as shown in F4.11.

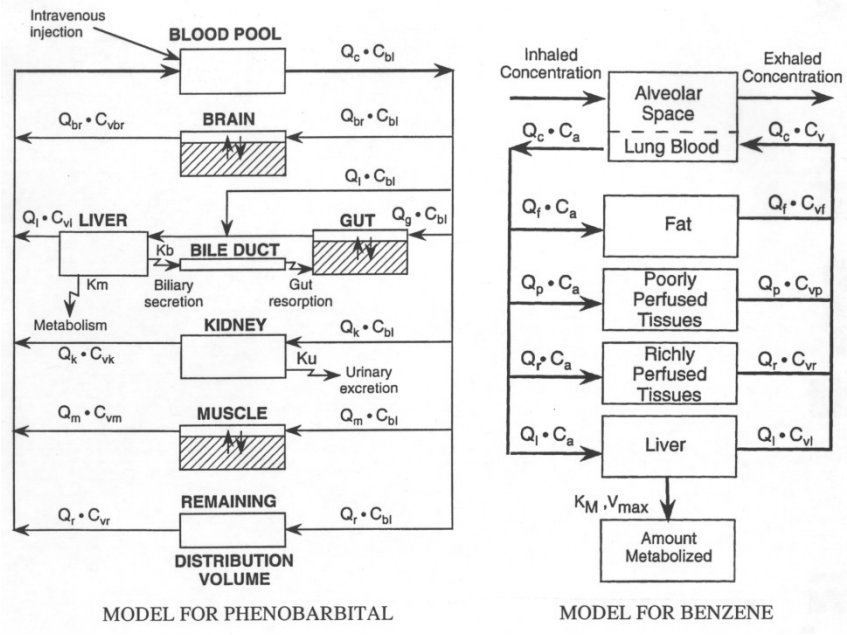
Toxicokinetic computerized models can become very complex with a multitude of compartments, as shown in F4.12.
But models of such complexity usually have limited usefulness "in the field"[1].

Calcium illustrates the importance of compartments. Living cells maintain an intracellular environment that is markedly different from the surroundings. Sodium chloride, abundant outside the cell, is poisonous inside the cell. *But few things are as poisonous as calcium:* molecule for molecule, it is many times more poisonous than cyanide. Both sodium and calcium are therefore vigorously pumped out of the cell. To eliminate calcium, many cells render it insoluble by combining it with carbon dioxide to form extracellular calcium carbonate. This can be discarded or used as armour, or various forms of structural support (i.e. a skeleton).

Many exposures are of the repeated (hourly, daily, weekly) or extended (years) variety, the level at any one time being insufficient to cause any overt toxicity. Successive exposures add to the body burden, with adverse effects perhaps appearing in the future.



F4.11. Two compartments, two elimination routes. Vc = Central Compartment (highly perfused, equilibrating within a few minutes), Vt = Tissue Compartment. Horizontal arrows are individual rate constants between plasma and tissues. Tissues in compartment 2 reach equilibrium with plasma at different rates, but these are sufficiently similar to be modeled by a single constant (pair of larger horizontal arrows). The surface of the tissues in this representation corresponds to the amount of drug contained in this tissue. Vertical arrows show elimination from both compartments. *Scientific American Medicine.*



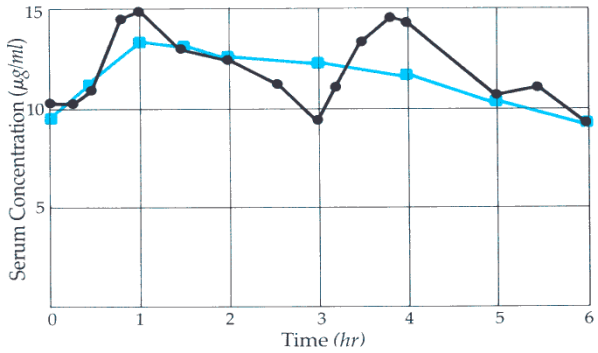
F4.12. Complex models for Phenobarbital and Benzene toxicity. Qs are blood flows (li/hr), Cs are concentrations (mg/li), Ks are elimination constants. Compartments that are diffusion-limited are separated in two, reflecting the fact that blood-borne movement of the toxicant is much faster than membrane crossing. *Casarett & Doull.*

4.7. Multiple Exposures

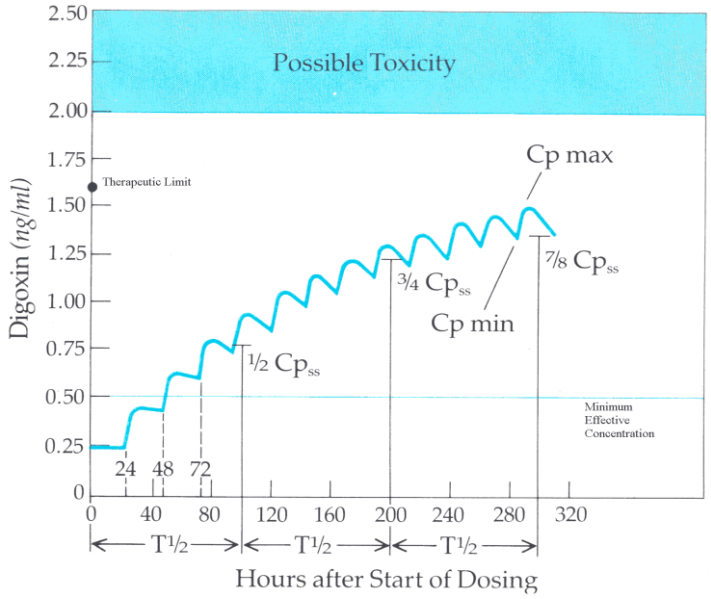
4.7.1. Repeated Exposure

Depending on the time interval between exposures, the subject may have had sufficient time to eliminate the earlier dose, but in many cases, there is a cumulation of the doses, leading to a climbing *body burden*.

As the concentration approaches the critical limit in the target locations, signs and symptoms of intoxication will appear in subjects. The signs and symptoms may reduce when people are protected from exposure, such as during the weekend.



F4.13. Repeated exposure. The black curve represents two administrations of the standard form of a drug, while the blue represents the slow-release form. Note the linear vertical axis. *Scientific American Medicine.*

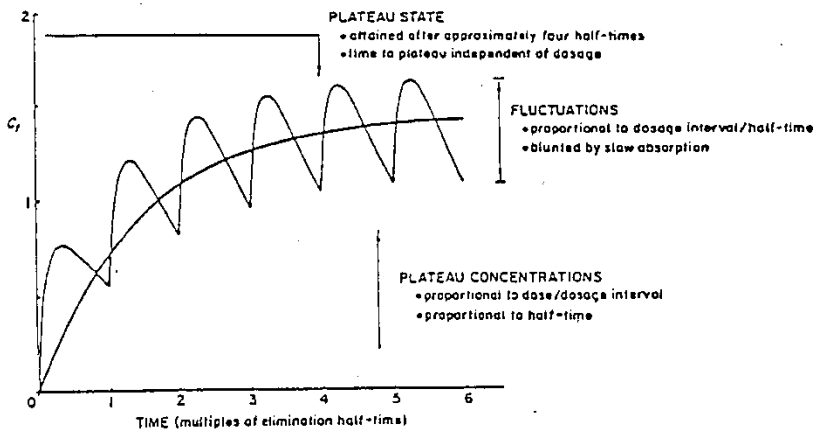


F4.14. Accumulation of body burden in the therapeutic administration of digoxin. A 0.125 mg digoxin tablet is administered daily to a 65-year old woman, 70 kg, treated for atrial flutter-fibrillation. Bioavailability is 0.65. Estimated half-life based on renal function is 100 hrs, and clearance is 2.1 liters/hr. Note the linear vertical axis. *Scientific American Medicine.*

4.7.1.1. Plateau

If one systematically exposed a subject to successive doses of an agent, retaining the assumptions that the β-half-life remains unchanged, the rising plasma concentrations would allow the subject to excrete more and more of the drug, until his excretion balances his intake.
After a certain number of exposures, we approach a "plateau", the blood concentration oscillating up and down during and after exposure but, within limits, remaining relatively constant.

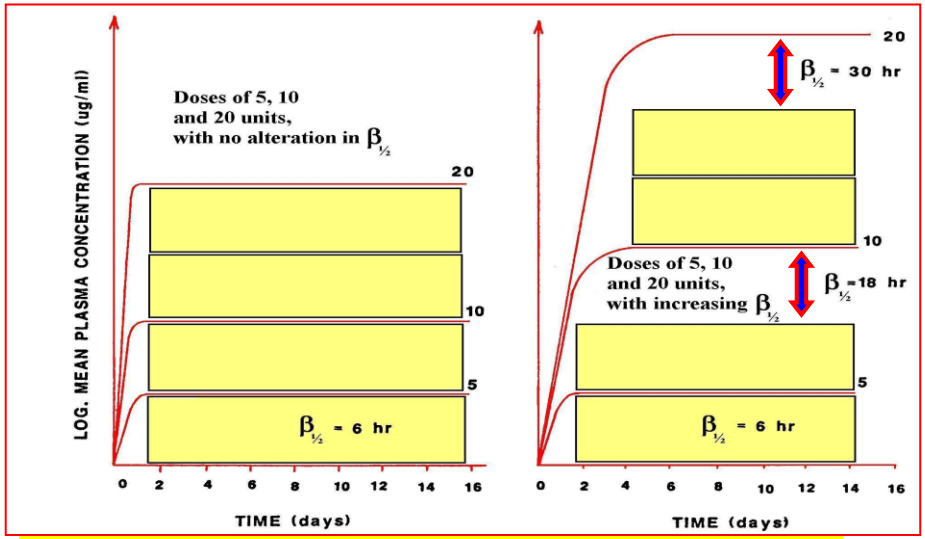
An exposure scenario familiar to physicians who want to obtain high therapeutic blood levels of an agent early is to give an initial large dose (bolus) followed by lower, *maintenance* dosage.



F4.15. Factors influencing blood concentrations under repeated identical exposures to toxicants. Assumes that the β½ does not alter with increasing concentration of agent in the body. *Basis of Toxicity Testing, 1997.*

The TIME TO ATTAIN A PLATEAU in the blood concentration is **determined as four to five half-lives of the agent** (see F4.15). The half-life ($\beta_{½}$) is inversely proportional to the **rate of elimination,** but is normally independent of the level of the exposure.

The CONCENTRATION AT PLATEAU is **dependent on the level of the exposure, the exposure interval and the $\beta_{½}$** (F4.15).



F4.16. Repeated exposures lead to plateaus of toxicant concentrations which are influenced by altered β½.

Example: We consume some 50 micrograms of Cadmium in our food each day (ubiquitous in its environmental distribution). Cadmium has a biological half-life (not blood half-life) of approximately 7 years. If one determines body burdens of Cd, there is considerable variability until one reaches 35 years of age, at which time, if there is no additional

source of exposure (industrial), the body burden becomes relatively constant.

An illustration of the influence of $\beta_{½}$ in repeated exposures is shown in F4.16, where the time needed to attain maximum concentration in the blood, as well as the plateau reached are altered.

In F4.16 *left*, the organism reacts similarly in excretion performance, irrespective of the dose. Three different continuous exposures with a $\beta_{½}$ of 6 hours all attain their plateau at 24 hours, and the blood level attained at 20 exposure "units" is 4-fold higher than that at 5 "units".

In F4.16 *right*, the organism excretes less effectively with increasing dose. As a result, $\beta_{½}$ increases (6, 18 and 30 hr) with the exposure level (5, 10, 20 units), and the time to attain the plateau shifts from 24 hr to 72 hr to 120 hr.
The stable blood concentrations attained are not "proportional" to dose (the gap is indicated by the blue-red arrows).

In workplace hygiene, toxicokinetic calculations may occasionally adjust the exposure standard (uniformly set for 8 hours) if unusual work schedules are involved. Calculation can be performed based on the number of hours worked each day, the number of hours worked each week, and the half-life of the agent. Adjustments are not required for agents with a half-life less than 3 hours (Short-Term Exposure and Ceiling Limits take over) or exceeding 400 hours[7].

4.7.2. Simultaneous Exposures: N-in-1

Pharmaceutical companies looking for drugs with desired pharmacokinetic parameters in animal models such as the rat, the dog and even some *in vitro* models (Caco-2 cell model) invest a lot of money obtaining concentration vs time profiles.

The *N-in-1* technique was introduced to reduce testing costs. It involves the oral or intravenous *co-administration of multiple drug candidates into a single model* and their separate analysis over time using gas chromatography and high performance liquid chromatography.

The *N-in-1* strategy is capable of significantly speeding determinations, using a fraction (1/N) of the animal resources. Data show pharmacodynamic profiles similar to those obtained for compounds in individual dosing.
Of course, compounds that can be converted to one another should not form part of a simultaneous pool. Even though there are some risks associated with the *N-in-1* method, they can be minimized by careful selection of the *N-in-1* pool.

4.8. Toxicokinetics Examples

4.8.1. Doping

The elimination of drugs from the blood and urine is a central variable in the evaluation of doping. In the table below, Drug Detection Times are expressed in terms of lower and upper boundaries. Many factors determine an individual's placement within these boundaries. In general, the following factors will increase a drug's detection time: chronic use, high levels of use, high drug potency, high urine pH, slow body metabolism, high body fat count (for Marijuana and PCP), low fluid intake, and overall poor health.

In the case of hexacarbon neuropathy, a certain body burden of n-hexane is required to produce the characteristic central and peripheral distal axonopathy, and the neuronal damage is advanced before the diagnosis can be confirmed.
Removal of the individual from the exposure site frequently results not in a lessening of the signs and symptoms, but in a worsening of the condition, much to the horror of the attending physician, who now doubts the accuracy of his diagnosis.
What should be appreciated is that it is not n-hexane that is toxic, but a biotransformation product. The body burden of the agent must be eliminated via this metabolic route, and so toxicity remains for about two weeks, during which the symptoms may remain stable or become more pronounced. They only begin to subside later. Depending upon the severity of the neurological damage, the patient may recover completely, make a partial recovery or remain permanently incapacitated.

T4.17. Drug Detection Times from *Drug Test Success*	
Alcohol	6 hours to 1 day
Amphetamines	1 to 4 days
Barbiturates	Short-acting (Allobarbital, Alphenal, Amobarbital, Aprobarbital, Butabarbital, Butalbital, Butethal, Pentobarbital, Secobarbital): 1 to 4 days
	Long-acting (Barbital, Phenobarbital): 2 to 3 weeks
Benzodiazepines	Short-term Therapeutic Use: 3 days
	Long-term Chronic Use: 4 to 6 weeks
Cocaine	2 to 5 days
LSD	1 to 4 days
Marijuana	Casual Use: up to 7 days
	Chronic Use: up to 30 days or longer
	Note: THC, Marijuana's primary psychoactive ingredient, is stored by the body in fatty lipid tissue. From there, it is slowly released into the bloodstream and urine for up to several weeks. Chronic users and individuals with high body fat count are at the greatest risk of long-term detection.
MDMA (Ecstasy)	1 to 4 days
Mescaline	1 to 4 days
Methadone	1 to 7 days
Methamphetamines	1 to 4 days
Methaqualone	up to 14 days
Nicotine	1 to 2 days
Opiates	1 to 4 days
PCP (Phencyclidine)	Casual Use: up to 7 days
	Chronic Use: up to 30 days Note: PCP is stored by the body in fatty lipid tissue. From there, it is slowly released into the bloodstream and urine for up to several weeks. Chronic users and individuals with high body fat count are at the greatest risk of long-term detection.
Propoxyphene	1 to 7 days
Psilocybin (Mushrooms)	1 to 3 days
Steroids (Anabolic)	Oral: 2 to 3 weeks
	Injected: 1 to 3 months
	Naldrolene: up to 9 months
Tricyclic Antidepressants	1 to 9 days

4.8.2. Examples of Clinical Toxicokinetics

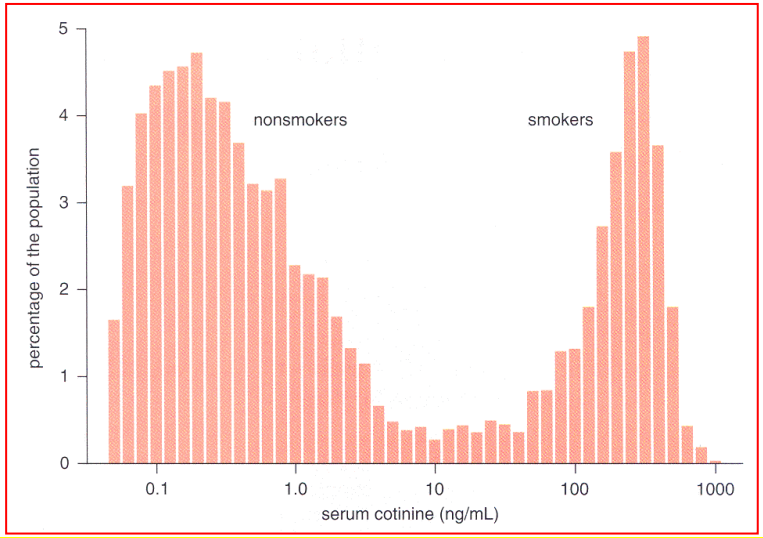
4.8.2.1. Toxicokinetics in the Blood and Urine

Toxicants or their metabolites can be sampled from body gases, body fluids or body tissues. Toxicants in exhaled gases are often detectable only for relatively short periods after intoxication, as is the case for alcohol. Therefore, one may need to be present on the spot for useful sampling. Because of this, and because of the discomfort of obtaining most body tissues (with the exception of nails and hair), sampling is often done using accessible body fluids, specifically urine and blood. Sensitive and specific biomarkers are available for metals, dioxins, furans, PCBs, pesticides, volatile organic compounds, phthalates, phytoestrogens and tobacco smoke.

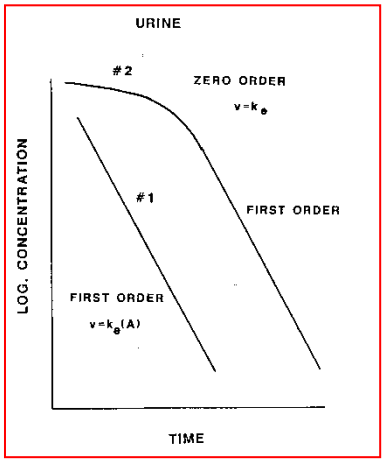
What classes of toxicants can we expect to find in either of them?

Dioxins, PCBs and organochloride pesticides reside for months or years in the body, either because they are metabolized slowly or because they are hidden in lipophilic (fatty) compartments. For example, the half-life of TCDD is 2–4 wks in rodents, while it is approximately 10 yrs humans. These properties tend to lower excretion rates.

Organophosphate pesticides, alcohol and volatile organic compounds, on the other hand, reside in the body only for hours or days, because they are metabolized quickly. They may be favorite targets of enzymes, or enzymes may have good access to them because they are not hidden in lipophilic compartments. This tends to heighten excretion rates.



F4.18. Exposure to tobacco smoke is measured as blood (serum) cotinine in this histogram. It clearly discriminates between smokers and non-smokers. *American Scientist, 2004*



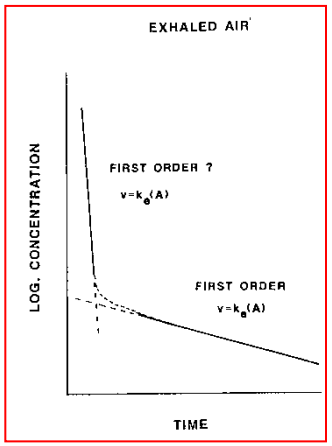
F4.19. Kinetics in urine.
Basis of Toxicity Testing, 1997.

Therefore, *persistent chemicals are often measured in blood*, while *transient ones are generally measured in urine*. Bio-transformation pattern is also a factor, some compounds (benzene) forming urinary metabolites, while others do not (dioxin).

F4.19 shows typical curves for urine. In #1 (lower exposure), a first-order linear relationship, where excretion half-life is dependent on an elimination constant (k_{elim}), not on the concentration of the chemical. In #2

(elevated exposure), the initial part of the relationship is curvilinear, but becomes linear with time. The curved portion represents saturation kinetics or a zero-order reaction (excretion of a certain weight per unit time). Progressively, as the concentration of agent diminishes, the mechanisms in the renal nephron (active proximal tubular secretion) is no longer saturated, and the elimination becomes first order (k[Tox]).

4.8.2.2. Toxicokinetics in the Lung



F4.20. Kinetics in the lung.
Basis of Toxicity Testing, 1997.

In the case of breathed volatiles[3,4], the lung is usually a relatively efficient route of elimination. Loss of the agent will frequently be observed in the blood as an initial very steep, first-order reaction (F4.20). We are looking at an initial excretion of circulating volatile chemical, plus any loosely "compartmentalized" agent (easily equilibrated back into the blood), followed by a slow excretion of compartmentalized (adipose tissue, etc.) chemical. Examples would include such anesthetics as halothane and isoflurane, where initial post-surgical recovery is rapid, but the patient may continue to exhale low levels of the agent for hours afterward.

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Hemato- and Vascular Toxicity

5. Hemato- and Vascular Toxicity

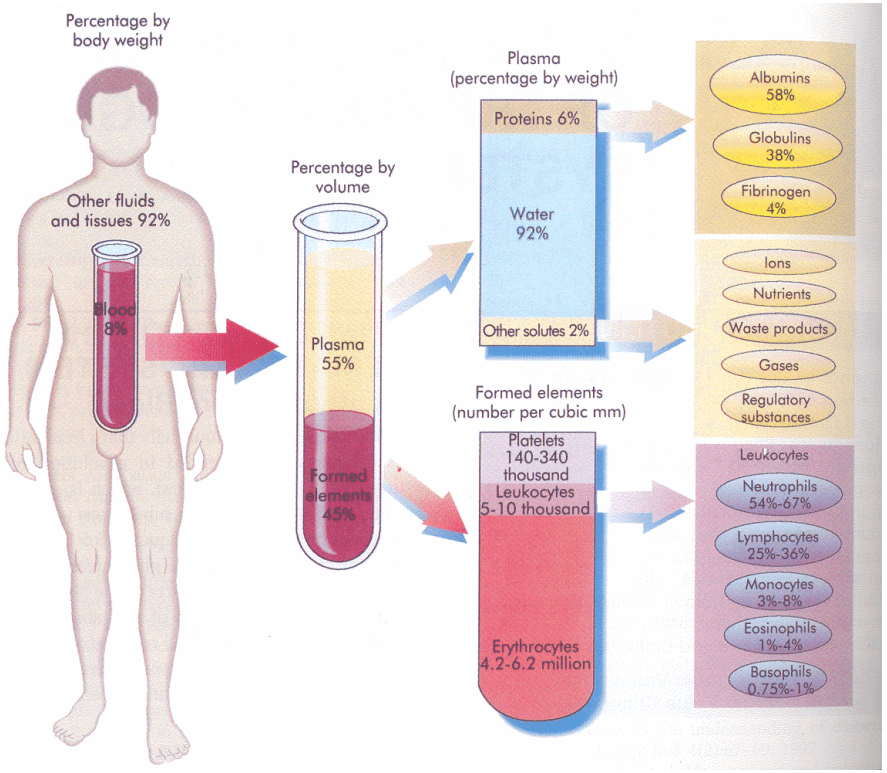
5.1. Blood

F5.1. THE COMPOSITION OF BLOOD

PLASMA (55 PERCENT)

CELLS (45 PERCENT)

CONSTITUENT	MAJOR FUNCTION
WATER	SOLVENT FOR CARRYING OTHER SUBSTANCES
SALTS SODIUM, POTASSIUM, CALCIUM, MAGNESIUM, CHLORIDE, BICARBONATE	OSMOTIC BALANCE, pH BUFFERING, REGULATION OF MEMBRANE PERMEABILITY
PLASMA PROTEINS ALBUMIN FIBRINOGEN IMMUNOGLOBULINS	OSMOTIC BALANCE, pH BUFFERING CLOTTING DEFENSE (ANTIBODIES)
CELL TYPE	**CELL FUNCTION**
ERYTHROCYTES (RED BLOOD CELLS) 5 TO 6 MILLION PER CUBIC MILLIMETER OF BLOOD	TRANSPORT OXYGEN AND HELP TO TRANSPORT CARBON DIOXIDE
LEUKOCYTES (WHITE BLOOD CELLS) 5,000 TO 10,000 PER CUBIC MILLIMETER OF BLOOD BASOPHIL NEUTROPHIL EOSINOPHIL MONOCYTE LYMPHOCYTE	PRODUCTION OF ANTIBODIES FOR DEFENSE AGAINST INFECTION
PLATELETS 250,000 TO 400,000 PER CUBIC MILLIMETER OF BLOOD	BLOOD CLOTTING
SUBSTANCES TRANSPORTED BY BLOOD	
NUTRIENTS (FOR EXAMPLE, GLUCOSE, FATTY ACIDS, VITAMINS), WASTE PRODUCTS OF METABOLISM, RESPIRATORY GASES (OXYGEN AND CARBON DIOXIDE), HORMONES	



Insects carry oxygen in their body using *diffusion* through internal air pipes. However, there is a critical insect size above which diffusion is not effective enough.

In crabs and some fish, the blood contains a free solution of *hemocyanin*, which binds oxygen using copper. It turns blue when oxygenated, and has only 25 % of the binding ability of mammalian hemoglobin, the molecule sequestered inside red blood cells for the purpose of binding oxygen. Horseshoe crab blood coagulates instantly when contacting *E. coli* and *Salmonella*, providing a useful diagnostic test.

There is only one vertebrate known to lack hemoglobin and red blood cells: the blackfin icefish, *Chaenocephalus aceratus*. The extremely high oxygen content of the Southern Ocean allowed it to survive the loss of globin genes, and to rely on oxygen dissolved in the plasma, a loss of 90 % in oxygen-carrying capacity. The fish has a much larger heart, blood vessels and volume of blood, and far denser capillary beds.

In mammals, an extensive capillary network supplies tissues with oxygen and removes carbon dioxide throughout the body. The difficulty of *diffusion* of oxygen and other nutrients through tissues limits the gap between adjacent capillaries to only 4 cell thicknesses.
Mammal-type circulation in an animal smaller than a shrew would be inefficient because of blood flow friction in the tiny capillaries.

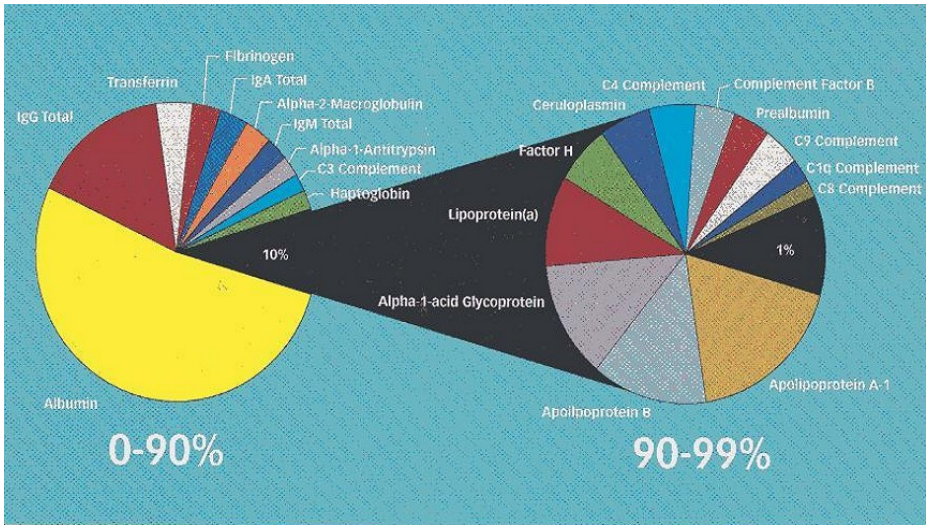
5.1.1. Plasma Protein

Exogenous agents such as oxygen, once into the bloodstream, may bind more or less loosely to **plasma proteins** (F5.2), which affect distribution to the blood's cellular components and to perfused organs. Plasma protein can have a substantial role in sequestering toxicants and in slowing their release to body tissues, thereby attenuating toxic shocks.
Venoms are animal poisons evolved from saliva, that are injected and distributed in the body by the victim's circulation. *Hemotoxins* from lizards attack blood vessel walls. *Stonustoxins* from the stone fish and platypus create incredible pain. *Necrotoxins* from spiders destroy flesh. *Neurotoxins* from the black mamba paralyze victims. *Myotoxins* from rattle snakes or sea snakes attack muscle specifically. *Atraxotoxins* from funnel web spiders over-stimulate nerves. *Cardiotoxins* from jellyfish attack the heart muscle. *Batrachotoxins* used on arrow tips by some hunters induce total body system shutdown. Antibodies are useful in treatment against these bio-molecules.

Mongooses, opossums and ground squirrels produce plasma protein (protease inhibitors) that neutralize the venom of snakes found in their habitat, showing an adaptive capability of this detoxification mechanism.

5.1.1. Types of Blood Cells

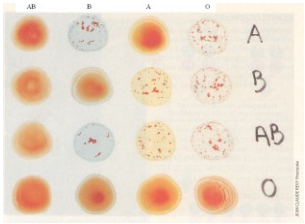
The various cell types in the blood, erythrocytes, leucocytes or platelets, are differently affected by various toxicants because they are so different.
Leucocytes come in many forms, and the role of the various forms is not completely known. T lymphocytes are involved in many cellular activities that define "self" within the body (as



F5.2. Identification of 99 % of the protein found in plasma. Blood contains one million different protein types, with abundances that span 12 orders of magnitude.

auto-immunity), while B lymphocytes produce anti-bodies that tag foreign materials for destruction. Monocytes are immune agents dispatched by the spleen and are the first responders to trouble sites.

Even the comparatively simple red blood cells have their own complexity. For example, erythrocyte blood types define compatibility for transfusion, according to the presence of A, B and Rh♣ antigens, but these characteristics have little to do with hemotoxicity.

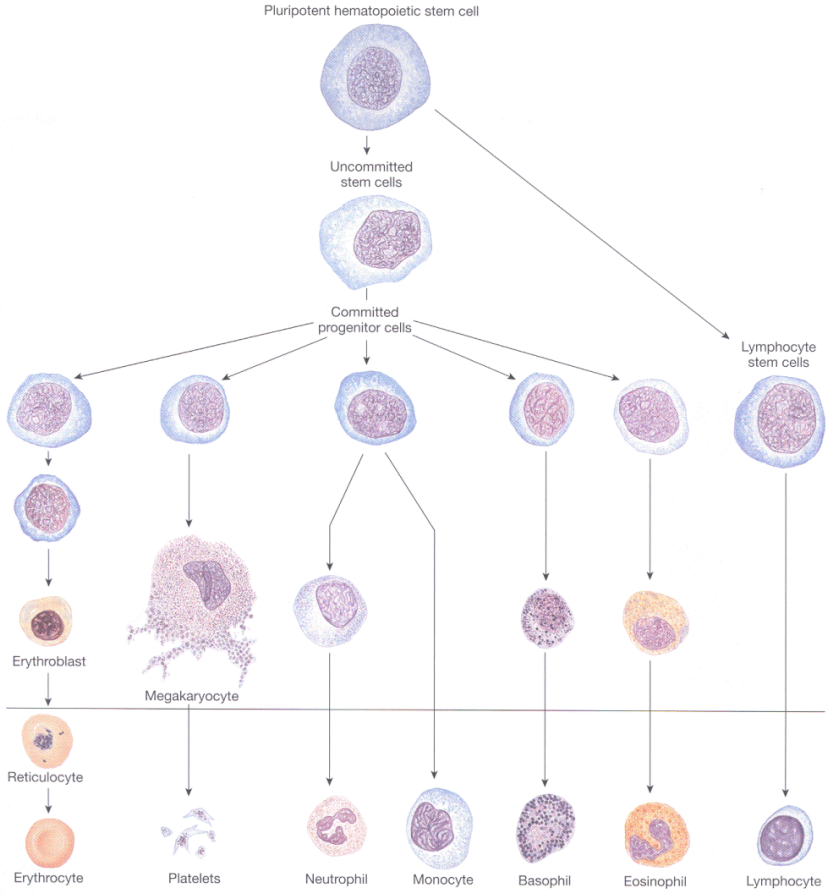


F5.3. Blood transfusion incompatibilities.
Scientific American.

5.1.2. Formation of Blood Cells

The 5 to 6 liters of blood in the human body originate from undifferentiated mesenchymal cells or pluripotent stem cells found primarily in the bone marrow (long bones, ribs, sternum) and, in some species, in the spleen. These stem cells give rise to erythrocytes, a series of "white" blood cells or leukocytes, and thrombocytes or platelets (F5.4). Stem cell proliferation is under the influence of specific cytokines (*poietins)* arising from different tissues in response to changes in physiological status. Cytokines have a major role in stabilizing blood cell populations by stimulating the activity of cells, much like hormones do.

The difference between hormones and cytokines is circumstantial. Hormones circulate in nanomolar (10^{-9}M) concentrations that usually vary by less than one order of magnitude. Cytokines (ex., IL-6) circulate in picomolar (10^{-12}M) concentrations that can increase up to 1,000-fold during trauma or infection. Some hormones are homeostatic control systems that keep constant the concentration of hemoglobin. Others function in a transient, pulsatile fashion, triggering physiological entrainment (testosterone, cortisol, LH, FSH). Neuro-transmitters could be seen as the ultimate short-term controllers.



F5.4. Blood cells types arising from pluripotent stem cells.
Silverthorn, 2004.

♣ **RH FACTOR**: antigens (agglutinogens) such as the Rh factor are present on the surface of red blood cells, leading to incompatible blood types.

An Rh- mother having her first Rh+ child is usually not a problem. But on the second child, problems start to appear because she has developed antibodies to the Rh factor, and the problems grow with each successive child. In the newborn child, hemolysis can lead to anemia and even death.

The supply of red blood cells in mammals is controlled by the cytokine erythropoietin (EPO). Hypoxic conditions, anemia, or the presence of cobalt ions will result in the release of erythropoietin from the kidney to stimulate the production of more erythrocytes. In contrast, hyperoxia or polycythemia will inhibit (or shut down) the release of erythropoietin, decreasing erythrocyte synthesis[1]. In physiological situations, the concentration of EPO can change by 1000-fold. 2 million new red blood cells per second are needed to maintain cell numbers in the adult.
Any reduction in the populations of needed cells will induce an attempt by humoral regulators to restore it. There are a large number of such molecular regulators, which act on long production chains for various needed cell types.

The stem cells are at the root of the tree (F5.4). Until recently, it was assumed that many adult tissues had no stem cells and that if they had, the stem cells could only reproduce the tissue in which they resided. However, more recent evidence suggests that stem cells from the bone marrow can form nerve, muscle and other types on non-blood cells.
Implicit in this blood system, and probably in other body systems, is asymmetrical division. Stem cells given rise to two daughters, one of which replaces the mother, while the other differentiates into granddaughters.

5.1.3. Blood Tests

Blood tests are extensively used in evaluating the health status of animals and humans. Laboratory results may be outside of the *normal* range because of factors such as race, diet preferences, age, sex, menstrual cycle, degree of physical activity, improper collection and handling of the specimen, non-prescription drugs (aspirin, cold medications, vitamins, etc.), prescription drugs, alcohol intake, etc.

Labs set the normal result range for a particular test so that 95 % of healthy patients fall within. So, 5 % of healthy patients fall outside of the "norm". Statistically, if you have 20 or 30 individual tests run as part of a panel, chances are that 1 or 2 will be slightly outside the normal range. We briefly summarize below the meaning of common blood variables for human subjects.

Glucose: High values are associated with eating before the test, and diabetes. The normal range for a *fasting* glucose is 60 -109 mg/dl. Diabetes is associated with a *fasting* plasma glucose of 126 or more. A precursor of diabetes, Impaired Fasting Glucose (IFG) is defined as readings of fasting glucose levels of 110 - 125. Sometimes a glucose tolerance test, which involves giving a sugary drink followed by several blood glucose tests, is necessary to properly sort out normal from IFG from diabetes.

Potassium is controlled very carefully by the kidneys, critical to the proper functioning of nerves and muscles, particularly the heart. Any value outside the expected range, high or low, requires medical evaluation. This is especially important if you are taking a diuretic or heart medication (Digitalis, Lanoxin, etc.).

Sodium is regulated by the kidneys and adrenal glands. There are numerous causes of high and low sodium levels, but the most common causes of low sodium are diuretic usage, diabetes drugs like chlorpropamide, and excessive water intake in patients with heart or liver disease.

CO_2 is intimately related to blood pH. Low CO_2 levels can be due to either increased acidity from uncontrolled diabetes,

kidney disease, metabolic disorders, or to chronic hyperventilation.

Blood Urea Nitrogen (BUN) is a liver waste product excreted by the kidneys. High values may signal kidney disease. BUN is also affected by high protein diets and strenuous exercise which raise levels, and by pregnancy, which lowers it.

Creatinine is a waste product largely from muscle breakdown. High values, especially with high BUN levels, indicate kidneys problems.

Uric Acid is normally excreted in urine. High values are associated with gout, arthritis, kidney problems and the use of some diuretics.

AST, ALT, SGOT, SGPT, and GGT and Alkaline Phosphatase are enzymes that may be released into the blood following various forms of injury to muscles, liver and heart. Alcohol and a number of diseases cause high values.

AST/SGOT, ALT/ SGPT are liver and muscle enzymes. They may be elevated from liver problems, hepatitis, excess alcohol ingestion, muscle injury and recent heart attack.

GGT is also elevated in liver disease, particularly with obstruction of bile ducts. Unlike alkaline phosphatase, it is not elevated with bone growth or damage.

Alkaline phosphatase is an enzyme found primarily in bone and liver. Higher values are expected for growing children and pregnant women, or when damage to bones or liver has occurred, or with gallstones. Low values are probably not significant.

LDH is an enzyme present in all cells in the body. Anything which damages cells, including blood drawing itself, will raise amounts in the blood. If blood is not processed promptly and properly, high levels may occur. If all values except LDH are within expected ranges, it is probably a processing error and does not require further evaluation.

Bilirubin is a pigment removed from the blood by the liver. Low values are of no concern. If slightly elevated above the expected ranges, but with all other enzymes (LDH, GOT, GPT, GGT) within expected values, it is probably an insignificant condition known as Gilbert's syndrome.

Creatine PhosphoKinase is an enzyme useful for diagnosis of heart and skeletal muscle diseases. This enzyme is the first to be elevated after a heart attack (3 to 4 hours). If CPK is high in the absence of heart muscle injury, this is a strong indication of skeletal muscle disease.

Albumin and Globulin measure the amount and type of protein in your blood. They are an index of overall health and nutrition. Globulin is the "antibody" protein important for fighting disease. A/G Ratio is the mathematical relationship between the above.

Cholesterol is a fat-like substance associated with heart disease. Less than 200 mg/dl is recommended.

Total Cholesterol: A high cholesterol level is a risk factor for heart and atherosclerosis. 200 mg/dl or more are too high and 240 and above are considered high risk. A low fat diet and regular exercise are recommended. There are three major kinds of cholesterol, High Density Lipoprotein (HDL), Low Density Lipoprotein (LDL), and Very Low Density Lipoprotein (VLDL).

High LDL mark formation of deposits in the arteries. 100 mg/dl is ideal, less than 130 is recommended, greater than 160 is high risk.
High HDL is associated with low incidence of coronary heart disease.
VLDL is another carrier of fat in the blood.

Triglyceride is fat which, if elevated, has been associated with heart disease and pancreatitis, especially if over 500 mg/dl. Triglyceride levels over 150 mg/dl may be associated with other problems.

Calcium in the blood is controlled by the parathyroid glands and the kidneys. Calcium is found mostly in bone and is important for proper blood clotting, nerve, and cell activity. An elevated calcium can be due to medications such as thiazide type diuretics, inherited disorders of calcium handling in the kidneys, or excess parathyroid gland activity or vitamin D. Low calcium can be due to insufficient parathyroid hormone or drugs like Fosamax or furosemide type diuretics.

Phosphorus is also largely stored in the bone. It is regulated by the kidneys, and high levels may be due to kidney disease. When low levels are seen with high calcium levels, it suggests parathyroid disease. A low phosphorus, in combination with a high calcium, may suggest an overactive parathyroid gland.

Thyroid hormones, thyroxine (T4) and triiodothyronine (T3), are easily measurable in the blood.
Thyroxine (T4) high levels may be due to hyperthyroidism, however technical artifact occurs when estrogen levels are higher from pregnancy, birth control pills or estrogen replacement therapy. A Free T4 test (see below) can avoid this interference.
T3 Resin Uptake or Thyroid Uptake is not a thyroid test, but measures the levels of proteins that carry thyroid hormone in the bloodstream and is used to compute the free thyroxine index. A high result indicates a low level of the protein.
Free Thyroxine Index (FTI or T7) : A mathematical computation allows the lab to estimate the free thyroxine index from the T4 and T3 Uptake tests. Unlike the T4 alone, it is not affected by estrogen levels.
Free T4: This test directly measures the free T4 in the blood rather than estimating it like the FTI. It is a more reliable , but a little more expensive test. Some labs do the Free T4 routinely rather than the Total T4.
Total T3 is used when thyroid disease is evaluated. T3 is the more potent and shorter lived version of thyroid hormone. Some people with high thyroid levels secrete more T3 than T4. In these (overactive) hyperthyroid cases the T4 can be normal, the T3 high, and the TSH low.
Free T3: This test measures only the portion of thyroid hormone T3 that is "free", that is, not bound to carrier proteins.

Thyroid Stimulating Hormone (TSH): This protein hormone is secreted by the pituitary gland and regulates the thyroid gland. A high level suggests your thyroid is under-active, and a low level suggests your thyroid is overactive.

Glycohemoglobin (Hemoglobin A1 or A1c, HbA1c) : Glycohemoglobin measures the amount of glucose chemically attached to your red blood cells. Since blood cells live about 3 months, it tells us your average glucose for the last 6 - 8 weeks. A high level suggests poor diabetes control.

5.1.4. Natural Hematological Diseases

The major natural diseases of the blood are red blood cell insufficiencies (anemias) and leukocyte proliferation (leukemias).

F5.5 directly displays the hematocrit (% volume of red blood cells) for various conditions, which is normally 40-54 % for men and 37-47 % for women. Sports competitors may induce artificial polycythemia (last tube in F5.5) by re-injecting their stored blood cells before a competition.

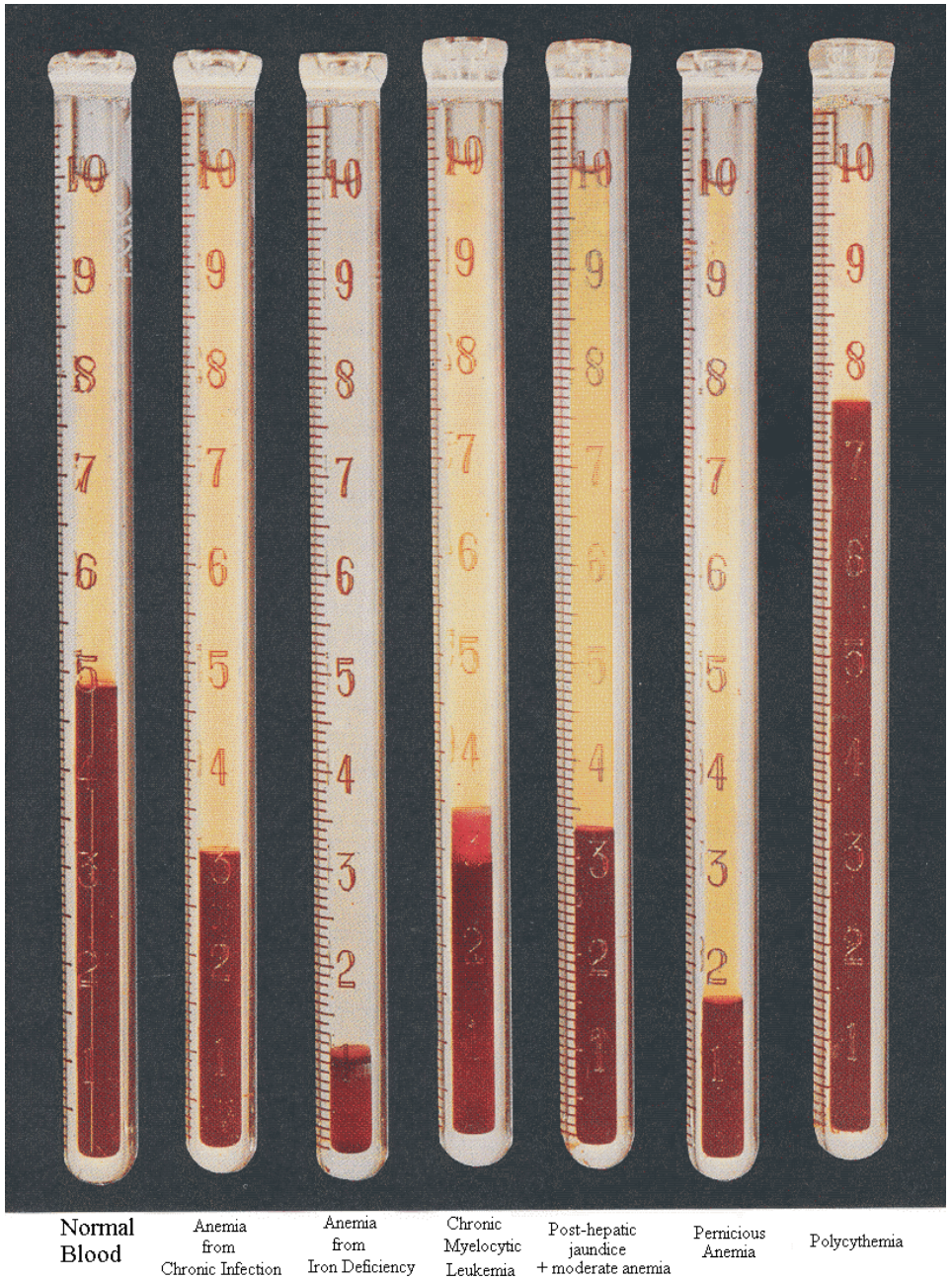
5.1.4.1. Anemia

Anemia results from insufficient delivery of oxygen to body tissues as a result of too few or defective red blood cells.

Chemical exposures can increase or decrease blood cell levels because the marrow-based process of generation of blood components is complex and delicate. Problems with red blood cells are common even without intoxication:

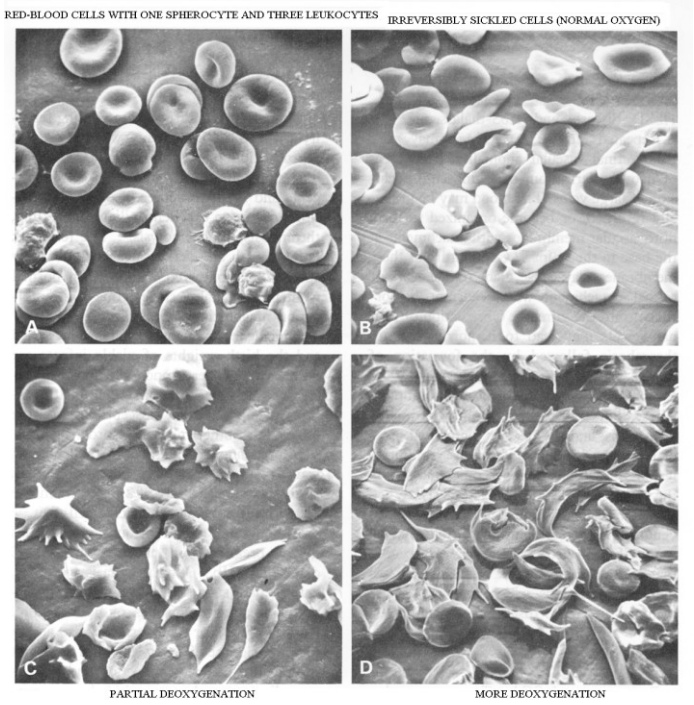
- Microcytic Hypochromic: too small RBCs from iron deficiency or hemorrhage. This problem can also be caused by *lead exposure*.
- Megaloblastic Microcytic: too small RBCs from folic acid or B_{12} deficiency.
- Aplastic: too few RBCs because of a dysfunction of blood forming systems.

Red blood cell destruction or fragility can result from genetic diseases (sickle cells), malaria or chemical exposure.



F5.5. Appearance of blood in various diseases. *Wintrobe, 1974.*

Chemicals can induce anemia, in part because red blood cells are exposed early to chemicals and have limited means of metabolic defense.
In mammals, they lack a nucleus and mitochondria. They produce ATP by anaerobic metabolism (glycolysis) because mitochondria would use up the oxygen that RBCs are intended to transport.
Damage to the erythrocytes may induce *hemolysis*.
Hemoglobin spilled into the plasma dissociates its four tightly bound sub-units and causes kidney toxicity by precipitating in the glomerulus. There is damage to the glomerular membrane, hematuria and a gel-like precipitation in the tubular lumen, thus impairing renal function. It also interacts toxically with a number of other molecules throughout the body.



F5.6. Sickle cell transformation. *Wintrobe, 1974.*

Destruction of red blood cells also impairs oxygen and carbon dioxide transport. The result is hypoxemia (insufficient supply of oxygen to tissues) and secondary toxicity in tissues which require large supplies of oxygen (CNS, heart).

5.1.4.2. Sickle Cell Anemia

Sickle cell anemia is a relatively common genetic condition, that amplifies problems related to oxygen insufficiency. It is thought to result from an adaptation giving immunity against malaria. The sickle mutation increases a protein, heme oxygenase-1, which increases CO concentrations. The binding of CO to heme reduces the toxicity that occurs when heme is released into the blood, as erythrocytes desintegrate[25].

The sickle cell transition is due to the spontaneous crystallization of the variant HbS, triggered by low oxygen atmospheres.

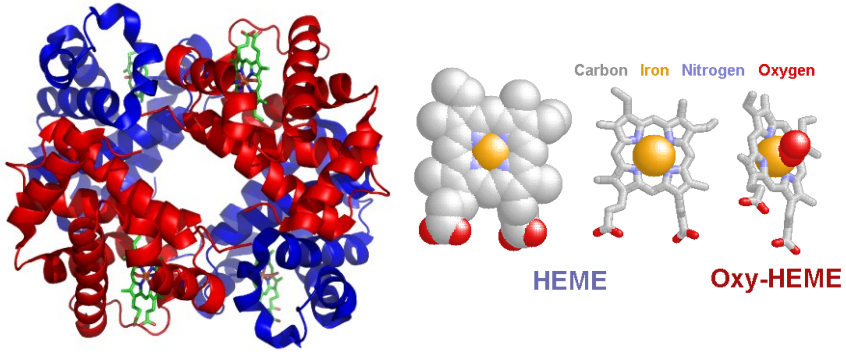
It appears that specific molecules on the deformed cells make them stickier than healthy red blood cells, contributing to painful sickle crises. Sickle cells are normally eliminated by the spleen. The condition is most prevalent in black women. Individuals that are homozygous for HbS are not usually in the workforce. Individuals that are heterozygous show almost no impairment in most situations. It may be appropriate to screen in special cases such as operation of aircraft and submarines. Genetic trials in rats aimed at correcting sickle cell disease have been successful[12].

5.2. Hematotoxicity

5.2.1. Oxygen Transport

The erythrocyte has a simpler metabolism than normal cells. There is no glycogen in the erythrocyte, therefore, glucose

must be available from the blood. It is metabolized by two routes: the Embden-Meyerhof pathway (anaerobic) and the pentose phosphate pathway (aerobic).
Routine detoxification of free radicals in the erythrocyte is heavily dependent on glutathione (page 3-19).

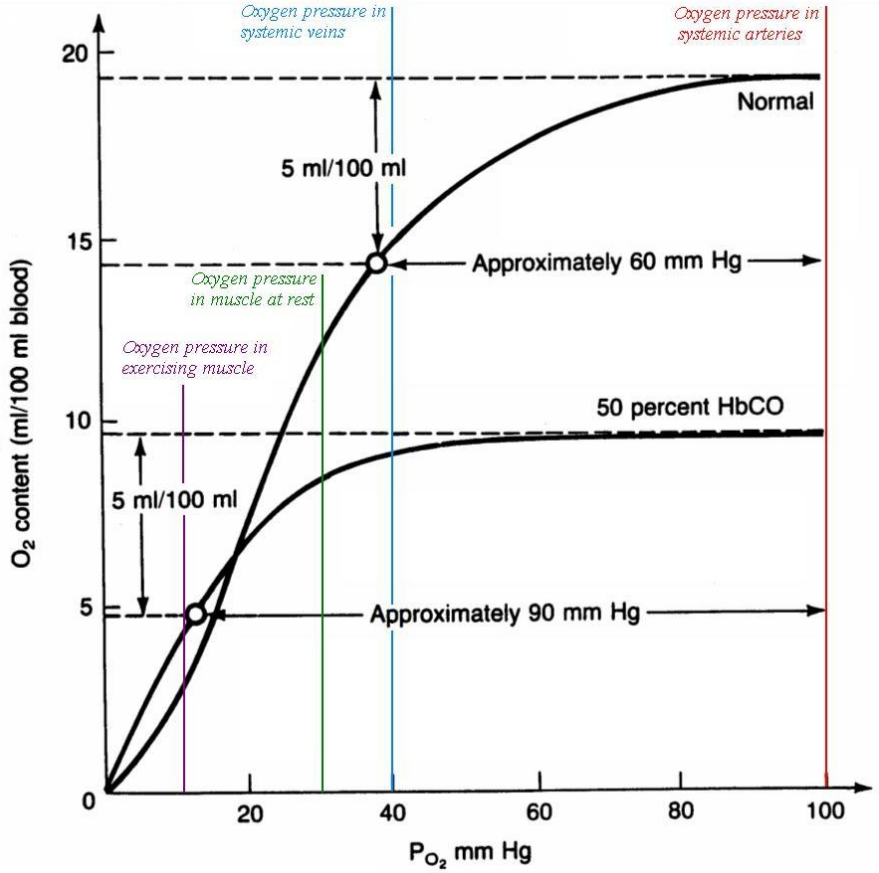


F5.7. Views of hemoglobin. Left, the complete molecule where the protein subunits are in red and blue, and the iron-containing heme groups in green. Right, the oxygen binding site (heme).

The erythrocyte carries **carbon dioxide** away from tissues. A small amount is carried in free solution, 75 % is transported as bicarbonate ion (HCO_3^-) converted by the enzyme carbonic anhydrase (a soluble enzyme in the intracellular fluid), the balance of the carbon dioxide is combined with free amino groups in the globin to form carbamino (R-NH-COOH).

30 % of the erythrocyte is made of the protein *hemoglobin*. **Hemoglobin** (Hb) is responsible for the transport of oxygen, attaching it to a ferrous (Fe^{2+}) ion which is held in position via chelating bonds to porphyrin ring N-groups. One free attachment of the Fe^{2+} is an imidazole group of the protein globin, which leaves one bond free for the oxygen (F5.7). *Heme* is a porphyrin chelate of iron in which the iron is Fe^{2+}, the oxygen-carrying, color-furnishing, non-protein part of Hb. In the disease called porphyria, mutated enzymes manufacture defective heme, which builds up to toxic levels.
The performance of Hb as an oxygen carrier is described by the oxygen saturation curve of blood. The curve defines the volume of oxygen released under various partial pressures of gaseous oxygen (F5.8). In order to prevent hypoxia in distal tissues, Hb *loses* its affinity for oxygen when the oxygen pressure is low.
Hemoglobin was originally a detoxification molecule used by simple animals as a defense against the oxygen "pollution" from plants. For example, the hemoglobin of *Ascaris lumbricoides* (a worm living in low oxygen environments) binds oxygen 25,000 more tightly than human Hb. Myoglobin in muscle has an even stronger affinity for oxygen than hemoglobin, making oxygen delivery to muscle tissues possible.
It has been demonstrated experimentally that there are alternative fluids to hemoglobin that can carry enough dissolved oxygen to sustain life (F5.9).

Without oxygen, animal metabolic pathways end up with excessive lactic acid. Turtles can cope with large amounts of lactic acid because they neutralize it using the calcium carbonate in their shell. Carps and goldfish can convert lactic acid to ethanol, which can be released from their gills[15].

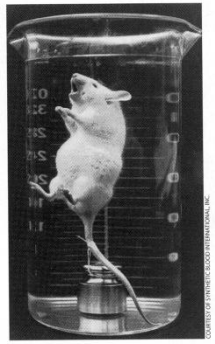


F5.8. Saturation curve of blood with air and CO. Atmospheric air has 160 mm Hg of O_2.
Modified from Williams and Burson, 1985.

5.2.1.1. CO Interference with Oxygen Transport

Carbon monoxide binds Hb 225 times more strongly than O_2, as shown in the equation below, where M = 225 and the Ps represent partial pressures. In the presence of CO, therefore, less Hb is available to carry oxygen.

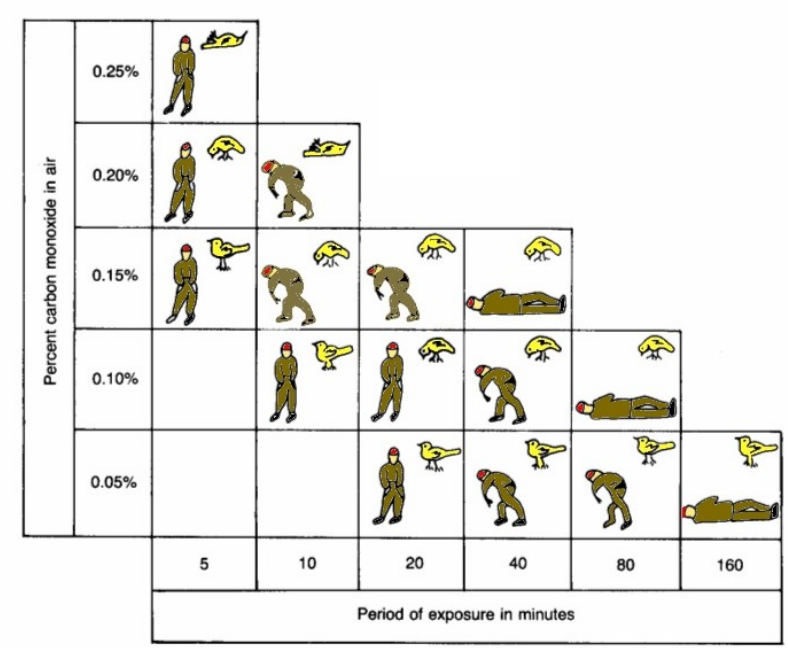
$$\frac{Hb - CO]}{Hb - O_2]} = M\frac{P_{CO}}{P_{O_2}}$$



F5.9. A rat breathing perfluorocarbons.

0.07 % CO can be lethal. Another effect of CO at 0.1 % is to alter the Dissociation-Saturation curve of Hb, as shown in F5.8. Saturation of Hb with CO inhibits oxygen transport by binding less oxygen more tightly: in the graph, a depression of 90 mm Hg in oxygen pressure (as opposed to 60 mm) is necessary to release 5 ml of oxygen.
The symptoms of CO poisoning are low blood pressure, fainting, dizziness, headache, weakness and nausea.

In a Toxicokinetic application, **canaries** have been used as warning devices to protect the health of miners, since coal mines are likely to contain pockets of CO. A canary is small, its metabolism is high, and it has a high breathing rate. Therefore, it will be in equilibrium with CO much faster than a human, and should serve as a warning for humans to leave the tunnel. However, the binding of CO to canary Hb is only 110 times (not 225) stronger than that of oxygen. Because of this, it is possible that the miner will die before the canary, usefully warning the canary to get out of the mine shaft. Therefore canaries as a warning device are only effective above 0.2 % CO.
Because the CO_2 chemo-receptors that increase respiration rate are not affected by CO poisoning, the lack of O_2 concentration into the tissues is not noticed until too late.



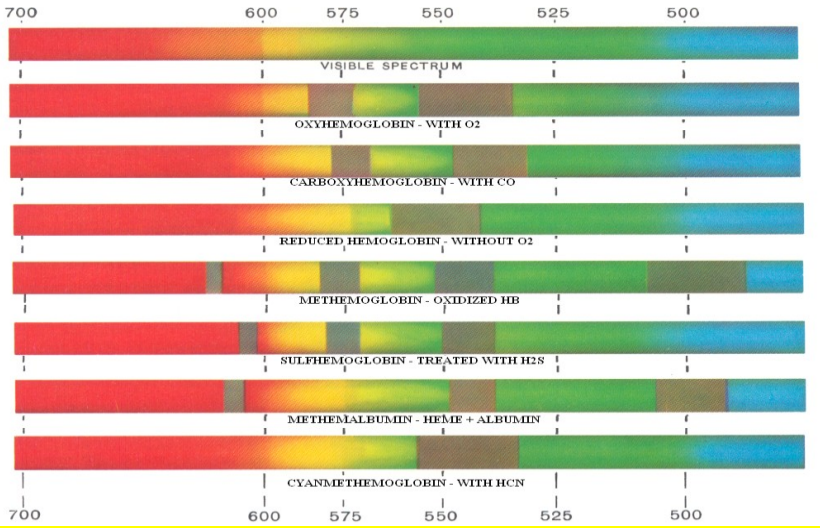
F5.10. Effects of Carbon Monoxide on Man and Canaries.
Ann. Occup. Hyg. 5: 1961.

Carboxyhemoglobin (CO-Hb)

- Individuals with previous coronary artery disease may develop angina with moderate activity at levels as low at 3-5 %.Cigarette smokers are chronically around 5 %. Exposure of 35 ppm CO for 10 hours is equivalent to 5 %.
- Cardiac compensatory effects seen at 8-10 %: heart attack, lightheadedness, chest pain.
- Electrocardiogram problems (extrasystoles, premature ventricular contractions, fibrillation) at 10-25 %.

Treatment: Carboxyhemoglobin pressure can be lowered by breathing oxygen through a mask for a few hours. It also combats nausea, headache and loss of consciousness commonly experienced by victims. However, hyperbaric oxygen should be the treatment of choice for seriously poisoned people to prevent memory lapses, confusion and language problems.
In the weeks following their poisoning by CO, some survivors develop concentration problems, personality changes or sensory impairments. These effects can be traced to misguided immune responses to a brain protein, myelin basic protein, that is altered by CO gas[14].
CO binds mitochondrial enzymes and myoglobin, increases platelet stickiness and decreases arrhythmia thresholds.

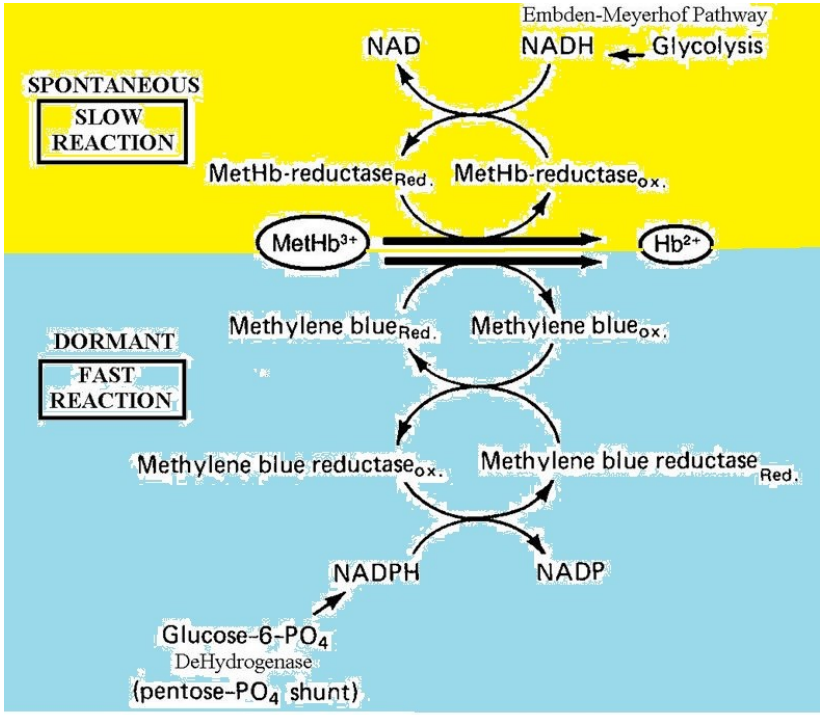
Methylene Chloride is a solvent used in degreasing and paint stripping which is absorbed by respiration or through the skin, and is metabolized in the blood to CO. It may elevate COHb to 10 % or more in poorly ventilated spaces. It is also a carcinogen.



F5.11. Spectrums of Hemoglobin. *Modified from Wintrobe, 1974.*

5.2.1.2. Chemical Attacks on Hb

Unfortunately, Hb is not immune to chemical attacks, as revealed by the dramatic changes in the color of hemoglobin (F5.11) in the presence of oxygen (ruby-red), carbon dioxide (blue-red), carbon monoxide (cherry red), and cyanide (bright red). The erythrocyte has limited ability to detoxify activated oxygen which, if present in excess, becomes toxic to the cell. *Hemoglobin can be pathologically oxidized* to **MetHemoglobin.**



G-6-PD in red blood cells converts methemoglobin back to hemoglobin. Humans genetically deficient in this enzyme can have 30% methemoglobin in their red blood cells. Treatment with reducing agents like ascorbic acid (Vitamin C) or methylene blue considerably lowers the amount of methemoglobin in their blood.
The main clinical manifestation of this deficiency is (induced) haemolysis. This is usually the result of the existence of an oxidizing factor from the environment: a drug, an infection, some foods, chemical exposure. In most cases this passes unnoticed because the organism is able to counteract. Most patients live a perfectly normal life without ever noticing the effect of this disorder.

METHYLENE BLUE

Methylene blue is an artificial dye first used as a marker in studying bacteria and body tissues. Methylene blue, which can be taken by mouth or injected intravenously, imparts a blue color to urine and feces.
It is also an antiseptic (disinfectant), although its bactericidal properties are very mild, and a mild urinary antiseptic and stimulant to mucous surfaces.
It has been used as an antiseptic in urinary tract infections and as an indicator dye to detect certain compounds present in urine. Methylene Blue has also been shown to minimize the occurrence of kidney stones, and may prevent their formation.
Its present uses are for its oxidation-reduction functions (methemoglobin regeneration to hemoglobin), tissue staining, and as an anti-aging drug. Methylene Blue activates a normally dormant reductase enzyme system which reduces the Methylene Blue to leucomethylene blue, which in turn is able to reduce methemoglobin to hemoglobin. It is believed to be reduced in the tissues to the leuco form, which is slowly excreted mainly in the urine, together with some unchanged drug. In large doses, Methylene Blue can *produce* methemoglobinemia.

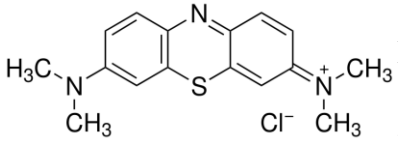
The iron in the Heme of Hb is transformed to the ferric state (Fe+++, an "electron" is lost) rather than the useful ferrous state (Fe++). MetHb is greenish brown to black in color and **does not bind oxygen or CO;** it binds water instead. Normal level in the blood are 1-2 %, it becomes dangerous at 15 %. Hb in erythrocytes can be reduced back to the ferrous state by two metabolic routes (F5.12):

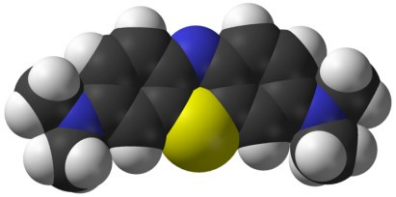
- a **spontaneous** system of detoxification based on reduced nicotinamide adenine dinucleotide (NADH) generated by the glycolytic Embden-Meyerhof pathway. Cytochrome b_5 and cytochrome b_5 reductase are the enzymes responsible for changing MetHb back to Hb by maintaining NADH supply. 95 % of MetHb is processed this way, and

F5.12. Reactivation of methemoglobin (MetHb) to hemoglobin (Hb) following chemical reduction.
Methylene blue can be used to reactivate Hb in emergencies.

- a **dormant** system based on glucose-6-phosphate-dehydrogenase (G-6-PD), involving methemoglobin reductase, otherwise known as the "glucose-monophosphate shunt" and generating the co-factor reduced nicotinamide-adenine dinucleotide phosphate (NADPH). This accounts for 5 % of the regeneration activity.

Both systems are dependent on glycolysis.





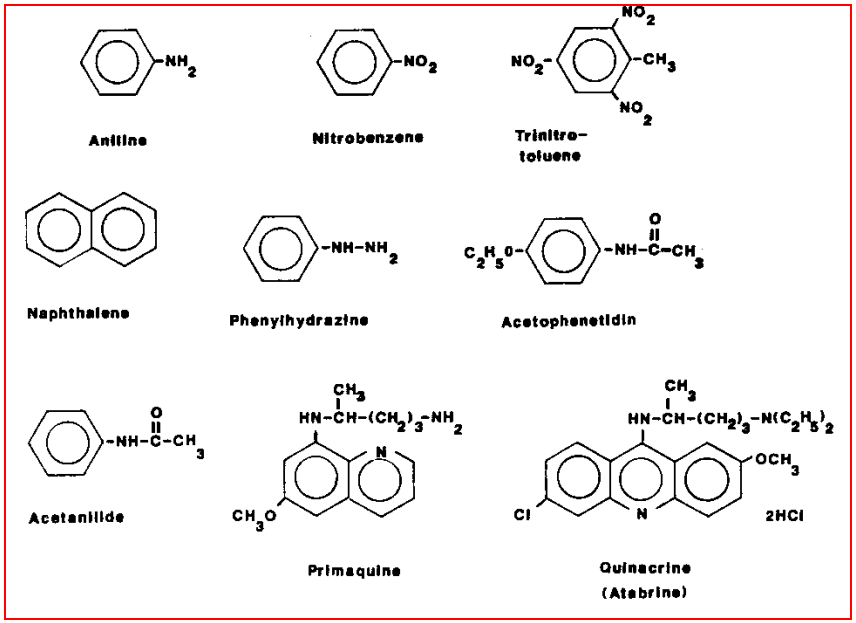
Detoxification of MetHb is also stimulated by addition of *methylene blue* (left), which is a classic **antidote for nitrite and cyanide poisoning**. Administered intravenously at doses of 2 mg/kg, methylene blue increases the rate of MetHb conversion to Hb 6-fold.

The vast majority of work-related hemolytic anemias are induced by exposure to benzene derivatives or aromatic ring structures. These agents are potential oxidation-reduction catalysts, acting as oxygen scavengers, water soluble enough to be carried in an aqueous medium but lipid soluble enough to penetrate the erythrocytic membrane, gaining access to a vast storehouse of oxygen. In the ensuing reaction, both the chemical and the oxygen become free radicals.

MetHb generating chemicals (oxidizers):

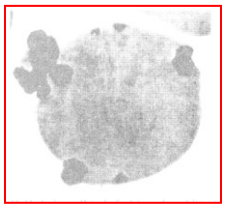
- nitrites (-NO_2),
- nitrates (-NO_3, need to be activated to nitrites in the gut), aromatic amines (NH_2-Benzene),
- chlorate salts (Na- O-O-O-Cl).

These chemicals can produce hypoxia in the exposed. See also figure 5.13.



F5.13. Structures of chemicals causing hemolysis or methemoglobinemia.

Hb can also be *irreversibly* denatured or oxidized by chemical agents. In this case, *Heinz bodies* are observed that are covalently bound to the inside of the Red Blood Cell membrane (F5.14). These cells are not toxic by themselves, but will usually hemolyze because of loss of flexibility, which can lead to anemia.



F5.14. Heinz bodies in an erythrocyte. x 26,000. *Rifkind, 1965.*

5.2.1.3. Exposure Examples

Chemical-induced hemolysis began in the 1800's German dye industry when coal tars were converted into basic industrial chemicals, including anilines, nitrobenzenes, quinones and naphthalene.
Hemolytic anemias can be caused by such metals as lead, mercury, copper, as well as arsenic (as arsine), all of these cytolytic metals are highly reactive with sulphydryl groups (-SH) that are abundant in erythrocytes in the form of glutathione, hemoglobin, etc. Cases...

1) Explosives workers, called "blue workers", due to chronic cyanosis resulting from methemoglobin formation as a consequence of exposure to nitro-benzenes and nitrotoluenes.

2) Antimalarial drugs related to 8-amino-quinoline such as pamaquine and primaquine, used in WW II in the Pacific theatre, resulted in massive hemolytic crises among black soldiers. It was recognized that there was a susceptible subgroup within this population.

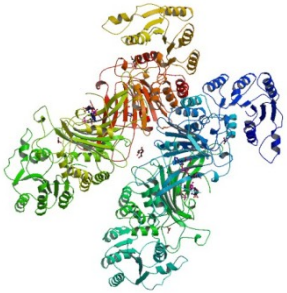
3) The analgesics acetanilide and acetophenetidin have been removed from the over-the-counter market because of patient hemotoxicity, particularly with abuse.

4) A defoaming agent in beer, an aniline-related chemical, caused "blue workers" in a Montreal brewery exposed to leaking defoamer[3-7].

5) Repeated development of new drugs based on phenylhydrazine.

5.2.1.4. The Genetics of Erythrocytic Hemolysis

In most individuals, the mechanisms of detoxification described previously are sufficient to deal with any MetHb and oxygen radical formation. However, the most common enzyme deficiency in humans is caused by a variety of mutations to band Xq28 of the "X" chromosome. Severe reductions of the enzyme glucose-6-phosphate dehydrogenase (G6PD, shown), impairs the "rapid" protective glucose monophosphate shunt system, leaving individuals unable to cope with oxygen stress. These subjects are particularly sensitive to red cell hemolysis induced by chemicals, because *glutathione* cannot be regenerated. The deficiency is rare among Caucasians but, among blacks, some 12-14 % of males, but only 2 % of females have the deficiency.



Humans genetically deficient in G6PD can have 30 % MetHb in their red blood cells. Treatment with reducing agents like ascorbic acid (Vitamin C) or Methylene Blue considerably lowers the amount of MetHb in their blood. The main clinical manifestation of this deficiency is (induced) hemolysis. In most cases this passes unnoticed, because the organism is able to compensate. Most patients live a perfectly normal life without appreciable effects of this disorder.
When it becomes noticeable, it is usually the result of an oxidizing factor from the environment: a drug, an infection, some food or chemical exposure.

The key cellular oxidant scavenger in this pathway is the tripeptide, reduced glutathione (GSH, gamma-L-glutamyl-L-cysteinyl-glycine), formed by the hexose-pentose monophosphate shunt. Relatively high levels (3-5 mM) of the reduced form are normally present in erythrocytes, with only miniscule amounts of the oxidized (GSSG) dimer.

Glutathione is a radical scavenger that works rapidly (with or without the enzyme glutathione-s-transferase, a Phase II conjugating enzyme) to neutralize highly reactive intermediates capable of damaging and weakening the erythrocytic membrane. The inability to maintain adequate levels of GSH destabilizes the cellular protective mechanism, resulting in weakened and easily ruptured erythrocytic membrane and enhancing hemolysis.
The syndrome leads, upon exposure, to methemoglobinuria, formation of sulfhemoglobin (only partially soluble in erythrocytes) which precipitates into spheroidal bodies (Heinz or inclusion bodies), alteration of the shape, rigidity, and viability of the red cell, causing hemolysis and resulting in massive hematuria.
It is important to point out that Heinz body hemolytic anemia can occur in normal (not enzyme-deficient) individuals, but only if exposure to a chemical oxidant is several fold higher than that producing hemolysis in the G-6-PD deficient person.

5.2.2. Metabolic Anoxia: Cyanide and H_2S

Hydrogen cyanide is a colorless gas or liquid. About 30 % of people are unable to detect cyanide gas's (HCN) bitter almond odor, but those who can often smell cyanide at autopsy of a poisoning victim. The lethal dose of sodium cyanide by mouth is 100 mg, and 2000 mg/minute x m^3 when inhaled. Effects are confusion, dizziness, increased breathing and heart rate,

Chemical-induced methemoglobinemia case history.
Levy and Wegman, Occupational Health, Little and Brown, 359-362, Boston, 1983.

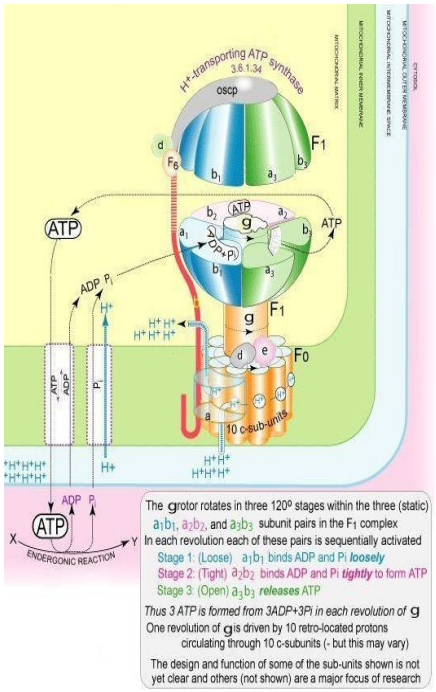
The patient was a well-built, vigorous, 50-year old man who had been employed for 21 years as a laborer in a dyestuff plant where he was in daily contact with aniline. Among his regular duties was the refilling each morning of a barrel containing industrial grade aniline. The barrel, in which aniline was stored under pressure, was located on the floor of a room shared by three employees. Aniline from this sealed barrel was pumped as needed via tubing into the dye-application work area by means of hydraulic pressure.

Beginning about two years prior to the accident, the man complained to his wife of a gradually worsening, unremitting headache. For two months she in turn had noted bluish discoloration of her husband's lips, ears, and fingertips, most evident on his return home from work each evening. One midsummer morning, just after the patient had filled the reservoir barrel with a fresh supply of aniline under sealed pressure, the bottom of the barrel blew out. Roughly 30 gallons of aniline gushed forth, flowing over the patient's clothing and inundating the floor of the work area. He, as well as the two co-workers standing nearby, rapidly experienced nausea, dizziness, and faintness, but after cleaning up the spilled chemical all resumed their work until noon. Two hours later the patient returned to work but grew dizzy, nauseated, and began to vomit. (The two co-workers had called in to report themselves too ill to return to the factory.) The patient's physician was called, and immediate hospitalization was arranged.

On admission, the patient was described as confused, agitated, breathing rapidly and deeply, and displaying dark cyanosis of the hands, feet, and ears. His mucous membranes also appeared grey-blue, particularly when viewed under light from a quartz-mercury lamp. Freshly drawn blood appeared dark maroon-brown. There was no anemia, but the reticulocyte count was 5.8 percent and, after supravital staining, 6.8 percent of his red cells displayed Heinz bodies. About 60 percent of the patient's hemoglobin was in the form of methemoglobin (ferrihemoglobin). Within less than 24 hours the hematocrit dropped moderately, the reticulocyte level rose (and continued to rise for several days), and the portion of red cells containing Heinz bodies increased to nearly 30 percent. The methemoglobin concentration fell abruptly during the initial 15 hours and by the second day only traces remained. However, 15 percent of the hemoglobin at that time was in the denatured form of sulfhemoglobin. By day 4, despite several transfusions, the anemia had become more severe, the reticulocyte count was 11 percent and the level of Heinz-body-containing cells was 28.8 percent. Nevertheless, the patient felt much improved and complained no longer of dizziness or nausea. Urine specimens on admission and during the several days thereafter contained high concentrations of the oxidant hydroxyl derivative of aniline, p-aminophenol.

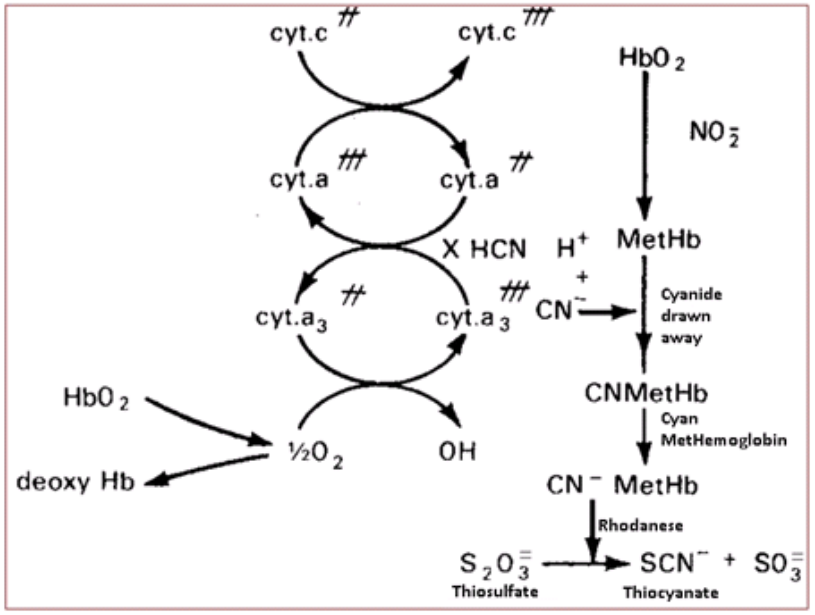
In the ensuing week the patient gradually recovered completely from his symptoms, and the only laboratory aberrations that persisted beyond ten days were elevated levels of reticulocytes and of cells containing Heinz bodies; traces of sulfhemoglobin were detectable for several weeks. All three abnormalities cleared entirely within two months of the accident.

convulsions and asphyxia. In HCN poisoning, blood delivery of oxygen is normal, but HCN impedes the use of oxygen by cells.



F5.15. Synthesis of ATP within mitochondria.
Donald Nicholson, 2002.

Exposed subjects have a red color, similar to CO poisoning, since their venous blood returns still loaded with unused oxygen. Ultimately, the brain cells controlling respiration die. Cyanide specifically inhibits (see F5.15) **cytochrome oxidase a_3**, the cytochrome that normally releases ATP. The therapy for cyanide poisoning is illustrated in F5.16. Oddly enough, the therapy involves the formation of a toxic product, MetHb. This is done by administration of inhaled amyl nitrite and by injections of sodium nitrite. The production of MetHb allows drawing CN away from cytochromes because ferric heme groups avidly bind ionic cyanide, forming cyanmethemoglobin. This is followed by the administration of thiosulfate. Thiosulfate ($-S_2O_3^{--}$) provides a substrate for the enzyme rhodanese which transforms cyanide bound to Hb to thiocyanate. The thiocyanate, much less toxic, is excreted.



F5.16. Therapeutic management of cyanide poisoning.
Casarett & Doull, 1995.

Zyklon B (shown), used in concentration camps and as an insecticide, is a crystallized form of hydrogen cyanide. Most resuscitations from cyanide poisoning occur in individuals who ingested soluble salts, where absorption is delayed.
H_2S, the gas with a rotten egg odor, is another chemical which impairs the use of oxygen by body cells (cytotoxic hypoxia). 80 ppm of hydrogen sulfide can reversibly bring the metabolism of mice to a standstill, lowering their body temperature from 37° to 11° C. The animals reduced their use of oxygen by a factor of 10, lowering their heart and breathing rates[20]. This characteristic might be used to attenuate ischemic damage during emergency situations.

C. elegans, when grown in 50 ppm H_2S, the OSHA human exposure limit, lives 70 % longer than worms living in normal air[19].
H_2S is naturally produced in the body, and manages many important biological functions, in particular blood pressure[21]
Few poisons are more rapidly acting than inhaled hydrogen sulfide, which is common to sewers and oil fields.
The treatment is similar to that of CN poisoning, but there are rarely trained individuals available, and prepared with an intravenous nitrite injection.

5.2.3. Alterations in Cellular Composition

Many chemicals can attack the blood, resulting in obvious changes in blood cellular composition. In most cases, the exact mechanism of toxicity is unknown. Toxicity to stem cells, which largely control the ultimate composition of the downstream differentiated cells, needs to be defined in a special way, different from toxicity to differentiated (somatic) cells, particularly in their relation to oxygen.
Differential blood counts can document imbalances of blood composition resulting from exposure to toxicants.

5.2.3.1. White Blood Cell Imbalances

Exposure to some chemicals (benzene) can induce leukemia. Chemicals that reduce white blood cell counts (leukopenia) lower resistance to infection (DDT decreases monocytes and lymphocytes).

5.2.3.2. Thrombocytopenia

Low platelet counts produced by certain chemicals may result in blood loss. There are other mechanism for blood loss. For example, warfarin inhibits fibrin formation from fibrinogen, while aspirin inhibits platelet aggregation.

5.2.3.3. Chemicals causing Thrombocytopenia

Acetaminophen (Tylenol), Aminopyrine, Aspirin and salicylates, Benzene, Bismuth, Chloramphenicol, Chlordane, Corticosteroids, Dextropropoxyphene (Darvon), Diazepam (Valium), Diethylstilbestrol, Digitoxin, Dimercaprol, Disulfiram (pesticide), Insulin, Isoniazid, Lindane$^{\Omega}$ (pesticide), Mercurials, Phenobarbital, Phenylbutazone, Potassium Iodide, Quinidine, Quinine, Stilbestrol, Tetracycline, Toluene diisiocyanate (manufacture of elastomers), Trinitrotoluene (TNT).

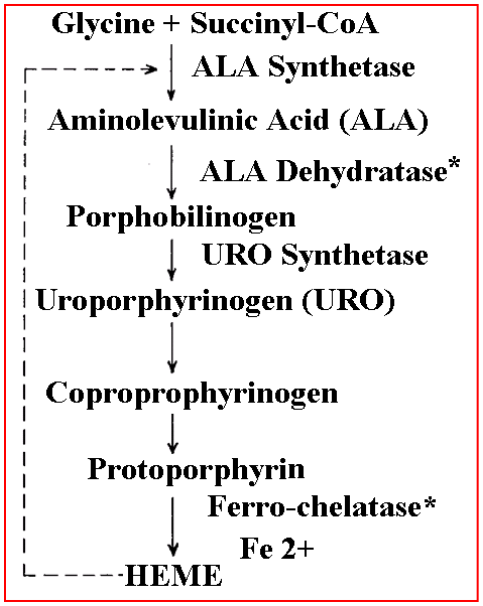
5.2.3.4. Chemicals causing Hemolysis

Arsine, Benzene, Butyl Cello solve (dry cleaning), Carbutamide, Chloramphenicol, Chlorpromazine, Dimercaprol, Lead, Mephenytoin, Methyl chloride, Naphthalene, Nitrobenzene, Phenylbutazone, Phenylhydrazine (dye synthesis), Primaquine, Quinacrine, Streptomycin, Tolbutamide, Trinitrotoluene.

5.2.3.5. Chemicals causing Anemia-Pancytopenia

Alkylating agents, Amitriplyline, Ampicillin, Arsenicals, Arsphenamine, Aspirin, Benzene, Carbon Tetrachloride, Chloramphenicol, Chlordane, Chloroquine, Colchicine, Diazepam, Gold compounds, Hydrochloroquine, Insecticides, Isoniazid, Lindane, Meprobamate, Methimazole, Oxyphenbutazone, Phenylbutazone, Potassium Perchlorate (explosive), Propylthiouracil, Quinacrine, Quinidine, Salycilates, Streptomycin, Sulfa drugs, Tetracycline, Trinitrotoluene.

$^{\Omega}$ Canada has banned lindane.



F5.17. Synthesis of Hb and mechanism of action of Pb, directly (*) and indirectly by feedback.

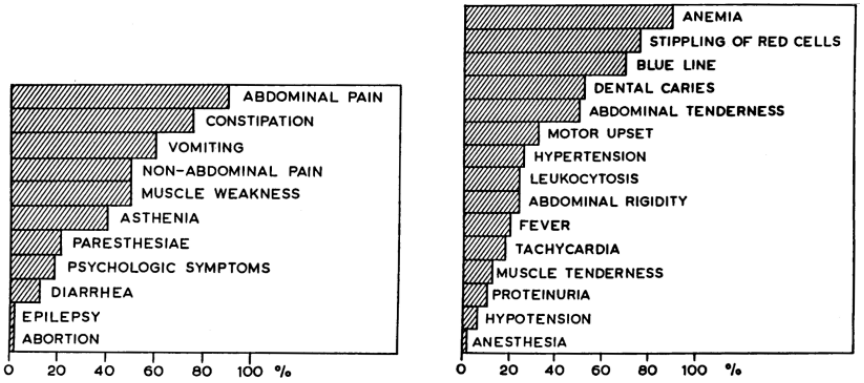
5.2.3.6. Example: Lead Poisoning

Lead intoxication is a on-going problem in numerous industries (smelters, brass foundries, painters, miners, garage workers, paint removal by blowtorch or power sander). Hypertension, reproductive problems, decreased kidney and brain function are common. Hematologic symptoms are generally chronic in nature, and marked by the insidious development of:

(1) chronic hemolysis,
(2) microcytic, hypochromic anemia, and
(3) sideroblastic (iron-related) changes in bone marrow.

Lead translocates to the long bones where erythropoiesis occurs, interfering with the synthesis of the heme (porphyrin) precursor to hemoglobin (F5.17), by inhibiting the key enzyme ferrochetalase, the last step in heme synthesis. In this scheme, the initial step in the heme pathway is controlled by a feedback mechanism on the enzyme aminolevulinic acid synthetase (ALA-S) based on the body's need for heme. With sufficient heme being manufactured, ALA-S does not function, whereas with the blocked ferrochelatase, there is no heme formed and ALA-S continues to make aminolevulinic acid, which is used by other enzymes to make porphyrin intermediates. Levels of these intermediates build up in the body, urinary excretion of ALA is elevated (one diagnostic test) as is excretion of a number of the protoporphyrins (both fecal and urinary excretion are diagnostic).

The symptoms and signs of intoxication are shown in F5.18. One specific sign of plumbism is the lead “line”, a deposit of blue-black lead sulfide deposited into the gums immediately above the teeth (F5.19).



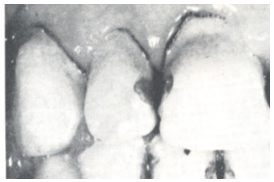
F5.18. Symptoms (left) and signs (right) in lead poisoning. *Dagg et al.*

Typical hematologic changes of lead poisoning in adults (F5.20) include:

1) hypochromic, microcytic erythrocytes,
2) basophilic stippling in erythrocytes: "granules" of semi-precipitated protein,
3) presence of siderotic granules (containing iron),

4) reticulocytosis - reticulocytes being released into blood,
5) increased osmotic fragility of erythrocytes, erythroid hyperplasia in bone marrow which causes #1 above.

F5.19. Lead lines. *Belknap, Wintrobe, 1974.*



	Normal	*Lead Poisoning*
Hemoglobin (g/dl)	♂14–18 ♀12–16	10.7 (8–13)
Volume Packed Red Blood Cells (l/l)	♂0.40–0.54 ♀0.37–0.47	0.35 (0.29–0.43)
Mean Cell Volume (fl)	89 (83–96)	79 (70–92)
Mean Cell Hb Content (g/dl)	34 (32–36)	31 (27–36)
Reticulocytes (%)	1.6 (0.6–2.7)	4.4 (1.5–11.6)
Stippled cells (%)	rare	1.8 (0.1–7.5)
Leukocytes ($\times 10^9$/l)	4–11	4–15

F5.20. Hematologic changes in the anemia of lead poisoning in adults.
Wintrobe, 1974.

If you have been intoxicated with lead, what can you do? EDTA can be used to chelate lead out of the body. For milder forms of intoxication, it appears that supplements of 1.2 g per day calcium can reduce blood levels of lead by 31 %[23].

5.2.3.7. Example: Benzene Poisoning

A study of hematotoxicity among Chinese workers heavily exposed to benzene (Rothman at al.) produced the results of F5.22: a decrease in white blood cells, absolute lymphocyte count and platelets.
Although drugs (chloramphenicol, sulphonamides, acetazolamide, phenylbutazone, mephenytoin, hexachlorocyclohexane) and anti-cancer agents (methotrexate, alkylating agents such as BCNU) can induce aplastic anemia (failure of bone marrow stem cells to produce an adequate number of erythrocytes, granulocytes, and platelets), benzene accounts for 5 to 10 cases of fatal aplastic anemia per year.

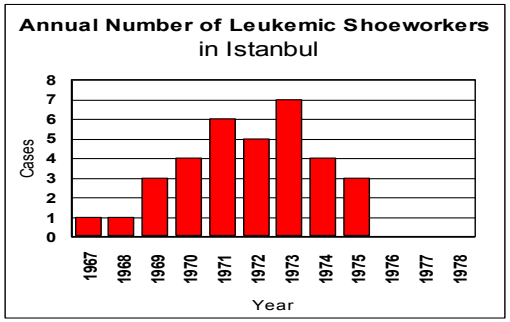
	WBC Mean ± sd (range)	Absolute lymphocyte count Mean ± sd (range)	RBC Mean ± sd (range)	Hematocrit Mean ± sd (range)	MCV Mean ± sd (range)	Platelets Mean ± sd (range)
Control (n = 44)	6.8 ± 1.7 (3.1–12.5)	1.9 ± 0.4 (1.1–2.8)	4.7 ± 0.6 (3.0–5.6)	42.0 ± 5.6 (27.7–50.7)	88.9 ± 4.9 (71.2–96.6)	166 ± 59 (62–313)
Exposed						
≤31 ppm (n = 22)	6.4 ± 1.8 (3.5–11.1)	1.6 ± 0.3 (1.1–2.5)[a]	4.6 ± 0.6 (3.2–5.7)[b]	41.2 ± 5.7 (29.5–49.7)	89.8 ± 3.9 (80.1–99.8)	132 ± 45 (59–204)[b]
>31 ppm (n = 22)	5.6 ± 1.9 (3.8–11.4)[a]	1.3 ± 0.3 (0.9–2.3)[c]	4.2 ± 0.6 (3.5–5.3)[c]	38.8 ± 5.3 (31.6–50.9)[b]	92.9 ± 3.4 (85.7–98.3)[c]	121 ± 43 (65–216)[a]

F5.22. Hematotoxicity among Chinese Workers heavily exposed to Benzene.

T5.21. Effects of Exposure to Benzene

Concentration in PPM	Effects
10,000	Death after a few hours
4,000	Narcosis
200-400	Risk of very severe pancytopenias Abnormal blood values in 50-80 % of cases
125-200	Considerable risk of leukemia Severe pancytopenias
65-125	Milder forms of pancytopenias & other cytopenias Acute, headache & fatigue after a few hours
40-65	Hemocytopenia
25-40	Blood cells affected Reduced blood Hemoglobin
10-20	Critical level for leukemia risk
1-10	Chromosome Damage
1	Odor Threshold & OSHA Exposure limit

Before 1950, benzene was responsible for approximately 60 % of secondary (toxic) aplastic anemias, of which 2 to 4 % progressed to acute non-lymphatic leukemia. Benzene is considered to be a human carcinogen, responsible for acute myelogenous leukemia (AML).
In Turkey, where benzene was a common constituent of glues for leather shoes, the incidence of AML climbed to a peak of 7 cases in a study population in 1973. Of some 44 patients with aplastic anemia, 6 died of AML. With introduction of restrictive measures on the use of benzene, the incidence of AML declined within three years to zero (F5.23).



F5.23. Leukemia and benzene exposure.

Occupational levels have been high in the past, but government regulations have forced their reduction to a TWA of 1 ppm.

In a study, Infante and White demonstrated that less than one week of exposure at 10 ppm resulted in chromosomal damage to bone marrow cells, significant depression of bone marrow and disturbances of immune function[4]. It was felt that these observations were highly significant, given the fact that most leukemias and related disorders seem to involve stem cell abnormalities and immuno-deficiencies.

5.3. ImmunoToxicity

5.3.1. Overview of the Immune system

The primary function of the immune system in mammals and lower species is protection from microbial pathogens (bacteria, virus, fungi and yeasts) and tumors.
Lymphocytes play an essential role in immune responses. They are divided into B and T lymphocytes, based on the presence of surface (CD) markers.
T lymphocytes constitute 55-75 % of circulating lymphocytes, and express specific surface markers (CD2 and CD3).
B lymphocytes first develop in the fetal liver, and later in the bone marrow. They constitute 10-20 % of peripheral blood lymphocytes, and express specific surface antigens (CD 21 and CD22).
Antigen-presenting cells (APCs) have the ability to present antigens to lymphocytes in a way that they can be recognized. B lymphocytes recognize self antigens, whereas T lymphocytes recognize antigenic peptides associated with major histocompatibility complex molecules, which includes both self and nonself antigens. The main types of APCs are Langerhans cells, dendritic cells and macrophages.

5.3.2. ImmunoToxicology

Immunotoxicology is concerned with the detrimental effects of foreign substances (xenobiotics) on host immunity, either as a consequence of the activity of a substance or its metabolites on the immune system or as an inappropriate immunological response to that substance. It includes immune responses to xenobiotics that result in allergy and other forms of immune injury.

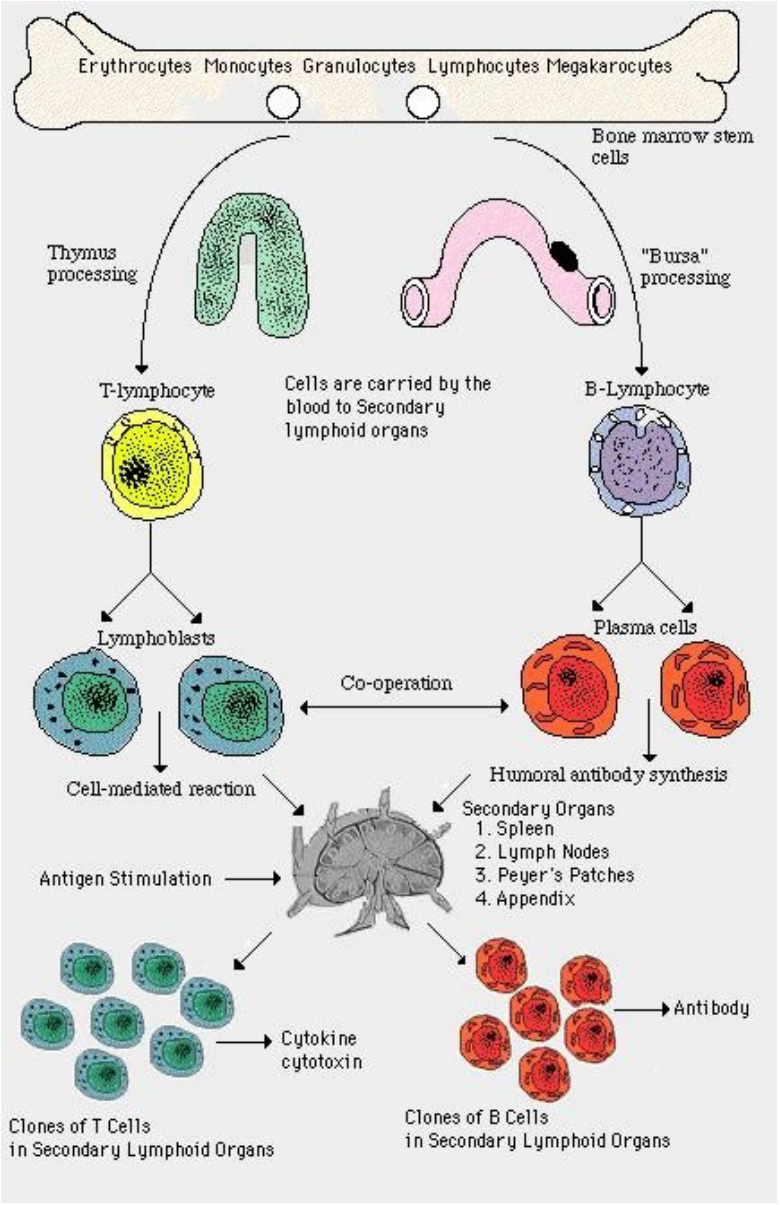
5.3.3. Immune Bioactivation of Xenobiotics

Xenobiotics can modulate the function of the immune system at several levels and can have two opposite consequences: immunosuppression or immune overstimulation. Immunosuppression can result in a diminished resistance against infection, while immunostimulation can result in hypersensitivity reactions, allergies, and autoimmune disease.

5.3.3.1. Immuno-Suppression

A number of xenobiotics can suppress the maturation and development of immune cells and cause immunosuppression. For example, benzene is myelotoxic, because its reactive metabolites can severely damage progenitor cells in the bone marrow, leading to a pancytopenia. This general hematopoietic cell destruction also includes the lymphocyte precursor cells (T and B cells), so most specific immune system cells will be compromised.

Immuno suppression can also be caused by other mechanisms occurring in other parts of the immune system. A widely cited example is the dioxins. The cause of dioxin-induced T cell depletion is found in the thymus, the major site where T cells differentiate. Indeed, after small doses of TCDD, animals exhibit a decrease in thymus mass, leading to thymic atrophy.



F5.24. Immune System.

5.3.3.2. Immuno-Stimulation

In contrast to xenobiotics such as TCDD, which suppress the function of the immune system, other xenobiotics can enhance the function of the immune system. Such an immuno-stimulatory effect can get out of control, and result in harmful reactions. These forms of reactions are rare, but they are toxicologically relevant because the degree of the adverse effects can be severe.

5.3.3.2.1. Hyersensitivity Reactions and Allergies

Two basic forms of such xenobiotic-induced immunostimulation are toxicologically relevant in humans. The first type comprises allergic reactions, which are most commonly known as chemically induced respiratory tract allergies or contact allergies in the skin.
In allergic reactions, the immune response is directed against toxicant-modified antigens, involving antibodies and T cells directed against toxicant and antigen.

F5.24a. A worker with protective breathing gear unloads a truckload of barley. The 1000 $ helmet is battery powered with filters.

5.3.3.2.2. Autoimmune Reactions

Multiple chemical sensitivity (MCS) is an adverse reaction to low levels of many common chemicals. Symptoms may occur after toxicant exposure, by inhalation or contact. Clinical ecologists believe MCS is a form of immune dysfunction caused by the insidious accumulation of exogenous chemical exposures over a lifetime. Another biologically oriented hypothesis is that MCS represents an atypical biological consequence of chemical injury, or injury to the respiratory tract after an acute inhalational episode. A more recent concept has focused on the relationship between the mucosa of the upper respiratory tract and the limbic (nervous) system.
Autoimmune diseases, such as *lupus,* disproportionately strike women.

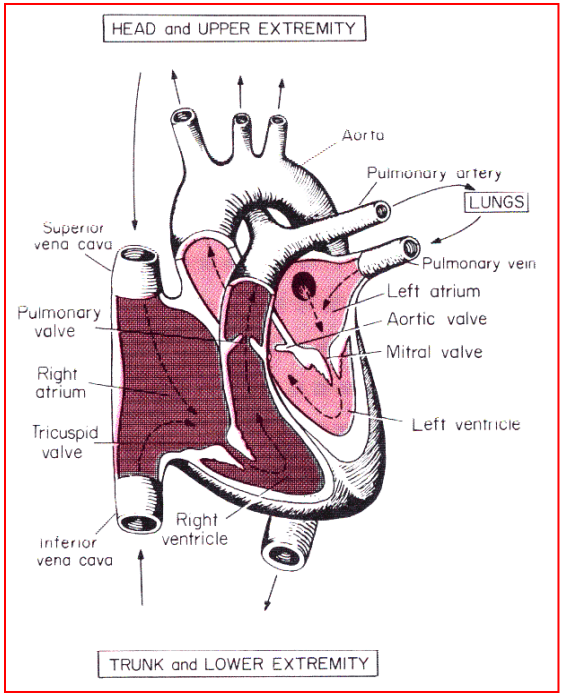
5.4. CardioToxicity

Toxicity of chemicals on the heart must include effects not only on the musculature of the heart (F5.25) but on the nervous tissue that controls the ventricular muscle (Purkinje system), the vasculature of the heart, and the peripheral blood vessels throughout the body.

5.4.1. Cardiac Physiology

The actions of agents on the heart are usually designated as:

1) chronotropic effects - relating to "time", a slowing or speeding up of the heart rate,
2) inotropic effects - relating to the force of contraction,
3) dromotropic effects - relating to nerve fibers and signal conduction.



F5.25. Basic cardiac morphology.
Guyton, 2001.

The major problems encountered in the heart itself usually involve interference either

(1) with the normal electrical activity of the heart musculature (atria, ventricles), thereby altering regular beating, or

(2) with the blood supply to the heart, resulting in ischemia (lack of oxygenation), with subsequent damage to the cells, and an interruption of normal electrical flow. Such damage is usually monitored by the electrocardiogram (ECG), specifically the PQRS complex and the T-wave repolarization, with measurements being taken as "point-to-point" and "time-to-points distances" in the ECG to identify the abnormalities (both timing and activity).

Many chemical agents found in industrial settings exert a direct effect on the peripheral blood vessels (constriction or vasodilatation), with subsequent changes in blood pressure causing hypertension or hypotension (severe headaches, dizziness or fainting). Many workers may be on medication and exposures in the workplace may either antagonize the effects or potentiate them, further confounding the issues.

5.4.2. Chemicals causing Cardiac Toxicity

Many of the *arrythmogenics* listed below are also *anesthetics*. Disturbances of the heart rhythm can be life-threatening, but note that occasional heart beat irregularities are also common in health, as shown by Holter monitors: chaotic behavior may be a hallmark of life, with rigidly periodic rhythm being pathognomonic…

Carbon tetrachloride, Chloroform Methyl bromide, Chloropentafluoroethane, Methyl chloride, 1,2-Dibromotetrafluoromethane, Methylene chloride, Dichlorodifluoromethane, Monochlorodifluoroethane, cis-Dichloroethylene, Monochlorodifluoromethane, trans-Dichloroethylene, Octafluorocyclobutane, 1,2-Dichloropropane, Propyl chloride, Dichlorotetrafluroethane, 1,1,1- Trichloroethane, Difluoroethane, Trichloroethane, Ethyl bromide, Trichloroethylene, Ethyl chloride, Trichlorofluoromethane, Fluorocarbon 502, Trichloromonofluoroethylene, 1,2- Hexafluoroethane, Trichlorotrifluoroethane, Isopropyl chloride, Trifluorobromethane.

T5.26. Arrhythmogenic Halogenated Hydrocarbons.

5.4.2.1. Gases

High concentrations of some gases (Table 5.27) affect hemoglobin in erythrocytes, reducing their oxygen-carrying capacity. Hypoxia ultimately affects the heart. Hypoxia (reduction of oxygen in body tissues below physiological levels) may cause angina (cardiac pain without tissue destruction), arrhythmias, and myocardial infarction (pain with destruction of cardiac tissue). With increasing concentrations of CO, for example, peripheral vascular effects occur prior to any serious action on the heart[2].
Industrial scenes are related to incomplete combustion of fuels: furnaces, boilers, automobiles, with hazards increased in cold weather, because of closed doors and windows.

T5.27. Cardiovascular toxicants.

GASES
CO_2, CO, CS_2, CN, H_2S, NO_x, SO_2, etc.
produce anoxia, hypoxia, with effects on hemoglobin and tissues hemoproteins.
ALCOHOLS/ALDEHYDES
ethanol-acetaldehyde, aldehydes in general, dihydroalcohols are cardio-depressants causing negative inotropic effects, cardiomegaly.
METALS
Cd, Pb, Co, Mn, Ba
produce negative inotropic and dromotropic effects as well as structural damage to the heart.
HALOGENATED ALKANES/ALKENES
degreasing solvents, freons, anesthetics (halothane, chloroform), dry cleaning fluids
elicit negative chronotropic and inotropic effects, resulting in dysrythmias, sensitization of cardiac muscle to catecholamines.
ORGANIC NITRITES/NITRATES
cardiac drugs (amyl nitrite, glyceryl trinitrate), room deodorizers (butyl and isobutyl nitrite), explosives (nitroglycerine, trinitrotoluene)
have as a dominant effect vasodilatation of peripheral blood vessels, pooling blood in extremities, production of methemoglobin.

5.4.2.2. Alcohols/Aldehydes

Ethanol (and its metabolite acetaldehyde), dihydroxyalcohols (propylene glycol, polyethylene glycols) and other relatively volatile chemicals cause a *negative inotropic* effect (decrease in force of contraction). Ethanol is effective at 75 mg per 100 ml, just below the "legal limit". Arrhythmias may result from chronic exposure to ethanol, sometimes resulting in ventricular fibrillation and sudden death.

Chronic exposure to industrial alcohols may induce
(1) cardiomegaly (heart enlargement, dilation of chambers),
(2) interstitial fibrosis, and
(3) increased lipids in myocardial cells.

Aldehydes at higher concentrations may stimulate the release of catecholamines, with a sympathomimetic acceleration of the heart rate (positive chronotropic effect).
It is believed that the cardiodepressant (acute) effects of alcohols and aldehydes are due to an inhibition of intra-cellular calcium transport. Calcium is essential for sodium-potassium transport, inter-cellular communication in the transmission of electrical impulses, and the release of neurotransmitters.

However, aldehydes might stop the formation of *advanced glycation end products*, which would explain the positive effect on cardiovascular health related to wine consumption[16].

Various phenols (phenol and dinitrophenols) are excellent inhibitors of sodium-potassium adenosine triphosphatases

(ATPases). The enzymes pump sodium out of depolarized cells (nerve cells as well as cardiac muscle cells) in order to achieve repolarization. Such agents have a *negative chronotropic* effect and a *negative dromotropic* effect, the heart beating more slowly.

5.4.2.3. Heavy Metals

Lead, cadmium and cobalt have selective cardiotoxic properties, producing *negative inotropic and dromotropic effects*, as well as structural (degenerative) changes in cardiac muscle. The cardiomyopathy seen in drinkers of illicit "moonshine" during prohibition was attributed to lead. The metal originated in the homemade condensing coils soldered with lead, or use of old automobile radiators.

A study in women 65 and older indicates that higher blood lead increases the risk of premature death. Women with less than 8 µg/dl of blood are used as a control, while those with higher lead had a 60 percent increased risk of dying, after accounting for factors such as age, smoking and drinking[24].

A bizarre cardiomyopathy affecting habitual beer drinkers in Quebec City was related to the presence of a chemical additive containing cobalt sulphate. The mortality, related to congestive heart failure, was 46.1 %[3-7]. A divalent cation, cobalt can substitute for calcium but, perhaps more importantly, it can interfere with hemoprotein (hemoglobin, cytochromes) synthesis, resulting in hypoxic conditions both in the blood and tissues, with impairment of Phase I bio-transforming enzymes (mono-oxygenases).

Other heavy metals (manganese, nickel) can block calcium channels in heart muscle.

Barium is a potent arrhythmogenic agent, as little as 5 mg/kg (intravenous) causing ventricular tachycardia (it is used as a model for screening antiarrythmic drugs).

5.4.2.4. Halogenated Alkanes/Alkenes

Degreasing solvents, freons, trichloroethylene, perchloroethylene and anesthetics (chloroform, halothane, methoxyflurane) exert similar *negative chronotropic, inotropic, and dromotropic* effects on the heart. Medically, chloroform was the first agent demonstrated to sensitize the cardiac muscle to catecholamines[δ] (epinephrine, norepinephrine) during surgical procedures. Halothane possesses the same property, as do most other halogenated (fluorinated and chlorinated) solvents. The negative inotropism increases with up to 4 chlorines and chlorinated alkenes (trichloroethylene) are more potent than trichloroethanol.
Dysrhythmias result and, if the ECG is done at an appropriate time, one can see skipped beats, irregularly spaced beats, extra beats, all out of phase with the regular electrical activity. The irregularities may be spasmodic (periodic), necessitating the monitoring of patients for a length of time before the effects are seen. Frequently, the arrhythmias will be related to excessive excitation, which produces an epinephrine "storm", triggering the responses.
Polychlorinated biphenyls (PCBs) are used as electrical insulators, as lubricants, as additives to make plastics more pliable, in adhesives, even as a component of some inks. Being lipophilic, these pollutants settle into fats and oil. David Carpenter of the University of Albany and his colleagues discovered a dose-dependent climb in risk of both systolic and diastolic hypertension with rising blood concentrations of PCBs. Those with less than 1.24 parts per billion were

[δ] Cathecolamines accelerate the heart rate.

designated the reference group. The middle third hosted contaminant levels between 1.25 and 3.64 ppb. Group three: everyone higher than that. The chance of having unhealthy systolic blood pressure — that maximum pressure, which occurs as the heart muscle contracts — was tripled in the middle PCB-contamination group and almost quadrupled in the highest PCB group.[26]

5.4.2.5. Organic Nitrates

The treatment of anginal pain with amyl nitrite is well known. Crushing a "pearl" of this highly volatile liquid in a cloth and inhaling it produces a rather dramatic relief of pain. Similar effects can be achieved with glyceryl trinitrate tablets placed sublingually and causing a longer action (10 to 30 minutes). By both routes, the heart and coronary vessels receive a relatively high dose of agent which causes localized vasodilatation of the coronary vessels and an improvement of local circulation with a relief of the ischemia. Longer-acting (6-8 hr) organic nitrates (erythrityl tetranitrate, pentaerythrityl tetranitrate) are also used. There are a number of nitrated compounds, particularly in the explosives industry, that cause the same effects.

With restrictions on prescription amyl nitrite, butyl and isobutyl nitrite have been used as room deodorizers in spray applicators, but also as drugs of abuse for enhancing sexual pleasure. The inhalation route produces a "rush". Butyl nitrite has a greater hypotensive effect than lower analogues but not as great as the isobutyl derivative. The toxic effects of nitrite inhalation are shown in Table 5.28, most of them from the peripheral vasodilatation. In addition, nitrites will diminish cardiac contraction without affecting heart rate (decreased efficiency). Other studies noted bradycardia (vagal nerve stimulation) and heart block at higher doses. Exposure has also been associated with tachycardia-hypotension, sometimes bradycardia-hypertension. Tolerance develops with repeated use, methemoglobinemia becoming a complication.

Fatalities have occurred in workers exposed to organic nitrates after strenuous exercise, 1 to 2 days following cessation of exposure. There is a loss of vascular tone with pooling and trapping of blood in the veins of the lower extremities. Hypertrophy of the left ventricle occurs in workers handling nitro-glycerine (glyceryl trinitrate). Considerable amounts of methemoglobin are formed with chronic exposure.

F5.28. Toxic effects of nitrite inhalation.

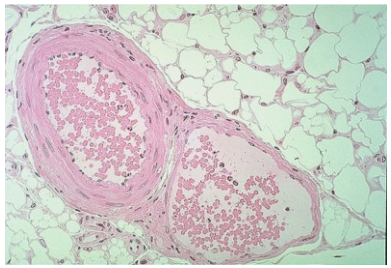
Rapid flushing (face)
Pulsation in head, headache
Cyanosis, shallow respiration, respiratory failure
Confusion, weakness, vertigo, fainting, hypotension
Motor unrest
Thready pulse
Clammy skin

Trinitrotoluene (TNT) is an excellent example of toxicity in the explosives industry. In a Japanese munitions plant, workers complained of dizziness, severe headaches, lethargy, and general malaise. The measurement of blood pressure showed marked decreases in systolic/diastolic ratios before and after work, from lowered systolic pressure. When handling the gel-like mixture, the workers wore surgical rubber gloves, but never changed the gloves, using them day after day, and not discarding them. With a fresh pair of surgical gloves at the beginning of the shift, no change in blood pressure was seen later in the day. Even more impressive was the fact that regularly replaced cotton (washable) gloves protected the workers. The concentration of nitrated compounds were building up over time in the gloves. The compound was being

absorbed by the hot and sweating skin, leading to vasodilatation.

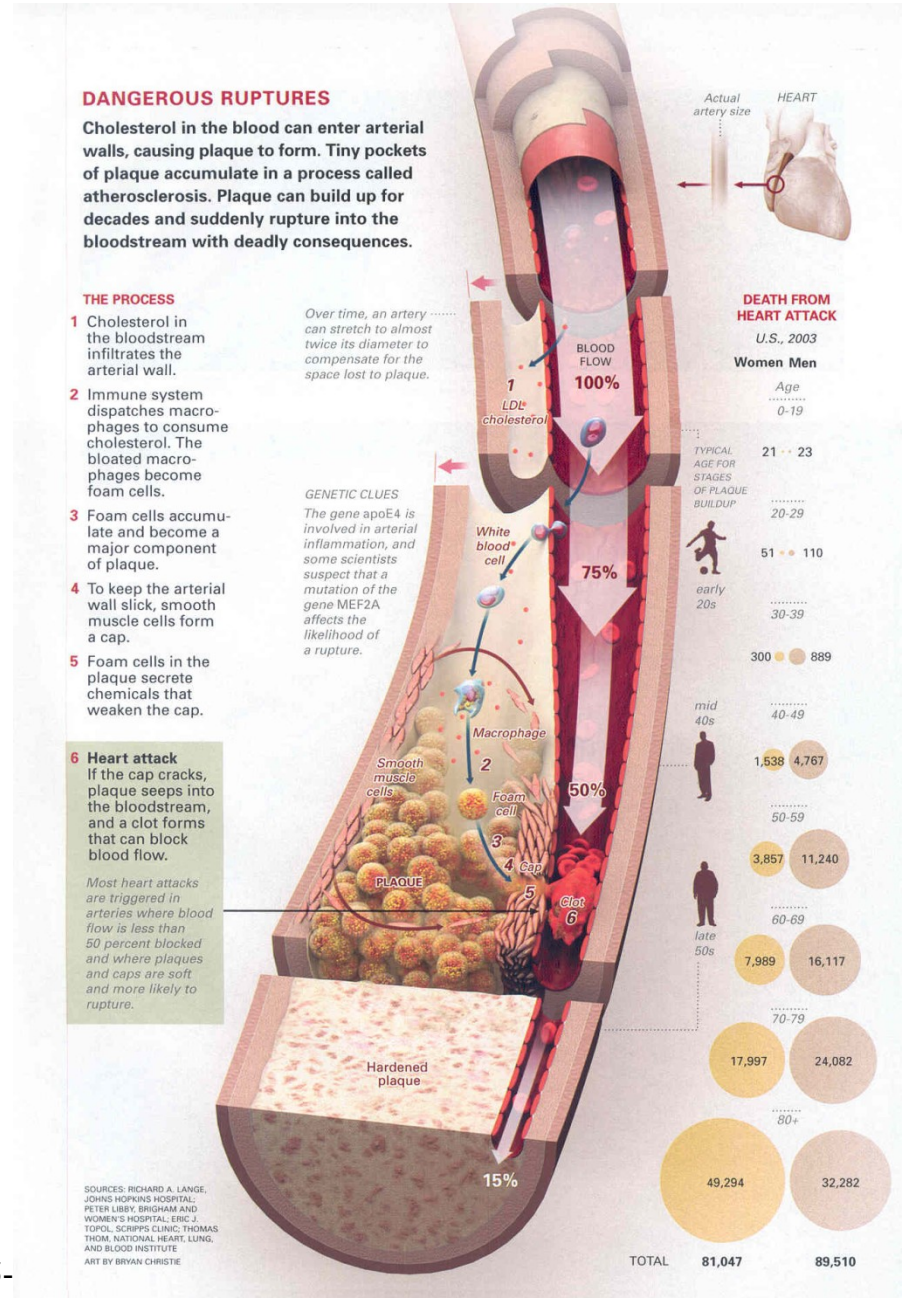
There are a large number of different nitrated compounds used in present-day military and civilian explosives. Miners, re-entering an area in which blasting has taken place, frequently experience severe headaches unless they wait for the ventilation system to clear the area of volatiles. Military personnel found that a "high" could be achieved by putting a small piece of explosive "putty" under the tongue or in the cheek. Nitrate workers can die suddenly after exposure ceases (on weekends and holidays), and may exhibit “Monday morning angina”. Re-exposure may trigger rebound vasospasms and fibrillation.

5.5. Vascular Toxicity



F5.29. Cross section of an artery and a vein. Notice the musculature around the artery.

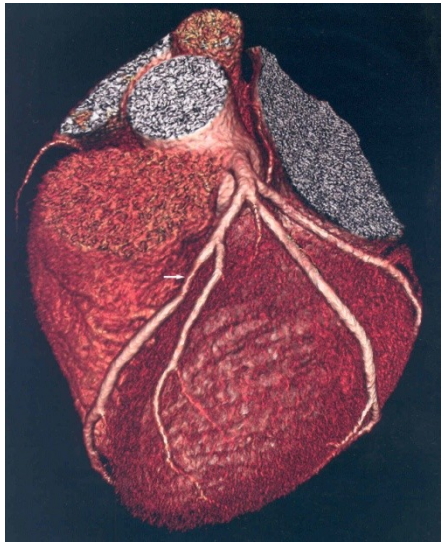
Since arteries must withhold more blood pressure, and control delivery of blood to tissues, they have a more substantial wall than veins (F5.29). The Smooth Muscle Cells in the arterial wall can be injured after the endothelium has been damaged by a toxicant. In response, they may proliferate, creating a bulge which may be invaded by macrophages. A toxicant may also penetrate through the endothelium to create a mutation in the smooth muscle cells, followed by proliferation. Both mechanisms trigger chronic inflammation and narrowing of the artery. The physiological mechanisms leading to a vascular accident are shown in F5.30.



F5.30. Mechanisms of endothelial injury.
National Geographic, Feb.2007.

Exposure to environmental chemicals can also disable vascular control. Phenanthraquinone, on oily component of diesel soot, inhibits vessel dilatation in older rat muscle, but not in younger ones[22].

5.5.1. Atherosclerosis



Some degree of arterial obstructions are found even in young men. Since coronary arteries are not that large (F5.31), it is not surprising that by-pass operations are becoming common.

F5.31. Narrowing of a coronary artery (white arrow).
National Geographic, Feb.2007

It appears that mitochondrial damage foreshadows the onset of atherosclerosis. Narrowing of arteries due to atherosclerosis can be seen in a beating heart using a 10-second advanced imaging technique (Computed Tomography).

5.5.2. Agents Toxic to Blood Vessels

There are four large classes of agents capable of intoxicating blood vessels: gases, heavy metals, dust particles and others.

5.5.2.1. Gases

Auto exhaust, Carbon monoxide, Nitric oxide, Oxygen, Ozone

5.5.2.2. Heavy Metals

Arsenic, Beryllium, Cadmium, Chromium (deficiency), Copper (Chronic and Acute), Copper (deficiency), Germanium, Indium, Lead, Mercury, Selenium, Thallium

5.5.2.3. Dust Particles

Animal and human studies show that dust particles less than 2.5 µm in air pollution irritate the lungs and provoke inflammation of the blood vessels. Over time, this leads to thickening of the artery walls[17]. Long-term exposure to air pollution enhances one's chances of dying from heart attack or stroke. The more pollution there is around your home, the thicker the walls of your carotid artery becomes[13]. Such particles, irrespective of their chemical makeup, are able to suppress secretion of endogenous vasodilators, such as nitric oxide.
Breathing 0.15 µg/m^3 of exhaust particles, typical of heavily polluted cities, is capable of immediately rising diastolic blood pressure by 6 mm of Hg. Once the exposure ends, the effect does not last more than a few minutes[18].

5.5.2.4. Others

Allylamine, p-Aminopropionitrile, Boron, Butadiene, Carbamylhydrazine, Carbon disulfide, Chlorophenoxy Herbicides, Dimethylnitrosamine, Dinitrotoluenes, 4-Fluoro-l0-methyl-12-benzyanthracene, Glycerol, Hydrogen fluoride, Hydrazinobenzoic acid, Paraquat, Polycyclic aromatic hydrocarbons, Pyrrolizidine alkaloids, Organophosphate
Pesticides, T-2 toxin.

Coronary Artery Disease can occur from exposure to volatile carbon disulfide (CS_2) through interaction with a number of enzymes.

A Case of Cardiac Failure

A 51-year old cement finisher spends 2 months spraying and mopping urethane coating in a parking.
He develops rash on his legs and quits because of health concerns. 2 days later, without a known history of heart disease, he is found dead by his wife.

Coroner: cardio-pulmonary arrest, probable acute myocardial infarction.
Two other workers testify of health problems which led them to quit the same job.

MSDS: two part mixture containing polyols, solvents, un-reacted methylene bis-4 cyclohexyl-isocyanate, xylene, 2-ethoxyethyl acetate, un-reacted Toluene Di-Isocyanate.
Measurements show as much as 1 mg/m^3 of TDI (7 times the allowed limit). Once sensitized to TDI, individuals can react to concentrations below the odor threshold.

Bronchospasm occurring from exposure to **toluene diisocyanate** can become permanent, resulting in poor lung ventilation. The ensuing hypoxia causes vaso-constriction*, pulmonary artery hypertension and finally right heart failure.

*Vascular reaction in the lung is contrary to reaction in other parts of the body. If vessels are isolated from the lung, they no longer show this reaction (an unknown substance is released by lung tissue).

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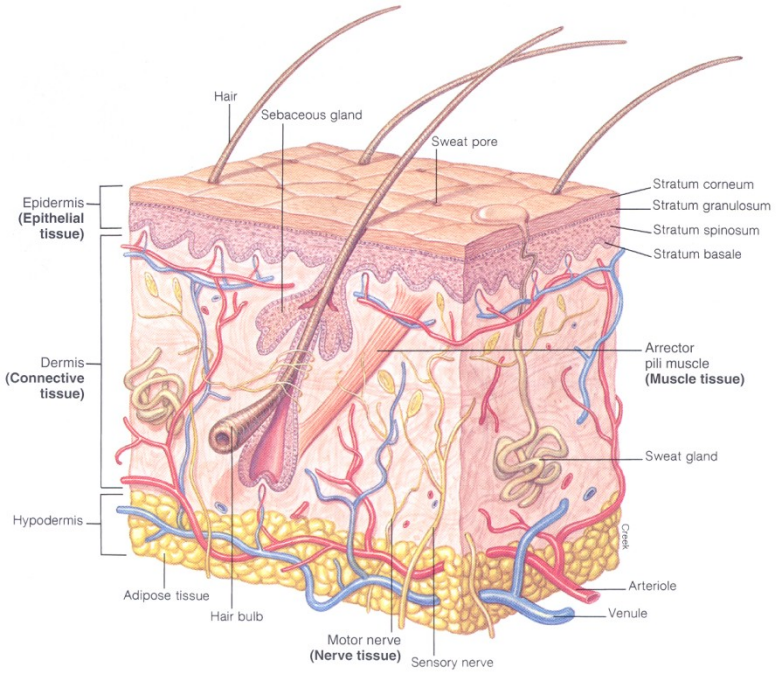
Dermatotoxicity

6· Dermatotoxicity



F6.1. Cross-section of the skin. *Fox, 2004.*

Skin is the largest "organ", weighing some 3.5 kg (6% of body weight) and covering an area of 2 m^2 in the average adult. 40% of occupational diseases involve the skin, resulting in prolonged discomfort and expense.

6.1. Skin Anatomy

There are tens of thousands of types of *spiders*, and practically all of them are venemous, because they require venom to kill and digest their prey. But *only a few* spiders are of lethal consequence to humans, because they possess fangs *large enough to penetrate human skin*.

The skin is composed of three layers (F6.1),

- the *stratum corneum* (*horny layer*),
- the underlying *epidermis*, the clear cell layer and
- the subcutaneous complex *dermis* containing nerve endings, muscle fibers, eccrine (sweat) glands, sebaceous glands, hair follicles, connective tissue, lymphatic channels, blood capillaries, lipids, etc.

The *stratum corneum* of dead keratinized cells is the principal barrier layer, composed of high lipid but low water content. The normal role of this layer is to retain water and heat (against dehydration and hypothermia), and to prevent the entrance of microorganisms and most toxic substances.

The *epidermis* is a compacted layer of epithelial cells with tight junctions in the *stratum granulosum*.

The *dermis*, underlying the epidermis, is composed of diverse types of cells including hair follicles, sebaceous glands, eccrine (sweat) glands with a dense capillary bed and good blood flow.

Heat control is managed by perspiration release, cooling of the surface and heat exchange through the capillary bed.

Dermatologists claim that **female** skin is more susceptible to dermatitis, although there is no specific measurement to document this. It seems also that women are more often sensitive to **allergens** as opposed to **irritants**. For example, contact allergy to nickel is found in 15% of women vs 2% of men.

6.2. Skin Penetration

Absorption of chemicals from the skin depends on **surface area** (T6.2), on **skin thickness** (T6.3) and on **exposure** (F6.4). Alcohol in the blood is known to increase toxic compound absorption through the skin. The barrier is imperfect, and substances can penetrate under unfavorable conditions. The efficient absorption in the scrotal area (T6.3, 29%), for example, brings to mind Sir Percival Pott's scrotal cancer cases in young chimneysweeps, the efficient regional absorption of polycyclic aromatic hydrocarbons causing the localized cancers ! The epidermis is replaced about once a month, the stratum corneum about once every two weeks. Therefore, should penetration be exceedingly slow, the toxicant will eventually be shed.

6.2.1. Mechanical

The stratum corneum is breached (by cuts, abrasions or pilar openings). In the occupational setting, workers' hands always have cuts, nicks, and scratches and, in any dermal irritancy test, both intact and abraded skin are tested, since the effects might be quite different. Intact skin is a reasonable barrier whereas "damaged skin" (repeatedly stripped with cellophane tape to remove the stratum corneum) allows 5-fold more benzene to penetrate.

6.2.2. Solvents

Potentially toxic chemical dissolved in an organic solvent (gasoline, oils, trichloro-ethylene, toluene, xylenes, ketones) can penetrate more easily. Rubber solvent, in which traces (0.35%) of benzene occurred, did not enhance the absorption of benzene on single exposure, but did upon repeated exposure or upon application to the palm of the hand[8].

6.2.3. Aqueous Solutions

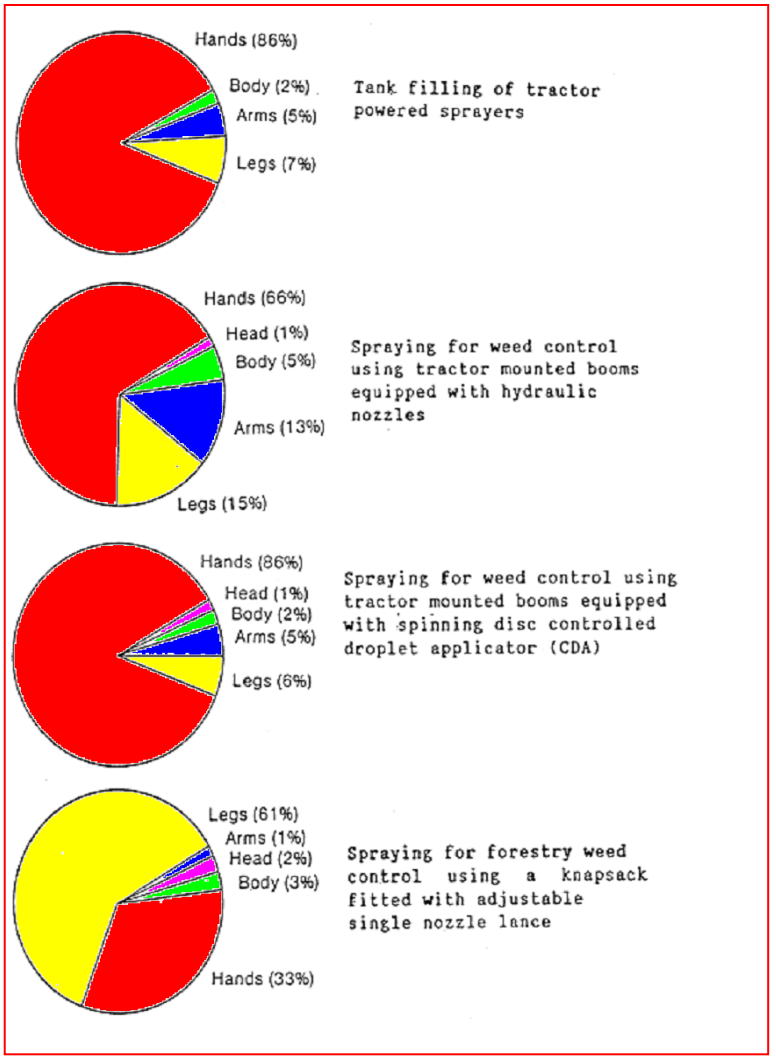
Little attention has been paid to the absorption of organic chemicals "dissolved" in aqueous solutions, e.g. wastewater, even potable drinking water used for bathing. The study of a component of gasoline, ethylbenzene, either pure or in aqueous solution, showed that the rate of absorption through human skin was higher for the agent (112 -156 mg/l) in aqueous solution (118-215 $\mu g/cm^2/hr$) than when applied pure (22-33 $\mu g/cm^2/hr$)[4]. More recent studies, examining the dermal absorption of toluene, ethylbenzene and styrene in aqueous solutions, representative of drinking water contamination, revealed that the dermal absorption of these contaminants has been consistently underestimated[5].
Skin penetration of chemicals in aqueous solution tends to decrease exponentially with molecular weight, and to increase with P_{OW} as well as with their water solubility.

6.2.4. Pesticides

Measurement of parathion absorption in pesticide application (F6.4) reveals that the forearms and hands account for 33 to 86% of the entire dermal dose[1]. On the basis of T6.2, T6.3 and F6.4, one could minimize exposure to pesticides by providing solvent-impervious gloves and appropriate headgear. This is what is recommended on many pesticide container labels, in addition to wearing an apron or coveralls while mixing and loading.
These experiments can be extrapolated to industrial chemicals such as benzene, PCBs, and nitroaniline, the results demonstrating a significant acquisition of agent in the epidermis (surface bound to stratum corneum) and dermis, as well as absorbed systemically[6].

T6.2. Surface of body regions in percent[1].	
Body Region	**Surface Area (% of total)**
Head	5.6
Neck	1.2
Upper arms	9.7
Forearms	6.7
Hands	6.9
Chest, back, shoulders	22.8
Hips	9.1
Thighs	18.0
Calves	13.5
Feet	6.4

T6.3. Urinary excretion of ^{14}C-parathion metabolites reflecting absorption rates from different anatomical sites[3].	
Region of Application	**% of dose excreted in urine over 24 hrs**
Forearm	1.77
Palm	2.45
Abdomen	3.86
Hand (dorsum)	6.03
Fossa cubitalis (elbow crease)	7.95
Scalp	8.88
Jaw angle	10.92
Post auricular (behind ear)	10.45
Forehead	10.96
Axilla (arm pit)	18.54
Scrotum	28.85



F6.4. Pesticide absorption by region: % contribution to total dose.

T6.5. Performance of Cutaneous Defense.		
Variable	**Black Skin**	**Caucasian Skin**
Abrasion resistance (strippings)	16	9
Stratum corneum layers	21.8	16.7
Density by sucrose gradient centrifugation	1.18	1.11
Thickness (µm)	6.5	7.2
Density (gm/ml)	1.68	1.39
Permeability to water (ml/cm^2 per 24 hrs)	0.4	1.4

6.3. Skin Metabolism

The skin has metabolic functions like any other organ, among which:

1. Synthesis of Porphyrins (cytochromes without the iron) and Heme (respiratory pigments),
2. Production of cytochromes P-450 and P-448,
3. Oxidation of Heme, Alcohols and Aromatic Rings,
4. Alicyclic Hydroxylation,
5. Deamination,
6. Reduction of Carbonyls and C=C,
7. Conjugation: Glucoronidation, Sulfation, Methylation.

Some of the skin enzymes, specifically

- aryl hydrocarbon hydroxylase (AHH),
- epoxide hydratase and
- glutathione-S-transferase,

can be strongly induced by repeated exposure to polycyclic aromatic hydrocarbons and coal tar through the aryl hydrocarbon receptor. This receptor is known for binding environmental toxicants, but it also regulates the balance of protein and anti-inflammatory responses of the immune system. Exposures to environmental toxicants such as dioxin may encourage the development of autoimmune diseases.

Many of the enzyme activities induced in the skin are directed against invaders such as bacteria and fungi, explaining why some have a toxic component. In the case of many petrochemical exposures, the enzyme AHH transforms chemicals landed on the skin into other compounds of stronger carcinogenic activity.
People who have psoriasis (a hereditary skin disease) cannot be induced for AHH. When exposed to coal tar, these people do not develop higher rates of skin cancer.

Skin tissue is rich in immune cells. Vaccines could be injected into the skin using about 20% of the dose used in intra-muscular injection, and still obtain the same protection.
A tight network of cells covering the entire body is formed in the skin by Langerhans cells. These cells ingest antigens present in the skin and transport them to lymph nodes, activating the immune system to protect the body against pathogens.

6.4. Contact Dermatitis

Contact Dermatitis, a most common injury, can be caused by any substance and takes a wide variety of clinical forms:

- Erythema (reddening of the skin)
- Purpura (bruise-like)
- Blistering
- Eczema, urticaria
- Rashes (weeping and oozing)

- Erosions of skin
- Hyperkeratosis (thickening, swelling)
- Pustules (small infections)
- Dryness and roughness (scales, itching)

6.4.1. Mechanism of Dermatitis

The effect usually occurs locally on the skin surface, from the degreasing, dehydrating, protein denaturation and osmotic pressure change induced by the toxic agent.
The resulting inflammation destroys the stratum corneum, allowing invasion by bacteria and a localized immune response. The condition may be difficult to treat, and persist for some time.
Once through the barrier, the toxicant may penetrate deeper into the subcutaneous layer and exert an effect on the pilosebaceous follicles, resulting in acneiform eruptions, aggravated by hot humid working conditions and exposure to grease and heavy oils.

There is a fair degree of **individual variation** among subjects. If DMSO (a test compound) in a concentration above 80% is put on the skin, stinging is produced in some subjects within minutes, in others within hours, with still others, you give up waiting…
Between 5 and 20% of the population can be classified as "stingers" because of their high susceptibility to many chemicals.

A large variety of compounds (T6.6) produce contact dermatitis. Generally (in increasing order of irritancy),

Alkanes < Alcohols < Aldehydes or ketones < Organic acids < Amines

Some compounds, like anthralin (a psoriasis medicine) induce an irritant response delayed by 8 to 24 hours.

T6.6. Skin Irritants in Industry

Water eliminates natural oils and swells live epidermal cells.

Cleaners solubilize-disorganize barrier lipids and natural moisturizing factors in the stratum corneum, denaturate protein, intoxicate membranes.
Soaps, Detergents, Bleach.

Oxidants and reducing agents (cytotoxicity, keratolysis).

Plant products, animal protein penetrate the skin through follicular shunts.

Alkalis denaturate barrier lipids, swell cells.
Inorganic: alkaline sulphides, barium hydrate and carbonate, sodium hydrate, carbonate, silicate and metasilicate, potassium hydrate and carbonate, ammonium hydrate and carbonate, calcium oxide, hydrate carbonate and cyanamide, trisodium phosphate.
Organic: Ethanolamines, Methylamine.

Acids denaturate protein, induce cytotoxicity.
Inorganic: Arsenious, Hydrofluoric, Chloroplatinic, Hydrofluosilic, Chlorosulphonic, Nitric, Chromic, Perchloric, Hydriotic, Phosphoric, Hydrobromic, Sulphuric, Hydrochloric.
Organic: Acetic, Maleic, Carbolic, Metanilic, Cresylic, Oxalic, Formic, Salicylic, Lactic.

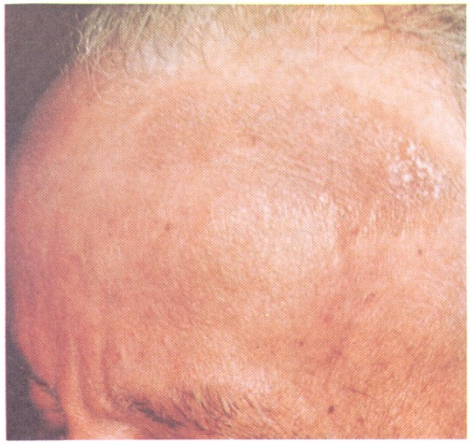
Elements and their salts:
Antimony and salts, Mercuric salts, Arsenic and salts, Nickel salts, Chromium and alkaline chromates, Zinc chloride, Copper sulphate, Silver nitrate, Copper cyanide.

Solvents solubilize barrier lipids, intoxicate membranes.
Petroleum solvents, Turpentine, Coal tar solvents, Terpenes, Chlorinated hydrocarbons, Carbon bisulphide, Esters, Alcohols, Ketones.

Acne producers disorganize barrier lipids.
Petroleum oils, Chloronaphthalenes, Cutting oils, Chlorodiphenyls, Pitch, Chlorodiphenyloxides, Tar, Solid chlorobenzols, Paraffin, Solid chlorophenols.

The natural pH of skin being 5.5, substances within the range of 5.5 to 7.4 are usually well tolerated by the skin.

In industry, the activities most likely to be associated with skin diseases are mixing, blending and grinding, painting coating and printing, dyeing, plastic processing, rubber compounding, mining, metal fabrication and woodworking.

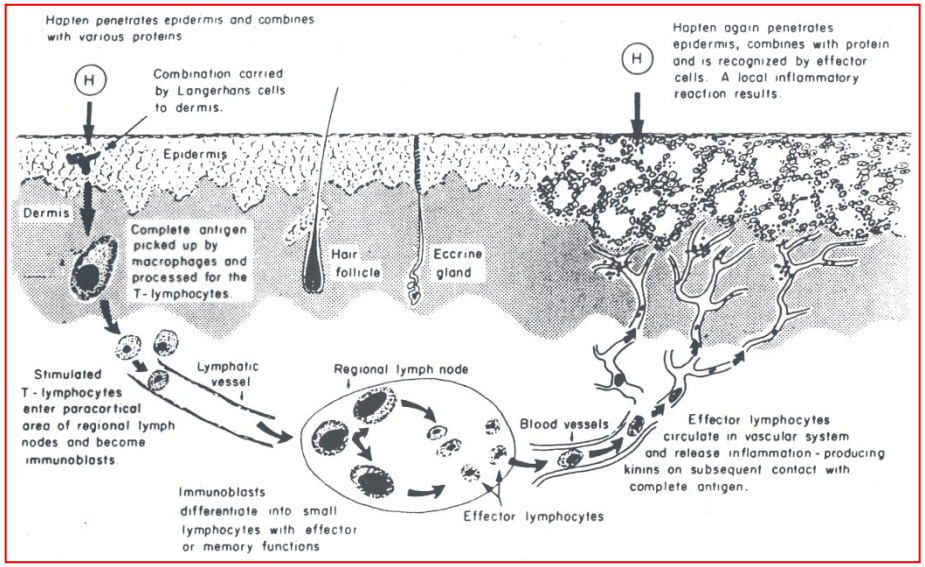


F6.7. Chronic eczematous contact dermatitis caused by leather components in a hatband.

6.5. Allergic Sensitization

Allergic Contact Dermatitis is a cell-mediated reaction (F6.8) that accounts for 30% of all occupational skin diseases. It may, in extreme cases, result in total permanent disability, in part because ordinary protective measures often prove ineffective in view of the small amounts of agent necessary to trigger reactions.

With so-called contact dermatitis "**irritants**", a previous exposure is *not* necessary, whereas for contact dermatitis "**allergy**", a previous exposure **IS** necessary. Typical delay in



F6.8. Mechanism of allergic sensitization.

sensitization is 10-21 days, and delay in reaction is 12-48 hours. With successive exposures to an allergen, an individual develops red spots often similar to **eczema** (with or without edema) which appear more rapidly and remain longer in later repeats.

Low molecular weight inorganics can cause allergic sensitization (ex: nickel, chromium in jewelry), but organics such as perfumes can also cause allergy.

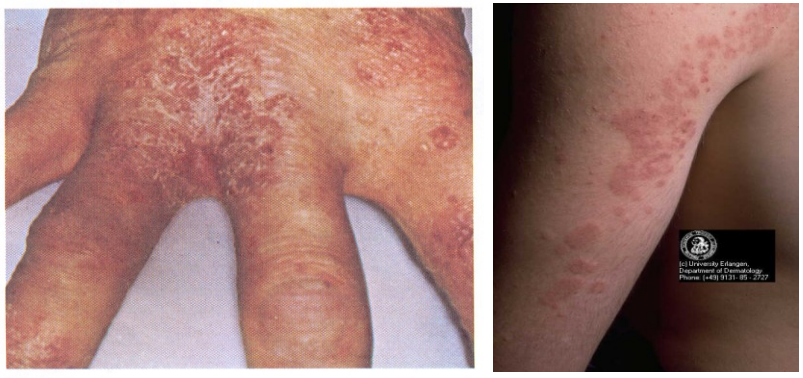
95% of substances in perfumes are actually synthetics derived from petroleum. 26 such contact allergens must be identified in perfumes, if marketed in the European Union:

Amyl cinnamal, Benzyl alcohol, Cinnamyl alcohol, Citral, Eugenol, Hydroxy-citronellal, Isoeugenol, Amylcin namyl alcohol, Benzyl salicylate, Cinnamal, Coumarin, Geraniol, Hydroxy-methylpentylcyclohexenecarboxaldehyde, Anisyl alcohol, Benzyl cinnamate, Farnesol, 2-(4-tert-Butylbenzyl)

Propionaldehyde, Linalool, Benzyl benzoate, Citronellol, Hexyl cinnam-aldehyde, d-Limonene, Methyl heptin carbonate, 3-Methyl-4-(2,6,6-trimethyl-2-cyclohexen-1-yl)-3-buten-2-one, Oak moss extract and Tree moss extract.

In digestive allergy, it is known that a larger time to breakdown in the gut for a protein means that it is more likely to be an allergen.
The term "**atopy**" designates an increased genetic susceptibility to the development of occupational dermatitis due to immune cell *hyperreactivity*. Atopic dermatitis is a T-cell mediated, eczematous skin disorder that affects 1% to 3% of adults.

Multiple chemical sensitivity is an adverse reaction to low levels of many common chemicals. Symptoms may occur after toxicant exposure by inhalation or contact. Clinical ecologists believe MCS is a form of immune dysfunction caused by the insidious accumulation of exogenous chemicals over a lifetime. Another biologically oriented hypothesis is that MCS represents an atypical biological consequence of chemical injury, or injury to the respiratory tract after an acute inhalational episode. A more recent concept has focused on the relationship between the mucosa of the upper respiratory tract



F6.9. Appearance of allergic reactions.

European Union Regulations 2010 on Cosmetic Products

Cosmetics include aromatherapy products, but exclude products used solely as medicines. The regulation forbids cosmetic products that may cause damage to human health when applied under normal conditions of use, or reasonably foreseeable conditions of use.

Animal Testing

Forbids testing a finished cosmetic, any of its ingredients or combination of ingredients, on an animal in order that the product may comply with Regulations and the manufacturer must not have tested or commissioned tests on animals of the finished product or its prototype or any of their ingredients. The cosmetic product must also not contain any ingredients that have been tested on animals by others for the purpose of developing new cosmetic products. These restrictions apply where there are authorized alternative methods of test (from 11 March 2013, to tests involving repeated dose toxicity, reproductive toxicity or toxicokinetics).

"Best Before"

If minimum durability is 30 months or less, it must be marked with a 'best-before' date. If minimum durability is more than 30 months, it must be marked with the period after opening for which the product can be used without harming the consumer.

Information available from the 'responsible person'

A responsible person is required to keep certain product information at the registered office address or the address detailed on the product. The product information must include the composition of the product. For perfume or perfume compositions in the product, you are only required to keep the name, code number and supplier identity. Qualitative information for all composites, and the quantitative information in relation to dangerous substances, must also be made easily available to the general public,

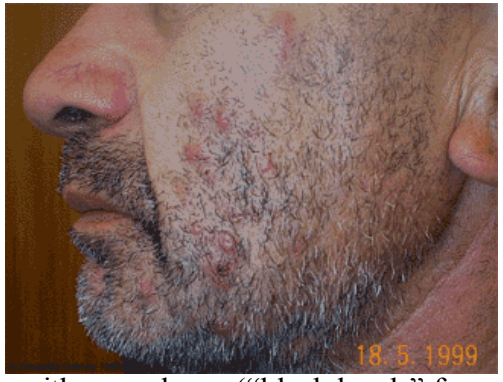
- the physico-chemical and microbiological specifications of the raw materials and the finished product, and the purity and microbiological control criteria of the cosmetic product,
- the method of manufacture, which shall be in accordance with good manufacturing practice,
- an assessment of safety for human health of the finished product, including the criteria as stipulated in the Regulations. There are additional criteria where the product is intended for use on children under three years old or exclusively for use in external intimate hygiene,
- the name and address of the person or persons, with the minimum qualifications as detailed in the Regulations, who carried out the assessments,
- existing data on the undesirable effects on human health resulting from use of the product. This information must also be made easily available to the general public,
- evidence to justify any claims made by the product,
- data on any animal testing performed by the manufacturer, his agents or suppliers, relating to the development or safety evaluation of the product or its ingredients,
- there is a procedure detailed in the Regulations that, subject to agreement, allows the confidentiality of some ingredients to be maintained.

and the limbic (nervous) system. Whatever the case, it seems likely that cures for these hypersensitivity reactions will be related to reducing by apoptosis the populations of rogue T-cells.

F6.10. The 1 and 2 Euro coins have been found to release large amounts of nickel in part because they contain two alloys, which create an electrochemical potential. The corrosion is apparent when coins are immersed in artificial sweat (right). Ni triggers allergic reactions[18].

The prevalence of natural rubber latex allergy has been estimated to be 5–18% in health care workers, and latex exposure has been one of the leading causes of occupational asthma in the last several years.

6.6. Chloracne

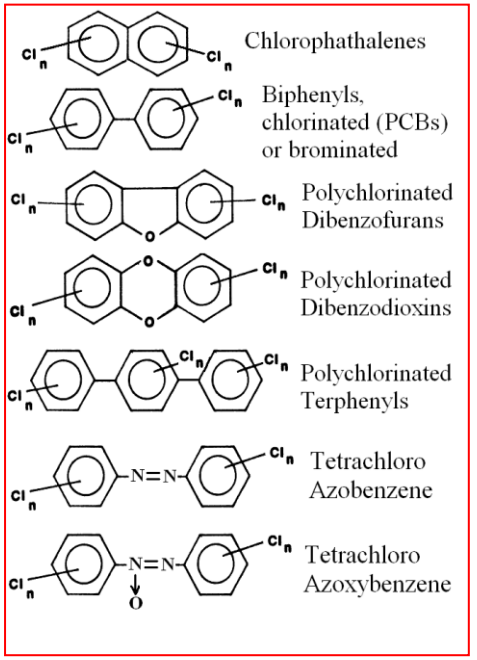


F6.11. Case of Chloracne.

Chloracne is a more severe and specific form of acne which could be labeled *environmental halogen acne*.
Clinically, it is similar to severe *acne vulgaris*, with comedones ("black heads" from obstruction of sebum evacuation, creating a harbor for bacteria), persistent papules (no pus) or pustules, with a frequent eczema component. Dioxins and Furans ( ), among other chemicals, are noted for producing chloracne (F6.12).

F6.12. Chloracneogens.

They bind to the AHH receptor, which is also involved, beyond skin metabolism, in a number of developmental pathways. Various vitamin-A derivatives such as tretinoin and retinoic acid have been useful in treating chloracne.



6.7. Skin Carcinogens

The substances currently accepted as Cutaneous Carcinogens are:

- Ionizing radiation: X-rays, γ-rays, β-rays, energetic particles, protons, neutrons,
- Ultra-Violet light at 256-320 nm,
- Physical trauma,
- Inorganic arsenic,
- Polycyclic hydrocarbons: Benzo[a]pyrene, 3-methylcholanthrene, dibenzanthracene, 7,12-dimethylbenzanthracene.

Chromium Skin Lesions

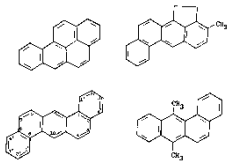
Workers performing chrome plating with exposure to hexavalent chromium from time to time will develop chrome ulcers on their skin (usually hands). Such ulcers have a very distinctive appearance—a "punched out" lesion, frequently with a remaining central core, or 'umbilicus', that seems to contain semi-necrotic tissue.

A typical practice would remove the exposure & treat any secondary infections. Healing may take weeks to months.

Some experienced platers (several with >30 years in the plating business) self-treat, shelling out the core of necrotic tissue using a crude, non-sterile excision, and they swear that their ulcers heal at least twice as fast.

Another strategy could be to reduce chromium (VI) to the relatively benign chromium(III) within the ulcer using topical ascorbic acid (effective in a guinea pig model).

"Within the chrome plating industry in the West Midlands (Great Britain) some platers treat their chrome ulcers with a substance known locally as 'Black Jack' which is an icthamol paste applied as an anti-inflammatory agent to the open wound." Icthamol is rich in ferrous iron, which would also reduce chromium (VI) to Chromium (III).



Anthracenes are a family of aromatics with 3 benzenes in line (left).

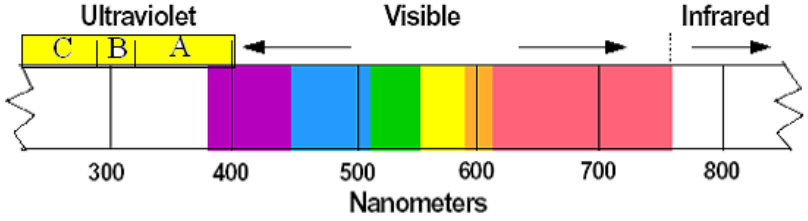
The polycyclic hydrocarbons are found in **soot, pitch, coal tar, creosote; shale, mineral, petroleum and cutting oils.** Petroleum products can be *activated* by skin enzymes or sunlight.

6.8. Barriers

Creams are relatively ineffective against dermatitis, perhaps except for the case of oil-based barrier creams against a wet environment.

6.9. Sunlight

6.9.1. Ultra-Violet Radiation



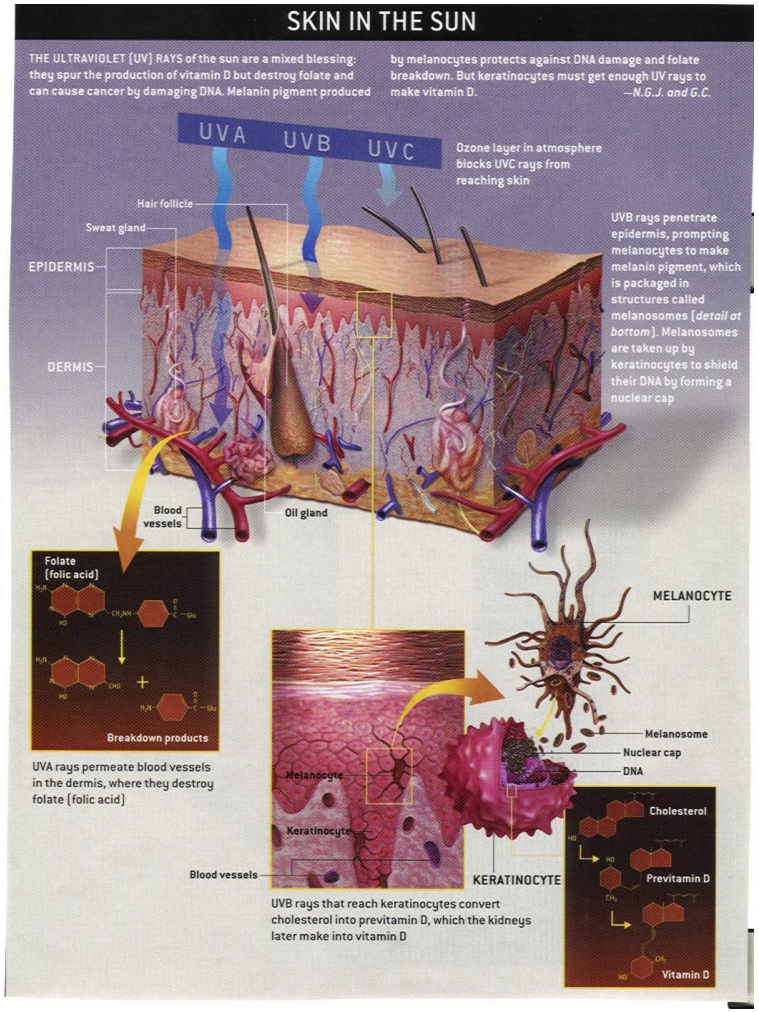
F6.13. "A", "B" and "C" Ultra-Violet radiation.

Sunlight is distributed according to frequency: infrared (> 700 nm, 49.4%), visible (400-700 nm, 42.3%, UVA (400-320 nm, 6.3%), UVB (320-290 nm, 1.5%), UVC (< 290 nm, 0.5%).

The intact skin is not a barrier to sunlight, particularly to the UVB radiation, the type that causes sunburn.

Sun burning cells using UV radiation triggers Fas-mediated apoptosis of keratinocytes[25].

Exposure to UVB when young seems very important in determining the incidence of the various forms of skin cancer later in life. This is why occupational compensation would be limited for such a disease.



F6.14. Penetration of Ultra-Violet Radiation into the Skin.
Scientific American.

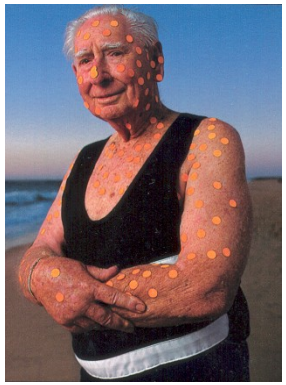
The various forms of skin cancer are:

Melanoma, the deadliest form, involves the pigment-producing cells, melanocytes, from the lower layer of the epidermis. It can develop from a mole or on unblemished skin, grows quickly and can metastasize.

Basal-cell carcinoma: The most common, usually caused by excessive sun exposure. From the lower layer of the epidermis, it develops slowly, rarely metastasizes and is nearly 100% curable if treated.

Squamous-cell carcinoma: from the cells in the upper layer of the epidermis, caused by UV rays, it is usually curable if treated early. Grows faster than basal cell carcinoma and can metastasize.

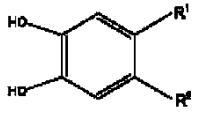
People with some history of artificial light tanning are 1.5 times more likely to have basal-cell carcinoma and 2.5 times more likely to have squamous-cell carcinoma. If they began before the age of 20, the risks are 1.8 and 3.6[25]. Young adults are experiencing a sharp increase in non-melanoma skin cancers, probably as a result of full body tanning[27].



F6.15. A former lifeguard in Australia shows the location of his removed skin cancer lesions (more than 532). Although such cancers are not often lethal, having them correlates with other types of cancers.
National Geographic, November 2002.

Although sunlight is the most frequent cause of skin pigmentation, coal tar and other petrochemical substances may increase pigmentation, while paratertiary

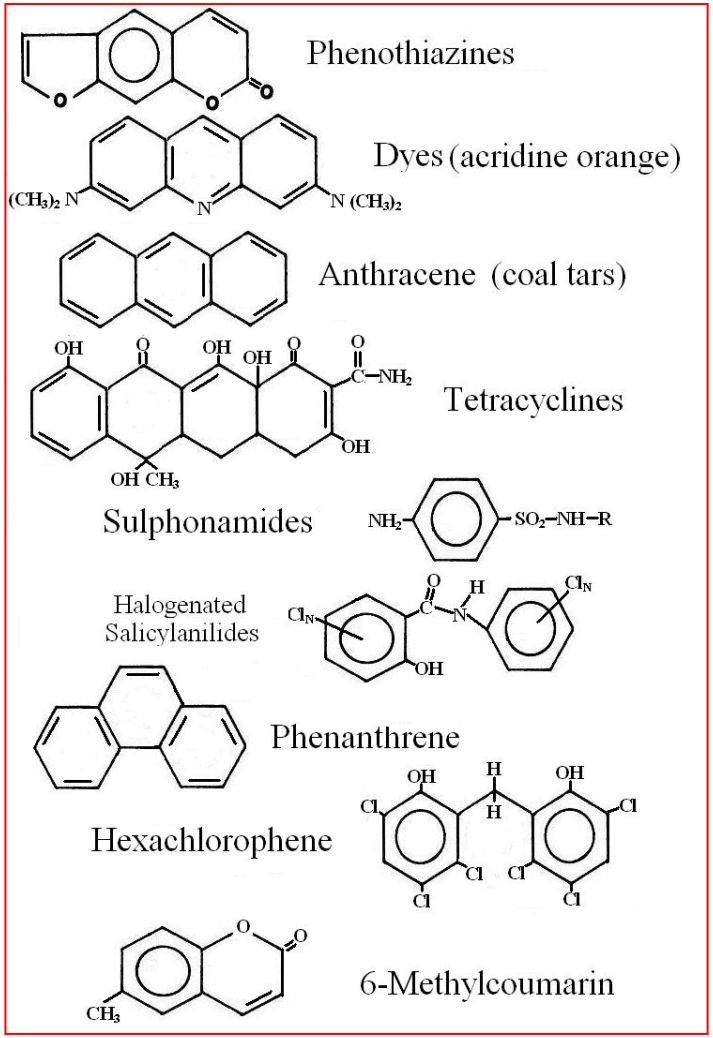
butyl phenols and cathecols (aside) can depigment the skin.
Uneven pigmentation can also occur from heat or microwave exposures.



6.9.2. Phototoxicity

In **phototoxicity,** a chemical is converted into dermally toxic compounds by sunlight in the skin.
UVA, 315-400 nm, can activate chemicals into phototoxic or photoallergic agents. The result is similar to contact dermatitis[7]. A phototoxic or photoallergic reaction can be produced with either a dermally acquired chemical or one taken systemically, such as a drug. The first photoreactive chemicals identified were drugs, the sulphonamides, phenothiazines, and tetracyclines. They caused acute dermatitis following ingestion and exposure to sunlight.

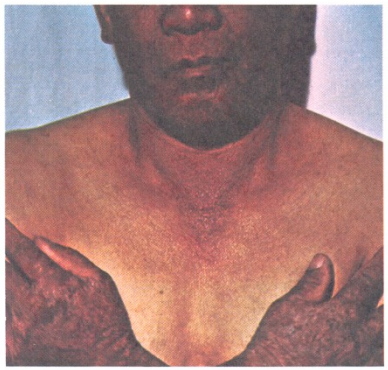
Phototoxicity is usually seen as a delayed erythema followed by hyperpigmentation and desquamation, e.g. a sunburn followed by more severe effects (photo-products with nucleic acids or membrane damage). As can be see in F6.16, these chemicals usually have large complex ring structures (halogenated benzenes) or interlocking, planar aromatic rings (phenanthrene, anthracene) or multi-rings. Such phototoxicity is independent of immune or allergic mechanisms and can occur in anyone if enough light energy and the concentration of drug in the skin is high enough.



F6.16. Phototoxic chemicals in humans.

6.9.3. Photoallergy

Photoallergy is an acquired, altered reactivity to UV irradiation that is dependent on an antigen-antibody or cell-mediated hypersensitivity response. While relatively uncommon, such reactions are similar to an exaggerated sunburn response and may be immediate (solar urticaria with transient wheal and flare reactions) or delayed (papular and eczematous). Most investigators consider that a cell-mediated (T-cell) immunity is involved, because the characteristic responses observed are similar to that seen in contact allergic reactions, e.g. poison ivy (active ingredient - urushiol). Eventually, as the individual becomes sensitized to the agent, spots will appear at other, often covered, sites on the body where contact with the agent has not occurred. Such conditions have all the appearance of a developing immunity, with time and frequency of exposure being the relevant factors.



F6.17. Photo contact dermatitis often involves areas exposed to the sun.

Since the chemicals that induce such reactions are ubiquitous and may be found in washing powders (brighteners), in perfumes, lotions, creams, etc. (bergamot oil containing bergapten), antibacterials (3,5-dibromo-salicylanilide), emulsifiable cutting oils (petroleum sulphonates, bactericides, rust inhibitors - nitrates and nitrites), it is extremely difficult to identify the toxicant. T6.18 shows a short list of chemicals used in identifying photoallergies.

There are a variety of well known drugs that are photoallergens, and more and more industrial chemicals are showing similar properties.

T6.18. Chemicals used in identifying photoallergy.

Dichlorophene
Bithionol
Hexachlorophene
4,4'-dichloro-3-(trifluoromethyl) carbanilide
3,4,4'-trichlorocarbanilide
3,5-dibromosalicylanilide
3,4',5-tribromosalicylanilide
2,3,4,5-tetrabromosalicylanilide
6-methylcoumarin
Musk ambrette

6.10. Testing

6.10.1. Ocular Irritation: Draize

Accidental eye exposure is a major concern. Irrigation of the eyes is the universal method of treatment for all serious eye exposures. It can be done in a shower, drinking fountain or by blinking in a full sink.
Although the lens includes structural proteins for neutralization of toxins such as glutathione-S-transferase, and heat-shock proteins, it is still very vulnerable.
If an agent is irritative dermally, there is no need to test it in the eye, since eye membranes are more susceptible than the skin's. There is no need to test strong acids and bases, as well as most moderate irritants in the eye. But marginal cases, like a baby

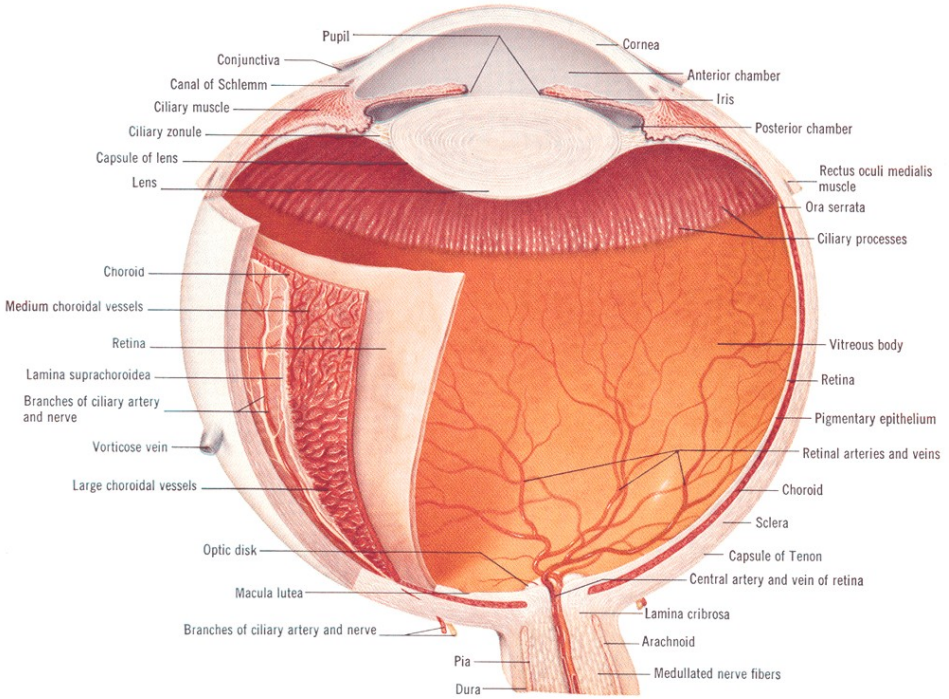
shampoo, where the chance of getting it into the eyes of a child at some time are 100%, should be investigated. If such a product must be safe, how do you test it ?

To provide appropriate safety messages on the labels and MSDSs, there is a testing requirement for chemicals. This need was first answered by the Draize ocular test (1944)[9]. It scores the physiological reactions in the cornea, conjunctiva and iris (T6.19). The test is:

(1) subjectively rated, the simple scoring system varying slightly with methodologies[9,10],
(2) vulnerable to interspecies differences.

There appears to be less interspecies variability with strong irritants or with non-irritants; the differences occur mostly with moderate irritants. Draize selected the rabbit as a test animal because it was more sensitive, thereby providing a margin of safety for human exposure[2], and could display a dose-effect relationship. This test was not satisfactory for all agents; for some, the rabbit eye was not as sensitive as the human eye.

The basic test has changed little in the past 58 years, although smaller volumes (0.1 ml > 0.01 ml) of chemical are now used to reduce adverse effects, while still allowing assessment of potential toxicity.
Various techniques to measure changes in corneal thickness have been tested, specifically a slit lamp perpendicular to the corneal apex. The measurements are accurate to 0.01 mm[4]. Five measurements are made at each time point (0, 24, 48, and 72 hr post-treatment).



F6.19. Anatomy of the lower half of the eyeball.

6.10.2. Dermal Irritancy

In view of the frequency of accidental splashes and spills, it is important to have MSDS data on the irritating or corrosive effects of a chemical on the skin. Again, the most successful test (T6.20) was proposed by Draize[9].
Most suitable animals possess a good fur coat which can be shaved, leaving only a fine stubble. There are strains of mice and guinea pigs that are hairless or "nude": sad looking, wrinkled things that generate sympathy. There is no agreement on which skin is morphologically closest to the human skin.

T6.20. Eye irritation test: grading ocular lesions. From Draize, J. H., Woodard, G., and Calvery, H. 0., J. *Pharmacol. Exp. Ther.,* 82:377-390 (1944).	
DESCRIPTION OF LESION	**#**
I. **CORNEA**: A. Opacity, degree of density	
Scattered or diffuse areas of opacity, but details of iris clearly visible	1
Easily discernible translucent areas, details of the iris slightly obscured	2
Opalescent areas. no details of iris visible, size of pupil barely discernible	3
Opaque cornea, iris invisible through opacity	4
I. **CORNEA**: B. Area of cornea involved	
One quarter (or less), but not zero	1
Greater than one quarter	2
Greater than one half	3
Greater than three quarters, up to complete area	4
Score = Part A x Part B x 5 --- maximum score = 80	
II. **IRIS**: A. Normal	
Folds above normal, congestion, swelling circumcorneal injection, iris light-reacting	1
No reaction to light, hemorrhage, gross destruction	2
Score = Part A x 5 ---- maximum score = 10	
III. **CONJUNCTIVA**: A. Redness (refers to palpebral conjunctiva)	
Blood vessels hyperemic (injected)	1
Diffuse, crimson color, individual vessels not easily discernible	2
Diffuse vessels, beefy red	3
III. **CONJUNCTIVA**: B. Chemosis (lids and nictitating membranes)	
Any swelling above normal (includes the nictitating membranes)	1
Obvious swelling with partial eversion of lids	2
Swelling with lids half closed	3
Swelling with lids more than half closed	4
III. **CONJUNCTIVA**: C. Discharge	
Not normal (ignore small amounts observed in inner canthus of normal animals)	1
Discharge with moistening of lids and hairs adjacent to lids	2
Discharge with moistening of the lids and hairs of considerable area around eye	3
Score = (Parts A + B + C) x 2 --- maximum score = 20	

Investigators use rodents, guinea pigs and rabbits, all of which have sensitive skin. Larger animals such as swine, minipigs, and dogs may also be used. For a wide range of chemicals, the skin of the rhesus monkey is considered to best mimic the human skin. However, dermal irritation studies are usually conducted in a specific animal species for simple practical reasons, selection usually having little to do with any similarity to the human skin.

T6.21. Skin irritation test: grading skin lesions.	
Skin responses	**Value**
Erythema and Eschar Formation	
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate-to-severe erythema	3
Severe erythema (beet redness) to slight eschar formation (injury in depth)	4
Edema Formation	
No edema	0
Very slight edema (barely perceptible)	1
Slight edema (edges of area well-defined by definite raising)	2
Moderate edema (raised approximately 1.0 mm)	3
Severe edema (raised more than 1.0 mm and extending beyond the area of exposure)	4

Two effects are observed on skin during the 24, 48 and 72 hours post-exposure: *erythema* (redness) and *edema* (puffiness or swelling).

It comes down to how red is red and to what degree is swelling seen. A numbering system or grading score has been developed for the two parameters which is dependent upon the skill of the investigator or technician to "grade" the severity of the biological response (See T6.21).

Having scored each animal for erythema and edema, obtained average scores for each parameter and incorporated them together, one is left with interpreting them for the products.

A scheme used by NIOSH is shown in T6.21. Based on these ratings, numbers generated in the test can be converted into label warnings and safety information in the MSDS (T6.22).

In the case of *dermal sensitization* studies, the animal of choice is usually the guinea pig, although rabbits can be used,

both being reactive and sensitive. Essentially, the chemical of interest is applied to the skin daily over a 14-day period and, following a resting period of 10-to-14 days, the animals receive a challenge dose of the agent, usually a lower concentration than was applied earlier. A positive flare-and-wheal reaction, usually dose-dependent, signifies that *sensitization* has occurred.

T6.22. NIOSH interpretation of skin testing ratings.
McCreesh and Steinberg, 1983

Rating		Interpretation
Intact Skin	Abraded Skin	
0-0.9		Nonirritant: probably safe for intact human skin contact
1-1.9		Mild irritant: may be safe for use but appropriate protective measures are recommended
2-4.0		Too irritant for human skin contact: avoid contact
	0-0.9	Nontoxic to cellular components of abraded skin: probably safe for human skin contact
	1-1.9	Mild cellular toxins: may be safe for abraded skin contact
	2-4.0	Cellular toxicant too irritant for abraded skin contact: avoidance of contact is advised

6.10.3. Irritection-Corrositex vs Draize

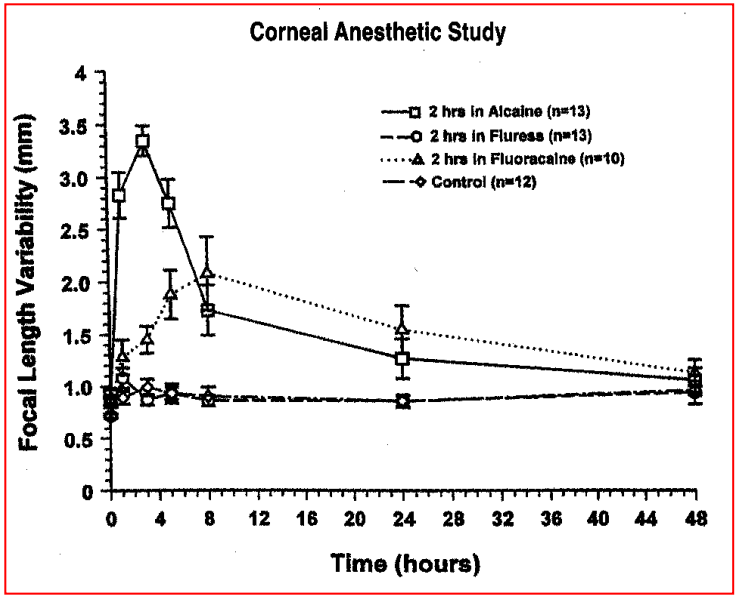
The descriptions of T6.20 and T6.21 have attracted the attention of animal rights activists, who have campaigned to eliminate such studies on the basis that they inflict needless pain, and use too many animals because of the variability in response.

6.10.3.1. Substitute Ocular Tests

Many promising alternative **ocular tests**, some of which are being accepted by regulatory agencies, have been developed, including isolated cornea, enucleated eyes, cultures of corneal and lens cells as well as non-animal preparations[11]. This still leaves the problem of a suitable species from which to obtain the eyes for the *ex vivo* tests. The results of a comparative study sponsored by the Commission of European Communities revealed that the enucleated chicken eye appeared to be the most practicable when compared with the eyes of swine and cattle and tested with a number of well known chemicals[15]. The Chicken Enucleated Eye Test (CEET) recognizes three levels of irritancy. Since large numbers of chicken eyes can be obtained at little cost from slaughterhouses, this test would appear to satisfy animal rights groups and still give a valid assessment of ocular toxicity. A competing technique uses isolated (enucleated) eyes following euthanasia of the rabbit, incubating *in vitro* (or *ex vivo*) with the test toxicant, with corneal thickness measurement with the slit lamp[12]. Comparing the *ex vivo* and *in vivo* tests has demonstrated that materials capable of causing ocular injury caused an intensification of the stromal image and swelling of the cornea with a good correlation between *in vivo* ocular damage and *ex vivo* results[14]. Since animal lenses can be cultured over days or weeks with their optics and repair mechanisms intact, even chronic changes as well as recovery can be documented. Variables used to gauge toxicity can be as simple as changes in the focal length of the lens, as shown below for three common corneal anesthetics.

6.10.3.2. Substitute Skin Tests

The development of synthetic **skin tests** has also been intensive. "Corrositex" is an *in vitro* test that determines chemical corrosivity and allows assignment to packing group specifications. It replaces the rabbit skin test. It is based on a glass vial filled with a chemical detection system, and capped



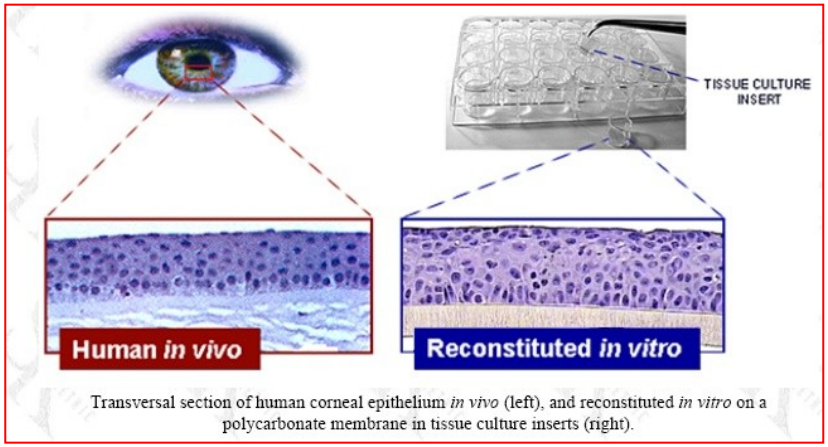
F6.23. Effect of focal length of bovine lens from three eye anesthetics.

by a bio-membrane. After a potentially corrosive chemical is placed on the membrane, the chemical detection system may become colored.

" Irritection" is a quantitative *in vitro* test that ranks ocular and dermal irritation potential of cosmetics, consumer products, pharmaceuticals and raw industrial chemicals.

The chemical under investigation is placed on a membrane that permits controlled delivery of the material to a reagent solution. The reagent solution contains proteins, glycoproteins, lipids and low molecular weight components that self-associate to form insoluble matrices. The toxic changes are measured over 5 hours using turbidity readings.

Most of the new *in vitro* tests offer considerable simplicity, economy and speed. Some things are lost from the animal models. The opportunity to observe many variables (as opposed to only a change in color or turbidity), to make chronic observations over time (as opposed to a few hours). Certainly the new tests are simpler to interpret. But simplicity of interpretation goes in hand with paucity of output information. The usefulness of the tests depends on how carefully they are designed and researched.

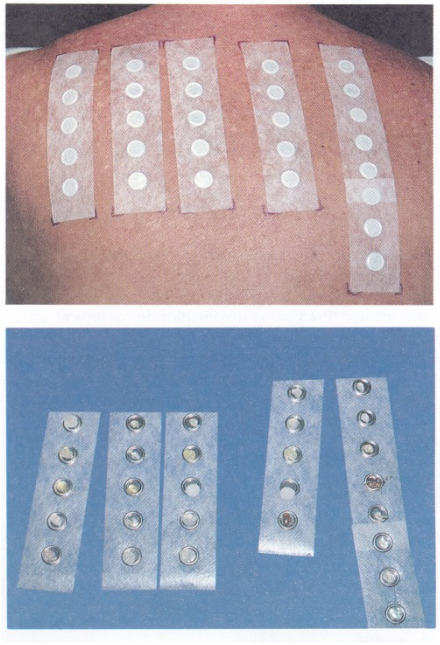


F6.24. When cultivated at the air-liquid interface in chemically defined medium, the transformed human corneal epithelial cells of the cell line HCE form a corneal epithelial tissue (mucosa), devoid of stratum corneum, resembling ultrastructurally (tissue morphology and thickness) the corneal mucosa of the human eye.

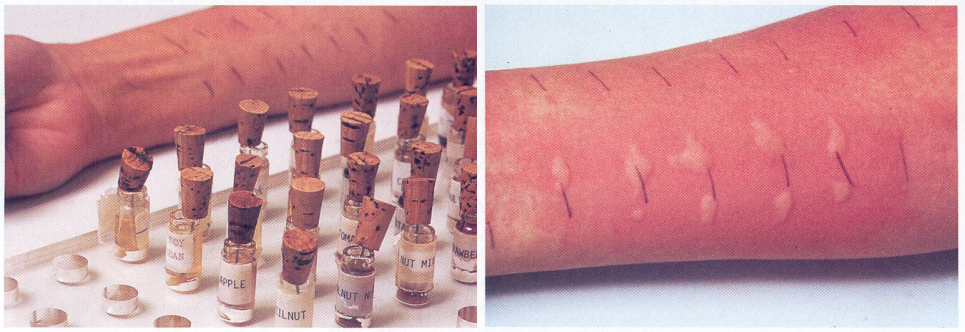
6.10.4. Clinical Patch Tests

People may need to be tested for their reaction to chemicals. Allergens are tested either in a petrolatum or aqueous vehicle in Finn chambers (F6.25), essentially a filter paper used to contain water preparations, The patches are maintained on the skin for 2 days. Reactions are graded one hour after the tape is removed. They are normally conducted with non-irritant concentration, to determine an hyper-sensitivity specific to a patient.

When there is greater reaction at the edge of the patch, this usually denotes irritation rather than allergy. Depigmentation can sometimes result from exposure to test chemicals. Sub-cutaneous injections (F6.26) can also be used.



F6.25. Finn chambers for allergic sensitivity tests in patients.



F6.26. Allergic sensitivity tests using sub-cutaneous injections.

The large number of chemicals that can be used in various versions of the clinical patch tests speaks to the variety of possible cutaneous reactions.

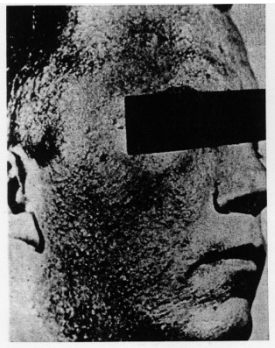
T6.27. Chemicals used in diagnostic patch testing. Unless noted, chemicals are in petrolatum in the first two columns. Chemicals present in more than one column are in bold.

	North American Contact Dermatitis Group	European Contact Dermatitis Group	Thin Layer Rapid-use Epicutaneous Test (allergens)
1	**Benzocaine**, 5%	Potassium **dichromate** 0.5%	**Nickel** sulfate 0.2 mg/cm²
2	**Mercaptobenzothiazole**, 1%	Paraphenylenediamine base, 1%	Wool **alcohols** 1.00 g/cm²
3	**Colophony**, 20%	**Thiuram** mix, 1%	**Neomycin** sulfate 0.23 mg/cm²
4	**Paraphenylenediamine** base, 1%	**Neomycin** sulfate, 20%	Potassium **dichromate** 0.023 mg/cm²
5	Imidazolidinyl urea, 2% aqueous	**Cobalt** chloride, 1%	Caine mix 0.63 mg/cm²
6	Cinnamic aldehyde, 1%	**Benzocaine**, 5%	**Fragrance** mix 0.43 mg/cm²
7	Lanolin alcohol, 30%	**Nickel** sulfate, 5%	**Colophony** 0.85 mg/cm²
8	**Carbamix** mix, 3%	Clioquinol, 5%	**Epoxy** resin 0.05 mg/cm²
9	9. **Neomycin** sulfate, 20%	9. **Colophony**, 20%	Quinoline mix 0.19 mix/cm²
10	**Thiuram** mix, 1%	**Paraben** mix, 8%	**Balsam** of Peru 0.80 mg/cm²
11	**Formaldehyde**, 1% aqueous	N-isopropyl-N-phenylparaphenylenediamine, 0.1%	**Ethylenediamine** dihydrochloride 0.05 mg/cm²
12	**Ethylenediamine** dihydrochloride, 1%	Wool **alcohols**, 30%	**Cobalt** dichloride 0.02 mg/cm²
13	**Epoxy** resin, 1%	**Mercaptomix**, 2%	p-tert-**Butylphenol** formaldehyde resin 0.04 mg/cm²
14	**Quaternium** 15, 2%	**Epoxy** resin, 1%	**Paraben** mix 1 mg/cm²
15	p-tert-**Butylphenol** formaldehyde resin, 1%	**Balsam** of Peru, 25%	**Carbamix** 0.25 mg/cm²

16	**Mercaptomix**, 1%	p-tert-**Butylphenol** formaldehyde resin, 1%	Black **rubber** mix 0.075 mg/cm²
17	Black **rubber** mix, 0.6%	**Mercaptobenzothiazole**, 2%	Cl+Me-**isothiazolinone** 0.0040 mg/cm² (Kathon CG)
18	Potassium **dichromate**, 0.25%	**Formaldehyde**, 1%	**Quaternium** 15 0.1 mg/cm²
19	**Balsam** of Peru, 25%	**Fragrance** mix, 8%	**Mercaptobenzothiazole** 0.075 mg/cm²
20	**Nickel** sulfate, 2.5%	Sesquiterpene lactone mix	**Paraphenylenediamine** (PPD), 0.090 mg/cm²
21		**Quaternium** 15, 1%	**Formaldehyde** 0.18 mg/cm²
22		Primin, 0.01%	**Mercaptomix** 0.075 mg/cm²
23		Cl + Me-**isothiazolinone**, 0.01% aqueous	Thimerosal 0.0080 mg/cm²
24			**Thiuram** mix 0.025 mg/cm²

6.11. Case Study: Carbamate and Propanil Pesticide case and Chloracne

(modified from Williams & Burson)

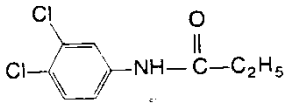


A plant in Arkansas manufactured several types of pesticides in rotation, manufacturing and packaging one pesticide for several weeks or months, and then doing the same with another pesticide, and then perhaps a third. The CDC became involved at this plant because there had been several hospitalizations of workers and also a number of complaints. OSHA inspected the plant and requested that a health hazard evaluation be done. Two physicians from the CDC then surveyed the plant. On investigation, they found that two types of compounds were produced.

One compound was a carbamate, methomyl (N-[(methylcarbamoyl)oxy] thioacetmidic acid methyl ester) which causes symptoms of toxicity similar to those caused by organophosphorous compounds-namely, nausea and vomiting, small contracted pupils, increased salivations, and muscle fasciculation. Methomyl is a highly toxic carbamate.

In addition to the methomyl, the plant was making and packaging an herbicide called propanil (3',4'-dichloropropionanilide), made from 3,4-dichlo-roaniline. A number of employees had signs traceable to propanil: "acne," which was really chloracne (see figure), and also a nonspecific rash and skin irritation. These skin problems were more of a problem than the toxic effects of the methomyl. In trying to evaluate the problems, we divided the workers into different groups. There were some differences in symptoms, but because the chemicals were so pervasive, it was not possible to demonstrate that very well. Workers also moved freely from one area into another, and many had been exposed to both compounds.

The herbicide propanil, which is an aniline-type chlorinated compound, has the following structure:



Propanil has a number of different commercial names. It is not very toxic, but it does cause the skin disease chloracne, which can be quite persistent and disfiguring. Propanil has this effect because it is contaminated with 3,4,3',4'-tetrachloroazoxybenzene. This compound is chloracnegic because of a peculiar chemical configuration. in which two chlorine atoms on one aromatic ring are connected either by another ring or by a double bond to two chlorines on another ring, reminiscent of TCDD (dioxin), 2,3,7,8-tetrachlorodibenzo-p-dioxin, which is extremely toxic It has only recently been established that the chloroazobenzenes also cause chloracne. Other herbicides-diaron, linuron, neburon-may also be contaminated with tetrachloroazoxybenzene. Other chemicals that cause chloracne are the chlorinated naphthalenes, biphenyls, dibenzodioxins, and dibenzofurans.
In a few documented cases patients have had active chloracne for up to 20 years after they were exposed to one of these chemicals. In many instances chloracne gradually improves over a number of years following cessation of exposure. Until we made our investigation it had not been known that propanil caused chloracne. Our findings were substantiated by measuring chlorinated azobenzene in the propanil and by testing the chloracnegic effect of this material on the rabbit ear.

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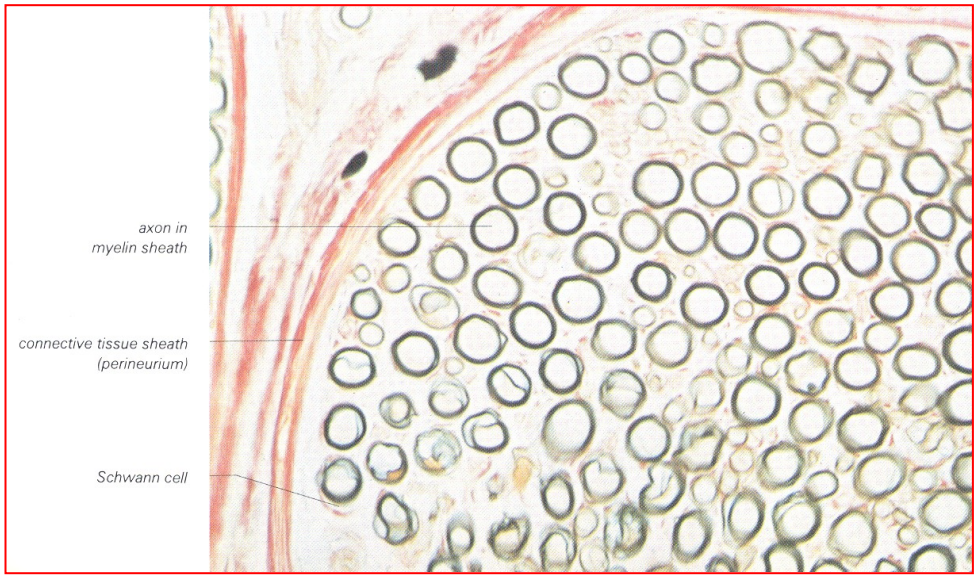
Neurotoxicity

7· Neurotoxicity

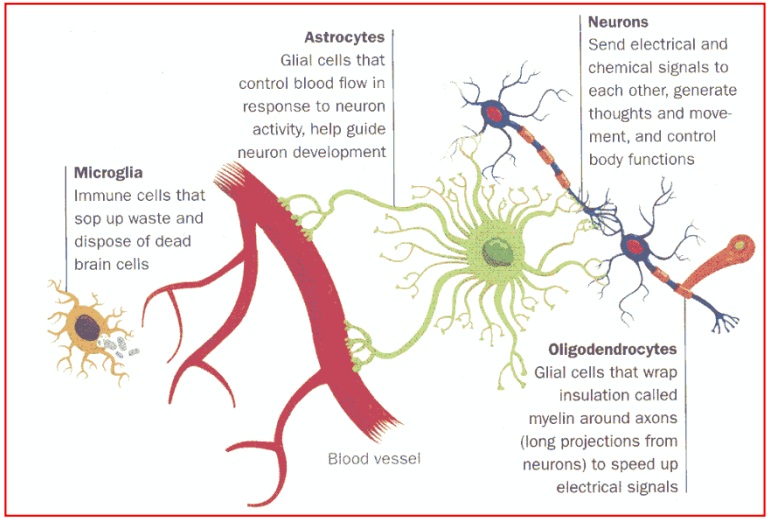


F7.1. Transverse section of a nerve. X700. The red tissue is a nerve sheath. The dark circles are nerves wrapped with myelin, the smaller light ones are unmyelinated nerves. *Biological Structures.*

7.1. Anatomy

The nervous system includes the brain, spinal cord, and the peripheral sensory and motor nerves. The *central* nervous system is the brain and spinal cord only.
Nerve cells normally grow when they are young, and stop when they are mature. Injured central nervous system cells are mostly unable to regenerate on their own (they do so to a very limited degree; there are very few absolutes in biology). In the peripheral nervous system cells, however, an injury can stimulate the cells to regrow, making it possible for severed limbs to be surgically reattached to the body, and regain function.

In the brain and spinal cord, *white matter* corresponds to the thick protective *myelinating sheaths* around nerves and nerve bundles. The *gray matter* is made of cell bodies and nonmyelinated or lightly myelinated fibers.
In higher vertebrates, the proportion of white to gray matter increases as one ascends the phylogenetic tree until, in man, more than 40 % of the cross-sectional area through the brain consists of glistening myelin.



F7.1a. Types of cells in the central nervous system.

There are various cell types in the brain, and the neurons have been the star, primarily because of their spectacular action potentials (F7.3). Neurons cohabit the brain with glia, which outnumber neurons by a factor of 10. This has led to the common misconception that humans "use 10 % of their brain",

underlining the opinion that glial cells contribute nothing to thinking. Specialized glial cells called *astrocytes* influence connections between neurons, the level of chemical messengers in the synapse, as well as the blood flow within the brain. They collect nutrients from the bloodstream, and purify them for neurons, protecting the brain from some toxic agents. When this protection fails, for example for solvents which can easily gain access to brain tissues, a variety of effects occur, some of which are reversible (for example, alcohol intoxication).

In humans and in many animals, a long childhood implies an adaptable neural structure that must be programmed through experience primarily in childhood, but even later in life. The processes needed for proper programming of such a complex structure probably increase the vulnerability of the brain to toxicants (lead is a good example).

T7.2. Physio-pathology of neuronal damage.

Vulnerabilities
Post-mitotic cells (little cell division)
Poor regeneration
Very vulnerable to oxygen depletion
Reasonable blood flow (toxicant access)
High lipid content (hydrophoic substances)
Unique shape and structure (long neurons)
Excitable membranes (receptors)
Low energy stores (glucose needed)
Axonal transport of nutrients (long distance)
Specific neurotransmitters (vulnerable)
Myelination

Protective mechanisms
Extra neurons (safety factor)
Local reorganizations (brain and peripheral plasticity)
Adaptive responses (re-programming)
Blood-brain barrier (denying access to toxicants)

The nervous system is a frequent target for a variety of chemicals: metals, solvents, plastics, monomers, agrochemicals…The protective mechanisms are limited, and damage often poorly reversible. The reasons for the nervous system's vulnerability are itemized in T7.2.

7.1.1. Blood-Brain Barrier

The endothelial cells of the vessels in the central nervous system are held together by *tight junctions* between cells, preventing access of unwanted substances. The cells of the barrier also have larger numbers of mitochondria and low pinocytotic activity.
The barrier is successful in preventing the following toxicants from entering: lead compounds, penicillin and staphylococcus toxin. But lipid-soluble compounds such as methyl mercury, pentachlorophenol, hexachlorophene, trimethyl tin and triethyl tin can penetrate cell membranes, and make it through the blood-brain barrier. The blood-brain barrier, like the Great Wall in China, is not continuous, but has gaps in the hypothalamus and the choroid plexa. In fetuses and very young animals, the barrier is under construction.

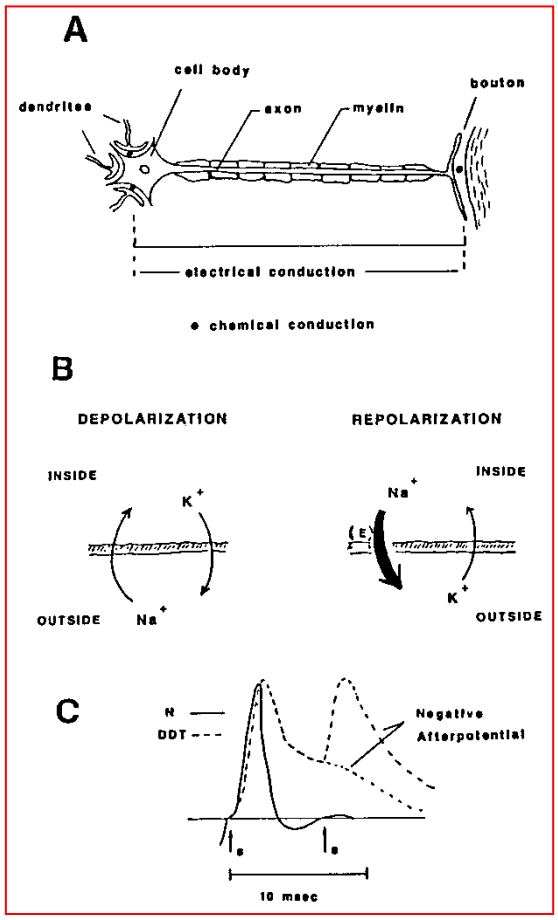
There is a vulnerable portal through the nose and the olfactory bulb that even allows small (0.1 µm) airborne particles into the central nervous system. Contrary to the lungs, the particles are not easily cleared from the CNS[13].
Mannitol opens the blood-brain barrier.

7.1.2. Electrophysiology

The basic structure and polarization of a neuron are depicted in F7.3. Anatomically, there is a cell body with dendrites, making close contact with the terminal portions of adjacent neurons. Communication between one neuron and another (across a synaptic cleft of gap) is chemical in nature, requiring specific *neurotransmitters* released from the terminal to a receptor.

Agonists substitute for naturally occurring *neurotransmitter* substances and produce a response.

Antagonist substances bind to receptors *without* producing a response.

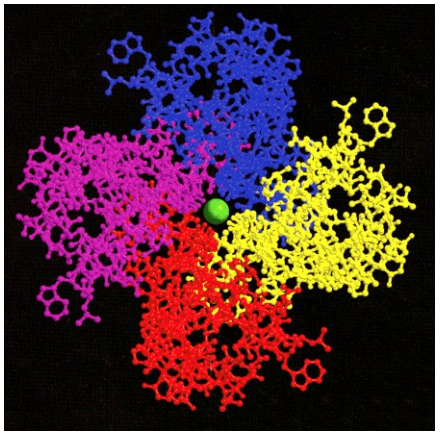


F7.3. Polarization of neurons.

Attachment of the neurotransmitter to a sensitive "patch" (receptor) on the cell body of the next neuron results in depolarization of the cell membrane and the propagation of an electrical impulse down the axon of the neuron.

With depolarization, there is an opening of pores in the cell membrane, permitting the influx of large quantities of sodium ions, with a corresponding loss of potassium ions to the exterior of the neuron (F7.3B) and a loss of electrical potential from a standing -80 mV to 0 mV (F7.3C).

With repolarization of the neuron in preparation for another stimulus, the open pores (gates) close, the internalized sodium is pumped out by specific enzymes (Na^+ - K^+ ATPase), allowing an influx of potassium and accompanying chloride ions.
All of this, under normal conditions takes no more than 10 milliseconds, but if the ability to repolarize is impaired (as with the insecticides DDT and pyrethrins), one sees a slow repolarization with a prolonged negative after potential (F7.3C), a state in which the nerve is only partially repolarized and is highly susceptible to rapid depolarization again.



F7.4. The specificity of ion channels is a size-matching and electrostatic effect. Here, a potassium ion (green) sits at the center of a bacterial ion channel (KcxA). The channel lets in one sodium for each 1000 potassium ions, stripped of its hydrating molecules.

7.1.3. Myelin

If you hit your finger with a hammer (you may try this at home), you may notice that the feeling of pain will "reach you" *later* than the hit detection. This is because these two signals are not propagated by the same neuron size.

The depolarization-repolarization is relatively slow in non-myelinated nerves, each small segment of neuronal membrane having to undergo the event as the pulse propagates down the

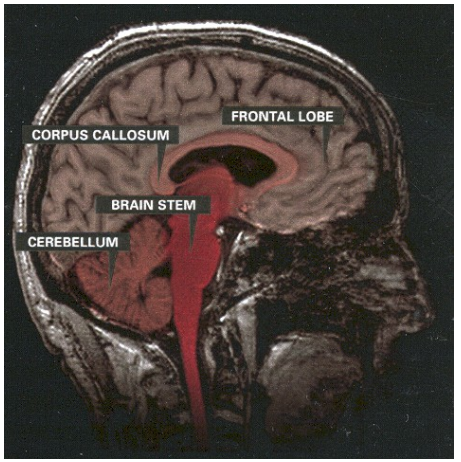
axon. To increase this velocity, a size increase proportional to the square root of the diameter of the neuron is required. So, if the rate of conduction is to be doubled, the nerve must be four times larger in diameter. This would lead to very large nerves when high speeds are needed.

A coating of *myelin* over axons reduces linear capacitance of nerves by 1000 and increases resistance by 5000, substantially increasing propagation speed (0.5 m/s in small nerves; 130 m/s in myelinated fibers of 20 µm).
The conduction velocity in *myelinated* nerves increases roughly in proportion to the diameter of the nerve e.g. a two-fold increase in diameter means a two-fold increase in conduction velocity.
Myelin reduces space requirements and makes nerves function faster. How? Rather than each small segment of the neuron undergoing depolarization-repolarization, this occurs only at the small junctions (nodes of Ranvier) between the myelin segments. Thus, the electrical impulse leaps down the axon from node to node.
Once the electrical impulse has reached the terminus (bouton) of the axon, the ionic changes stimulate the release of neurotransmitters from storage vesicles to diffuse (by exocytosis) out of the bouton membrane and across the synaptic gap to bind to a receptor on the surface of the next cell (neuron, muscle, etc.) to initiate a new electrical stimulus.

7.1.4. Oxygen Depletion

Although the brain accounts for only 2 % of body weight, it consumes 15 % of the oxygen. When oxygen levels in the blood become too low, neurotransmitter release in the frontal lobe is affected, leading to impaired memory and reasoning. Blood vessels in the brain allow plasma leakage into many areas of the brain (pictured here), leading to cerebral edema: a staggering gait, coma and death.

F7.5. Oxygen depletion in the brain (red regions). Anoxia in the frontal lobe causes impaired memory and reasoning. Plasma leakage in the corpus callosum creates internal pressure. When the cerebellum is affected, a staggering gait results. Coma and death result from further stress on the brain stem.



7.2. Classification of Neurotoxicants

Neurotoxicants are legion and come from all categories, as can be seen from T7.6. Solvent neurotoxicity is commonly reported in the literature.
Neurologists classify neurotoxicants according to their own functional nomenclature, as follows.
Blocking agents: botulinum (bacterium) toxin prevents release of acetylcholine, tetrodotoxin (puffer fish) blocks the sodium channels.
Depolarizing agents: batrachotoxin (frog skin) destroys the sodium gradient, DDT and pyrethrins (chrysanthenum flower) increase sodium permeability.
Stimulants: strychnine (from tree seeds, rat poison) reduces the effect of the inhibitor glycine, picrotoxin antagonizes the inhibitor GABA, caffeine inhibits the breakdown of cAMP (a second cell messenger).
Depressants: carbon tetrachloride, alcohol, barbiturates (mechanisms less well known).

Receptor antagonists: atropine (nightshade plant) competitively bind cholinergic receptors, propanolol binds receptors of adrenalin and norepinephrine.
Anticholinesterase agents: organophosphate and carbamate insecticides increase stimulation of cholinergic nerves by impairing the destruction of acetylcholine in the synapse.
Neuro-muscular blockers: curare blocks acetylcholine action, succynylcholine prevents membrane repolarization.

T7.6. A list of common industrial neurotoxicants.
Morbidity and Mortality Weekly Report, Vol. 35, No.8, p. 114, Feb. 26, 1986.

Acetyl ethyl tetramethyl tetralin	Acetyl pyridine	Acrylamide
Adiponitrile	Alkyl phosphates	Aluminium
Aniline	Arsenic, inorganic	Arsine
Aryl phosphates	Azide	Barium
Benzene	Boron	p-Bromophenyl acetylures
Cadmium	Carbon disulfide	Carbon monoxide
Carbon tetrachloride	Chlordane	Chlordecone
Chloroprene	Cobalt	Cuprizone
Cyanide	2,4-Dichlorophenoxy acetic acid [2,4-D]	Dichlorodiphenyl trichloroethane [DDT]
Diethyl ether	Diisopropyl fluorophosphate [DFP]	Dimethyl sulphate
Ethylene dichloride	Hexachlorophene	n-Hexane
Hydroquinone	Lead	Lead, tetraethyl
Leptophos	Malonitrile	Manganese
Mercury	Methanol	Methyl bromide
Methyl chloride	Methyl n-butyl ketone	Nickel [carbonyl]
Nitrogen trichloride	Organochlorine insecticides	Organophosphate esters
Organotins (triethyltin)	Paraquat	Phenol
Phenyl mercury	Phthalate esters	Polybrominatad biphenyls [PBBs]
Selenium	Styrene	Sulfur dioxide
Tetrachlorobiphenyl	Thallium	Toluene
Trichloroethylene	Triorthocresylphosphate [TOCP]	Vanadium, inorganic salt
Zinc	Zinc pyridinethione	

7.3. Sites of Attack

F7.3A points to the four vulnerable sites for neuronal damage by chemicals.

1. the dendritic end of the neuron, these fine, filamentous (hair-like) extensions having no protective myelination and only a thin membrane,
2. the cell body of the neuron is usually exposed, with very little protection other than the normal, thin, cell membrane,
3. the axon, either unmyelinated or myelinated, vulnerable because of its length and the necessity of transporting nutrients from the cell body to the bouton and the metabolic products back to the cell body. Attack can occur at either the axon or the myelin,
4. at the bouton itself, with an effect on the membrane or on the neurotransmitters released.

With a more *functional* view, the types of attack could be as follows:

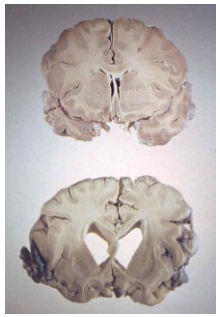
1. on protein synthesis,
2. **axonopathy,** electrical impulse propagation along axons and axonal transport of nutrients using the microtubular system. Axon degeneration usually starts at the end of the axon and grows proximally,
3. on neurotransmitter secretion, action and turnover,
4. **myelinopathy,** decay of myelin sheath. The classic natural disease is multiple sclerosis, which results in pain, loss of coordination and vision. Loss of myelin (death of the Schwann cells) affects nerve conduction velocity. For example, heavy use of organic solvents can lead to walking problems. Also, the bacterium that causes leprosy directly damages myelin sheating[15].

Agents: Cyanate, lead, chronic cyanide, carbon monoxide.
Remember Resdan shampoo, featuring *hexachlorophene*? Hexachlorophene is a good bacterio-static anti-biotic

against *Staphylococcus Aureus*. Unfortunately, it also produces myelinopathy. Fortunately, it does not penetrate the skin well, and its toxicity was detected in burn victims.
5. Blood-brain barrier alterations (glue sniffing, microwaves)
6. Astrocyte alterations (brain's cleaners disabled)
7. Peripheral nerve damage: acrylamide, arsenic, methanol.

Some culprits, such as n-Hexane, have been banned.

Polyneuropathy can involve more than one mechanism, in the same way that more than one organ can be intoxicated by a single agent. n-Hexane produces polyneuropathy.



F7.6a. At the anatomical level, neuropathy can be peripheral or central. A graphic loss of central neurons can be seen in Alzheimer's disease (bottom), where the temporal lobes formerly filled with neurons providing memory and language are now empty ventricles.
A normal brain is on top.

7.4. Reversible/Irreversible Effects

In mammals, a severed nerve in an arm or leg regrows and re-established functional connections. A similar injury in the spinal cord or within the brain will not be repaired, resulting in permanent disability and paralysis. Poor regeneration in the central nervous system has been attributed to proteins embedded in brain myelin (the membranes that wrap each nerve axon), which interact with an inhibitory receptor on neurons called NgR.

Many *acute exposures* with *low molecular weight* compounds produce **reversible** effects in nervous tissue.

Volatile organic solvents affect permeability of cell membranes, so that membrane potentials are altered. CNS depression and anesthetic effects result. Typical agents are nitrous oxide, halothane, methoxyflurane, chloroform, ketones and aldehydes, and solvents found in quick-drying glues. However, *repeated use* of any of these may result in damage so extensive that repair and regeneration mechanisms cannot cope.

According to some sources, the average adult loses 85,000 brain cells per day, but only regenerates 50. There are about 100 billions cells in the adult brain. This limited regeneration is an idiosyncrasy: adult canaries regenerate brain cells quite readily[24].

New brain cells are produced by exercise, estrogen, stimulating environments, high social status, electroconvulsive therapy[22], stroke and other injuries and antidepressants. It appears that cannabinoids promote neurogenesis, and produce anxiolytic and antidepressant effects.[16] This is very different from other drugs of abuse, which suppress neurogenesis (nicotine, heroin, cocaine).

Neurons involved in olfaction and memory are produced into adulthood. Fewer cells are produced as a consequence of aging, spikes in stress hormones, sleep deprivation, barren environments and Ritalin.

Developing brains may be more susceptible to chemicals. Common anesthetics administered to children (midazolam, nitrous oxide and isoflurane) induce apoptosis in baby rat models, which leads to lasting memory and learning deficits. Exposed rats took longer to learn and tended to forget quickly, probably because of observed cell deaths in the hippocampus. In humans, delicate periods for brain development cover from the third trimester of pregnancy to 2-3 years after birth[11].

Anoxia can result from these agents, as well as excessive stimulation with excitatory transmitters, leading to "organic

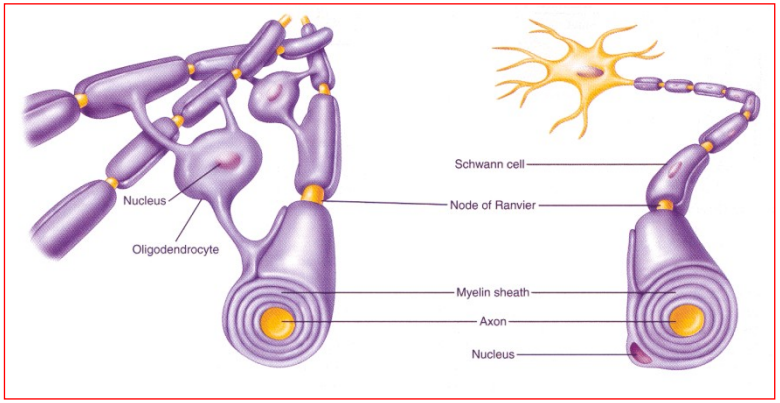
brain syndrome or disease". Mixtures tend to be far more toxic, due to inter-chemical potentiation.
For example, toluene potentiates the toxicity of all other solvents, while methyl ethyl ketone potentiates the toxicity of n-hexane and methyl n-butylketone.
In *chronic exposures*, myelinopathies, axonopathies or destruction of cell populations (Parkinson's) are more often seen. These effects are **irreversible**…

- Unborn children transplacentally exposed to **methyl-mercury** poisoning exhibit symptoms of cerebral palsy at birth. Histology of the brain tissue after their death shows absence of myelin and astrocytes ("infantile" brains without normal maturation).
- There is an inverse relation between a mother's milk level of PCBs (**polychlorinated biphenyls**) and the IQ of children. When at least 1.25 µg/per gram of milk is present, the IQ averages 6.2 points lower. Effects are developmental delays, distractability, short-term memory and planning skills impairment. PCBs were used as insulation for transformers and now taint most soils and water. These effects in the children are permanent.
- Utility workers used to wash their hands in PCBs, secure in the knowledge that PCBs were "only dangerous when burnt" (furanes are then generated). Polybrominated diphenyl ethers (PBDEs⊕), used as a flame retardant in foams, have a toxicity profile resembling PCBs[14] (birth defects, thyroid imbalance, neurological damage).
- Permanent damage is also produced by DDT and manganese. Children drinking manganese-tainted water at levels between 0.2 and 1 mg/l showed an inverse correlation between Mn and IQ scores[17]. Fortunately, Mn contamination of water at dangerous levels gives it a nasty smell.

The difficulty of assessing clinically neurological disturbances is illustrated by the Gulf War syndrome, where marginal exposures to toxicants produced headaches, forgetfulness and mood disorders. 60 % of these people show reduced blood flow in the cortex. There is great conflict and discussion over the existence of the syndrome.

7.5. Myelin and Myelinopathy

There are differences between myelination in the peripheral (both sensory and motor) and central nervous systems. This is important to understand the distinction between central and peripheral myelinopathies induced by chemicals[1].
The myelin[8] sheath in peripheral nerves is from a specific cell, the *Schwann cell*, which wraps itself around a portion of the neuronal axon in much the same fashion as a window roller blind. The nucleus of the Schwann cell resides on the outer surface of the rolled myelin layers. A number of Schwan cells, side by side, provide the myelin for one neuron.



F7.7. Myelin sheath anatomy in the peripheral and central nervous systems. *Human Physiology, Benjamin Cummings, 2002.*

⊕ Two PBDEs have been phased out of production.

The junction between the edges of two Schwann cell myelin projections form the nodes of Ranvier, the point of neuronal depolarization, a thin "tunnel" into the membrane of the neuron. Damage to a single Schwann cell can result in its death, with a gap of nonmyelinated axon surrounded by debris. The neuron conducts pulses rapidly down to the point of the damage, but pulses slow down over the defect, resulting in the disruption of smooth conduction.
In contrast, in the CNS, the role of the Schwann cell is carried out by a specialized cell, the *oligodendroglial cell* which can produce a plethora of sheaths around several adjacent axons, rather than just on one axon. Destruction of one oligodendroglial cell results in the changes in a group of neuronal axons, and may result in more than one observed adverse effect. Unlike peripheral myelinopathy which tends to be relatively specific or localized in effect, a centrally located myelinopathy may appear quite *diffuse*. Multiple sclerosis is one such myelinopathy.

7.5.1. Solvent Myelinopathy

The short-term alcohol-like symptoms of solvent neurotoxicity are headache, dizziness, sleepiness, agitation, euphoria and confusion[9].
The chronic symptoms are premature aging, memory impairment, mild depression and anxiety.
The solvents are typically chlorinated hydrocarbons, alcohols, esters and ketones. They are used for cleaning, degreasing, thinning, stripping and finishing.
The least toxic are alcohols, esters and aliphatic hydrocarbons. Aromatic hydrocarbons and most ketones are highly toxic, and halogenated derivatives of hydrocarbons damage the CNS, liver and kidneys. Benzene is both a neurotoxin and a carcinogen.

Among chronically exposed workers, the disease is common but difficult to recognize because of highly variable, inter-individual responses[3]. Individual diagnosis is difficult even with the best test batteries, each victim presenting one or a few symptoms[2,3]. Victims often present at a relatively young age with serious neurological sequelae from excessive exposure to solvents by inhalation, and are forced to retire from their work. Most of the neuro-psychological symptoms appear to be relatively minor, but still involve demyelination in the central nervous system. The effects are fatigue, sleplessness, headaches, irritability and nausea as well as memory and concentration difficulties.
Problems occur with other organs, such as liver, kidneys, skin, mucous membranes, heart, eyes and reproductive system.

The mechanisms by which solvents induce such effects is unclear and many theories exist. However, many chemicals are capable of nonspecific interactions with neurological membrane phospholipids, causing instabilities and interference with conduction, as well as causing damage to Schwann or oligodendroglial cells and subsequent alterations in myelinated pathways.

7.6. Hexacarbon-Induced Polyneuropathy-Axonopathy

n-hexane and methyl-butylketone cause classical peripheral nervous system effects. They were at one time used in fast-drying glues in the shoe industry and responsible for outbreaks in several countries such as Japan, Italy, Turkey and USA. These two chemicals can be considered together since they share a common toxic metabolite, 2,5-hexanedione[7].
The associated pathology, first detected in humans, was taken afterwards to animals models in rats and chickens. Scientists

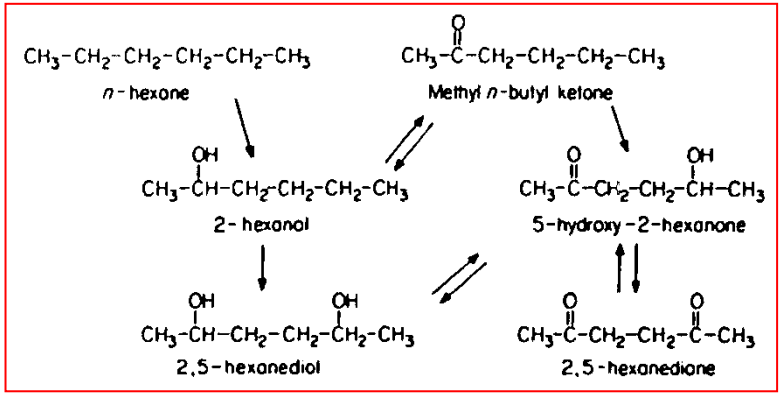
returned to the human situation with recommendations for modification of glue formulations, which produced fewer neurotoxic metabolites.

The neuropathy, reported among workers in the shoe industry, was always most severe in the early springtime, following a winter of closed doors and windows (poor ventilation). The signs and symptoms listed in T7.8 are matched morphologically with Ranvier paranodal swelling at the distal end of myelinated axons, with a progression of the swellings toward the proximal portion of the nerve axon. These swellings cause disruption of the microtubule and neurofilament system responsible for the transport of nutrients to the distal part of the axon from the cell body, with eventual death and disintegration of the nerve endings. If recognized early enough, the syndrome is reversible.

T7.8. Symptoms of hexacarbon neuropathy.

Weight loss
Gradual onset of distal paresthesia
Muscle weakness - distal (hands, feet)
No ataxia or muscle spasticity
Symmetrical dysfunction (hands, feet)
Loss of sensation - “stocking and glove”
Persistent muscular weakness
Atrophy of musculature

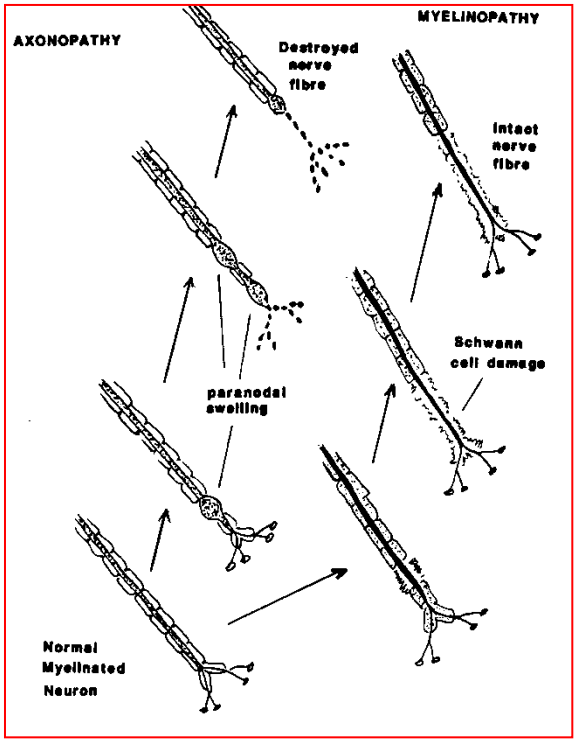
Experiments in animal models revealed that not only were the distal parts of fine nerves of the peripheral nervous system affected, but the distal portions of fine myelinated nerves in the spinal cord (lumbar, sacral) were also damaged, showing much the same morphology. In the paranodal swellings tangles of microtubules and filaments were observed, giving the appearance of a bird nest. Nutrients are no longer transported to the distal end.



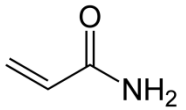
F7.9. Partial metabolic pathway for n-Hexane and methyl n-butyl ketone.

n-Hexane is transformed by two successive hydroxylations and carbonylations (F7.9). The central-peripheral distal axonopathy, so named by neurologists because it affects distal axons of both the central and peripheral nervous systems, is caused by a reactive metabolite, 2,5-hexanedione, found to cyclize during further biotransformation to form a 5-membered ring (imidazole) that interacts with protein structures to disrupt neural organization and cause the tangles. The complete mechanism has not been entirely identified, and interest has declined with the banning of n-hexane and methyl-butylketone and the substitution of other volatile ketones.

F7.10. Formation of axonopathies and myelinopathies.



Humans take a long time to bio-transform and excrete a single exposure to these agents, peak elimination in the urine occurring between 12 and 48 hr after exposure. The workers were exposed daily to high concentrations (estimated as high as 2500 ppm, TLV = 50 ppm) throughout the winter months when ventilation in homes and factories was poorest. When such workers were removed from exposure, the condition was not alleviated, but continued to worsen for approximately 14 days, after which recovery began slowly. The condition was only partly reversible. 2,5-hexanedione also attacks the gonads (testicular atrophy).



Acrylamide (shown) and the catalyst DMAPN (di-methyl-amino-propio-nitrile) cause the "stocking-and-glove" central-distal axonopathy by a somewhat similar mechanism.

This is seen in tunnel workers who inject an acrylamide resin using a pressurized lance into soil and rock to create an impervious, water-proof layer to prevent leaking.

7.7. Metals Neurotoxicity

The term "heavy metal" describes a class of agents in the periodic table possessing certain chemical similarities, including a divalent positive ion. Cadmium, cobalt, lead, magnesium, manganese, mercury, nickel, and vanadium are toxicologically important and all are neurotoxic.

7.7.1. Lead

7.7.1.1. Adults

A millenium ago, body concentrations of lead were typically one-hundredth to one-thousandth of post-industrial levels. The average blood lead level today in the US is less than 2 µg/dl. In the early 1970s, blood lead levels up to 40 µg/dL were considered safe. Today, in adults, levels of 30 µg/dL are tolerated. OSHA will permit a worker to return to work at 40 µg/dL, after being medically removed from work because of lead poisoning.

Long-term exposure to low levels of lead may result in the development of learning and behavior problems, cardiovascular and kidney diseases, decreased fertility, hypertension and cancer[25]. Blood lead levels ranging from as little as 20 to 29 µg/dL are associated with a 39 % increase in mortality from all causes, a 46 % increase in mortality from cardiovascular diseases, and a 68 % increase in mortality due to cancer.

Methyl-mercury is associated with the risk of myocardial infarction, and this partially offsets the protective effects of consuming fish[26].

Overexposure to inorganic lead continues among workers in battery manufacturing, mining of lead and zinc ores, and

painting and paper-hanging. In adults, acute lead exposure leads to renal proximal tubular damage, while chronic exposure can cause renal failure, hypertension, hyperuricemia, and gout[27]. Work-related lead exposure remains a problem, and prevention needs to be strengthened because of lead's low dose toxicity.

7.7.1.2. Children

Lead is especially toxic to the central nervous system, affecting mental development and intelligence in children. Behavioral disorders such as attention deficit disorder have also been attributed to lead exposure[28]. In children, 20 to 25 mg/100 ml can cause irreversible brain damage[29].
Studies of lead in city children initially drove the lowering of acceptable lead concentrations in the blood to 10 µg/dl. But IQ in children further increases as blood levels are lowered to 5 µg/dl[6]. Within the range 1-10 µg/dL, for each µg/dL increase in blood lead level in children, there is a 0.82 point IQ deficit. Above 10 µg/dL, each µg/dL increase only corresponds to a 0.13 point loss. It is probable that any lead in the blood decreases IQ.
2.2 % of children aged 1 to 5 are above 10 µg/dL, 10 % are above 5 µg/dL and 90 % are above 1 µg/dL[10]. The best predictor of lifelong IQ outcome is lead level in the blood at 2 years of age. So, be careful of "pica" !
Similar effects on cognitive developments in children have recently been found in relation to Polycyclic Aromatic Hydrocarbons (from combustion of fossil fuels)[21].

Acute lead poisoning kills children
in the state of Zamfara, Nigeria

A total of 163 children out of 355 cases from several remote villages have died of lead poisoning. The state of Zamfara retained a Chinese company to mine gold in ground that also had high concentrations of lead, but villagers attempted to capitalise by illegally digging for the precious metal themselves. The deaths were discovered during the country's annual immunisation programme, when officials realised there were virtually no children in several remote villages. Villagers said the children had died of malaria, but blood tests from local people showed high concentrations of lead, pointing to death from lead encephalopathy. It is likely locals became sick after lead removed during the process of refining gold ore contaminated local water systems.

For every 5 µg/dl increase in a child's average blood-lead level (ages 5 and 6), there is a 25 percent increase in the number of violent activities associated with criminal arrests later in life[23].

Lead acts by multiple mechanisms in the central nervous system[4]. In neurodevelopmental toxicity, interference with cell adhesion molecules causes a mis-wiring of the central nervous system during early development, and permanent dysfunction. This is a major concern for young children who absorb lead much more efficiently than adults, and who may come into contact with higher levels than adults.

Another toxic mechanism involves interactions between lead and other essential divalent cations (calcium and zinc), these ions having vital roles in neurotransmission[21].
Lead can also act on retinal tissue through apoptosis[12]. Early prenatal exposure to lead causes kids to have retinal deficits up to 10 years later.

7.7.2. Methyl-mercury

Mercury has no known metabolic function, and is the most toxic non-radioactive heavy metal. It affects nerves, muscles and all organs. In inorganic form, it is most injurious to the kidneys, while its organic form in very neurotoxic.

Mercury promotes the production of free radicals, and may bind selenium, which cannot then serve as a cofactor for glutathione peroxidase. Mercury may inactivate the antioxidant properties of glutathione, catalase, and superoxide dismutase. Mercury intoxication has been also known as *Minamata disease*, the name of a city in Japan where the offspring of mothers who consumed fish poisoned by local industrial discharges were victims of mental retardation and absence of limbs.

Metallic mercury is poorly absorbed from the digestive system, but mercury as a vapor (broken thermometer) can cross the blood-brain barrier and accumulate in the central nervous system as well as in kidneys, lungs and fatty tissues. It causes dysfunction, acute and chronic inflammation and results in a large variety of symptoms. Mercury-based dental fillings were a bad idea; the problems of toxicity were pointed out as early as 1840. Such fillings are unacceptable today, in view of alternatives.

Inorganic mercury as calomel (mercurous chloride) was used for centuries by physicians, until it was realized that it did much more harm than good. Recently, it was possible to purchase in Mexico a face cream named "Crema de Belleza", which had calomel is an ingredient. Urine levels found in users and their partners were up to 20 µg/dl. Only 10 % of inorganic mercury is absorbed by mouth, but it affects the digestive system at all levels. The salts also damage the kidneys, leading to reduced urine flow and possible need for dialysis. The familiar antiseptic mercurochrome (a disodium salt of dibromo-hydroxy mercuri-fluorescein) has caused death in children when applied to large burns.

Organic mercury can be 90 % absorbed by the digestive system (methyl mercury). Organic forms of mercury easily travel through organic tissues and accumulate in brain, liver, kidney, blood, skin, hair and breast milk. The body only excretes 1 % of its organic mercury load every day, and prognosis for this intoxication is very poor.

In the brain, methyl mercury inhibits acetylcholine synthesis, resulting in fatigue, memory loss, mood changes, tremors, pallor, weakness and loss of vision or taste. The symptoms are very similar to those of multiple sclerosis.

This toxicologist, Karen E. Wetterhahn of Dartmouth College, used dimethyl mercury, protecting herself with latex gloves. She spilled a tiny amount on her gloves in August 1996, became ill within a few months, bumping into doors and slurring words, slipped into a coma and died less than a year later. Dimethyl mercury penetrates disposable latex gloves in 15 seconds or less, is volatile and very lipid soluble.

As a result of this incident, MSDS sheets were updated.

7.8. Perfluoro compounds

Some chemicals, such as perfluoro-octanesulfonic acid (PFOS) and perfluoro-octanoinc acid (PFOA), used in non-stick fry pans, have been shown to adversely affect behavior of mice[19]. The probably carcinogenic compounds have also been shown to act as estrogen mimics[20].

7.9. Parkinson's

The motor manifestations of Parkinson's disease are related to a lack of the neurotransmitter dopamine in the brain.

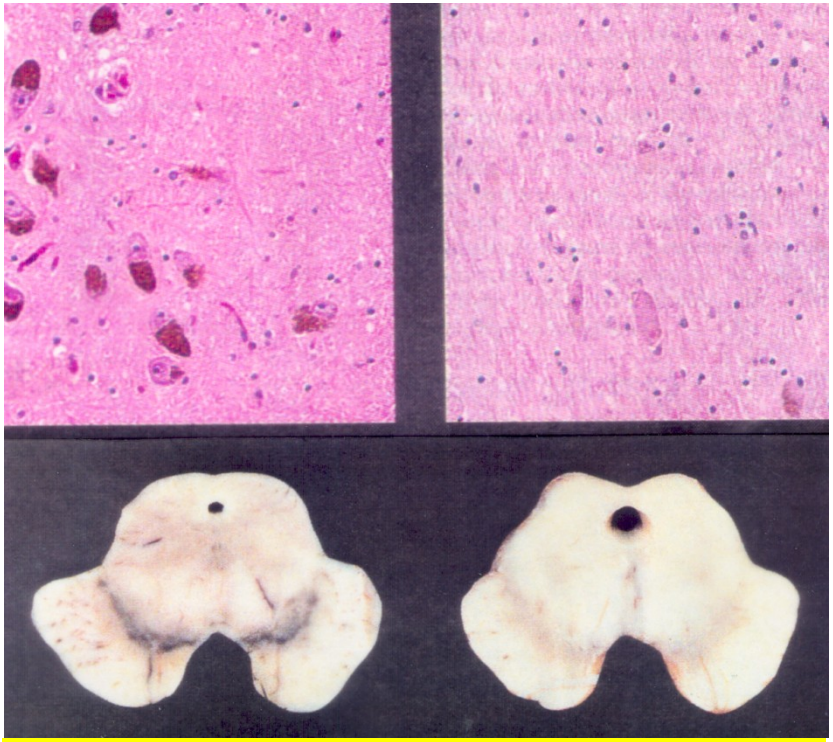
Within the brain, the *pars compacta* (F7.11) of the *substantia nigra* (purple in F7.12) loses neurons which normally provide dopamine signals to the *striatum* (blue). The *striatum* signals control motion in the brain's cortical regions.

The dark brown pigments at top left in F7.11correspond to dopamine-producing neurons which oxidize dopamine to the pigment neuromelanin. The normal brain at left shows more dopamine both in the histology (dark brown cells) and in the anatomical section (black pigment) than the Parkinson's brain at right

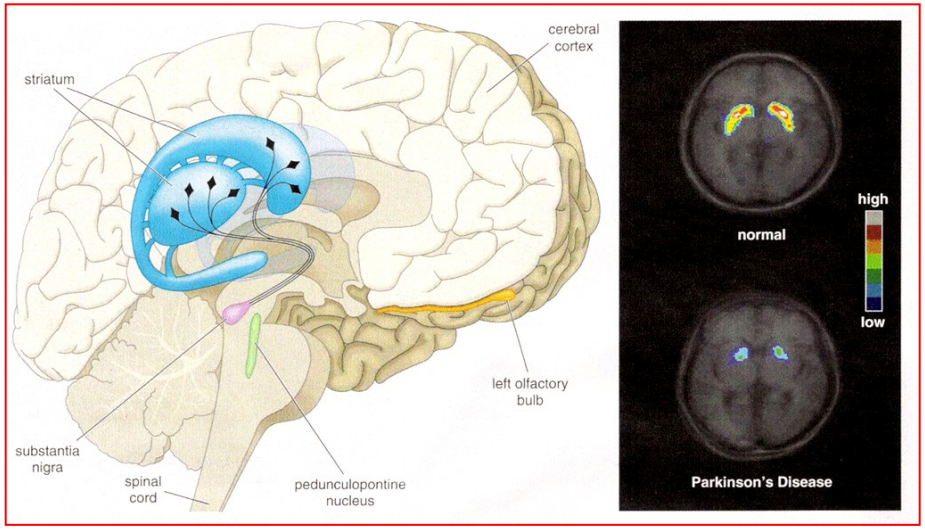
An effective therapy for the motor symptoms is the administration of *levodopa* which can cross the blood-brain barrier and act as a precursor of dopamine.

Although lesions in the dopaminergic system have been prominent in Parkinson's research, this brain disease is not restricted to the *substantia nigra*. Other areas of the brain, the spinal cord and the peripheral nervous system are affected in Parkinson's, as are serotonin, norepinephrine and acetylcholine neurotransmission. Postmortem studies suggest that the brain stem is first affected, then the substantia nigra-striatum-midbrain, and finally the cerebral cortex.

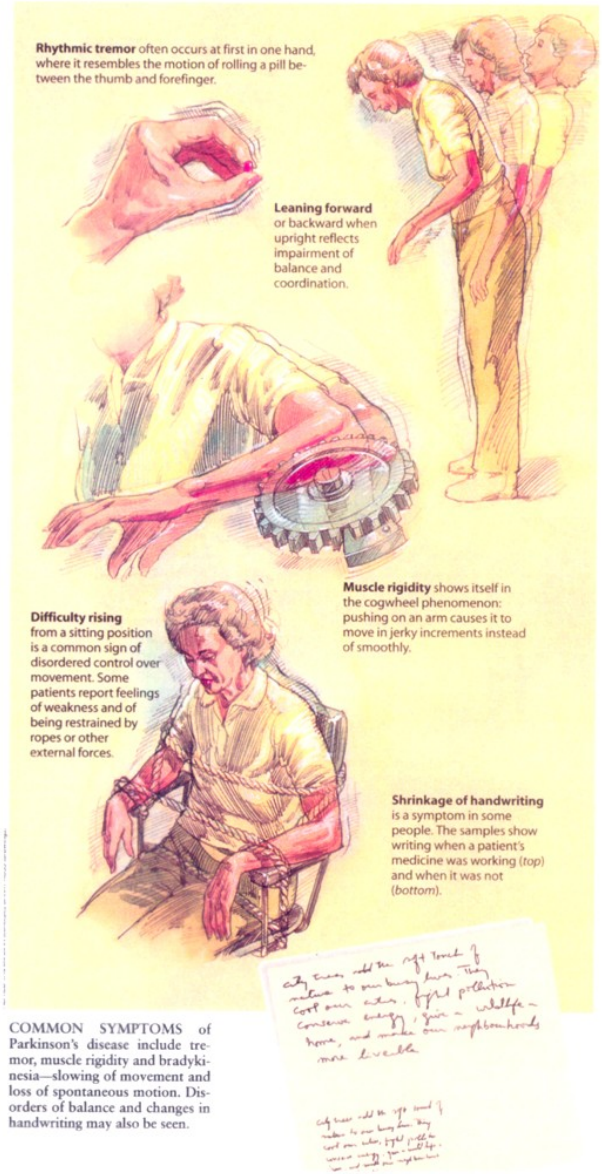
One strange feature is that in the same individual, some neurons are affected while others are spared, which underlines our incomplete understanding of this disease. Early Parkinson's may manifest by a weakened sense of smell. Late Parkinson's results in non-dopaminergic difficulties, such as dementia.



F7.11. Histology of the *pars compacta* in the *substantia nigra.* Section of the top of the brainstem (hole is the central canal) at the level of the *substantia nigra.* *Scientific American.*

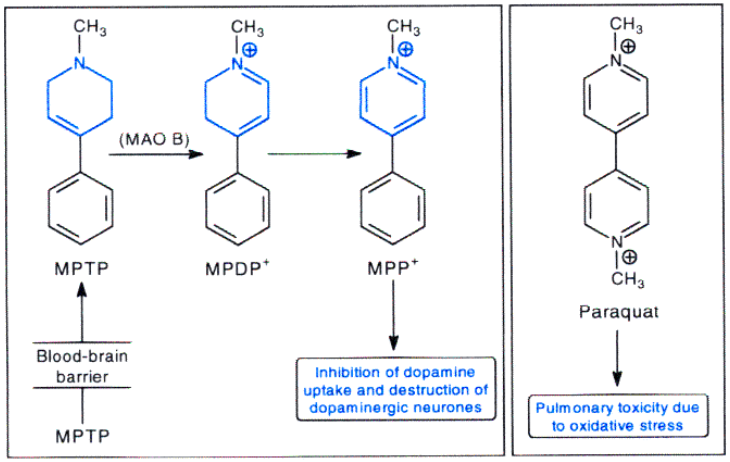


F7.12. Neuro-anatomy and PET scan of dopaminergic activity in Parkinson's disease.
American Scientist. Scan by Alain Dagher, McGill MNI.



7.13. Common symptoms of Parkinson's. *Scientific American.*

F7.14. The frozen addicts. *Scientific American.*



F7.15. MPTP metabolism and action. *Casaret & Doull.*

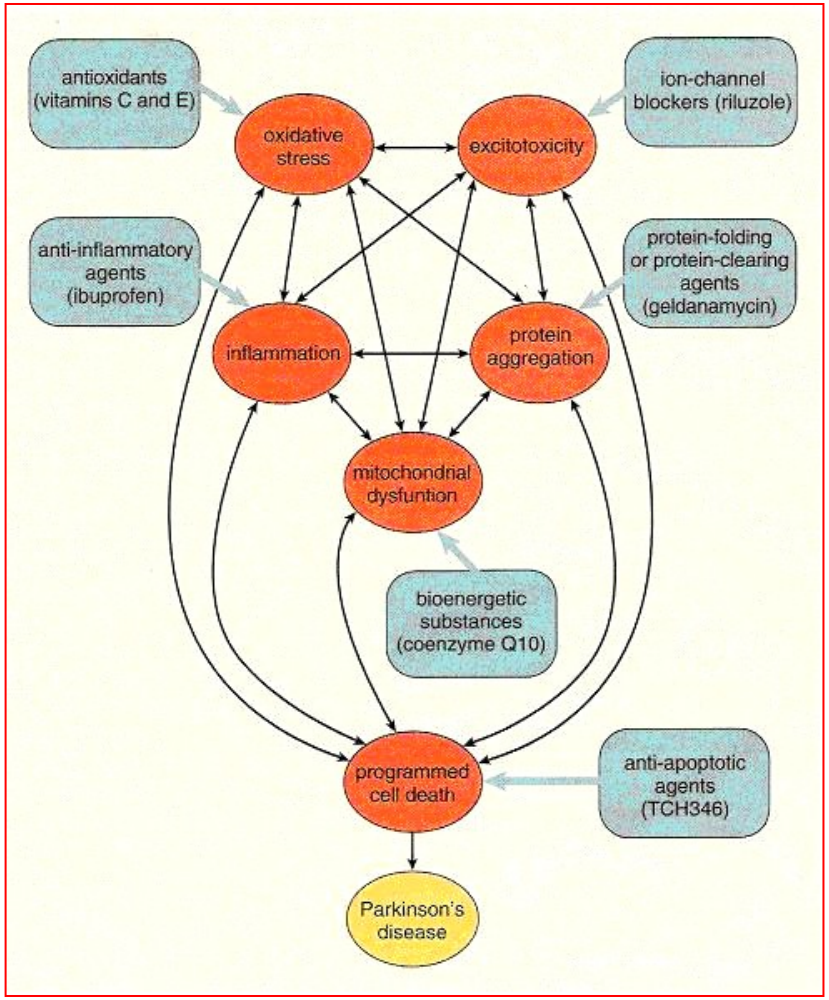
The exact causes of Parkinson's disease are not known. There are many indications that Parkinsons' s is related to toxic exposure in the environment, an association long suspected by epidemiologists.

The so-called frozen addicts posed for a picture in 1991 (F7.14) after having received treatment. Nine years earlier, all suddenly became immobile as if they had instantly acquired Parkinson's. They had consumed a narcotic containing the impurity MTPT (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) which inhibits mitochondria's electron transport chain. This episode provided a model for the disease. MPTP causes a condition similar to Parkinson's in animals and its molecular structure is related to that of several pesticides (F7.15).

Older people in Guam and in Guadeloupe are victims of a disease very similar to Parkinson's. The disease has been recently linked to eating sago palm (Guam) and soursop, custard apple and pomme canelle (Guadeloupe).
The fruits contain alkaloids (*alkaline-like*, loose group of compounds such as curare, atropine, physostigmine, morphine, codeine, cocaine, caffeine, LSD, quinine, serotonin, strychnine, nicotine). The disease does not respond to *levodopa*, contrary to Parkinson's.
Prolonged administration of rotenone, a plant-derived pesticide used in "organic" gardening produces symptoms of Parkinson's in rats[5].

As in many neurological diseases, it is difficult for clinicians to determine if a drug therapy actually slows (or reverses) the death of brain cells, or just improves brain function, so that it compensates for the loss of cells. Until this distinction can be

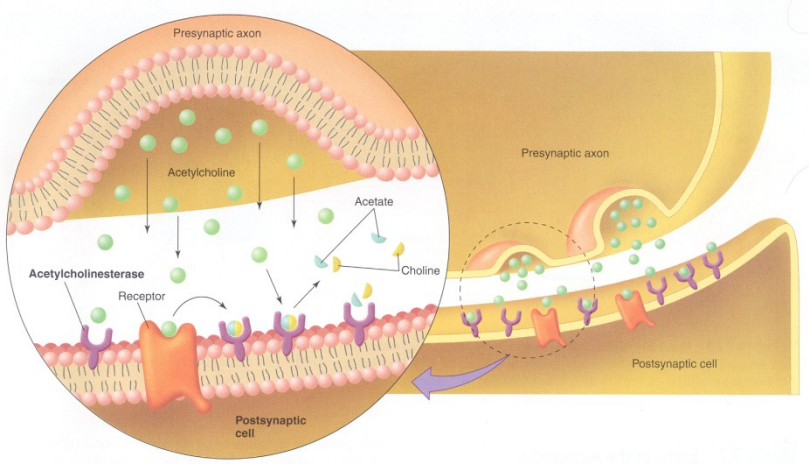
made, the various attempts to deal with the disease by preventing the death of neurons may not be productive.



F7.16. Therapeutic thinking for prevention of cell loss in Parkinson's disease (and many chronic ailments). The mechanisms for cell killing are in red, interventions to prevent cell loss are in blue-gray.

7.10. Acetylcholinesterase Inhibition.

There are 2 types of cholinergic receptors: muscarinic (heart rate, bronchoconstriction, arteriole dilatation, secretions of stomach and intestine, salivary and lacrimal glands) and nicotinic (neuro-muscular junction, autonomic ganglia). Acetylcholine will stimulate both of them, but muscarine and nicotine are selective.
The sites of action may be anywhere in the body, but the mechanism is very specific, as depicted in F7.17. Toxicants can have effects on muscarinic and nicotinic receptors and on acetyl-cholinesterase itself (the enzyme responsible for degradation of acetylcholine in the synapse).

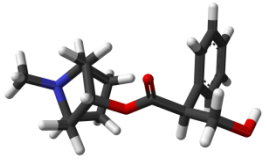


F7.17. Nerve synapse. Toxicants can interfere with neurotransmitter release, stimulation of receptors or the breakdown of acetylcholine by acetycholinesterase.

Organophosphates and carbamate insecticides inhibit acetylcholinesterase, producing over-stimulation of the cholinergic systems.

For example, organophosphates react with a serine hydroxyl group at the active site of acetylcholinesterase, inactivating the hydrolytic function. This causes an accumulation of acetylcholine esters, leading to cholinergic over-stimulation. In acute or moderate chronic exposures, neuromuscular weakness occurs because of down-regulation of receptor activity. The diaphragm is a red muscle dependent on acetylcholinesterase, therefore breathing can be affected. California has a long-standing formal blood cholinesterase monitoring program for mixers, loaders, and applicators of pesticides. The test looks at two substances, acetylcholinesterase (nerves and red blood cells) and pseudocholinesterase (liver). Normal pseudocholinesterase values range between 8 and 18 units per milliliter.

Intoxication is treatable with *atropine*, which competitively binds the receptors of cholinergic nerves. Atropine (shown), otherwise known as *Atropina Belladoña* has an interesting story. Women in times past would administer themselves atropine to dilate their pupils before going to court or meeting prospective important lovers. If you are in court, how others perceive you may be your best weapon, so-to-speak putting "your best eyes forward".



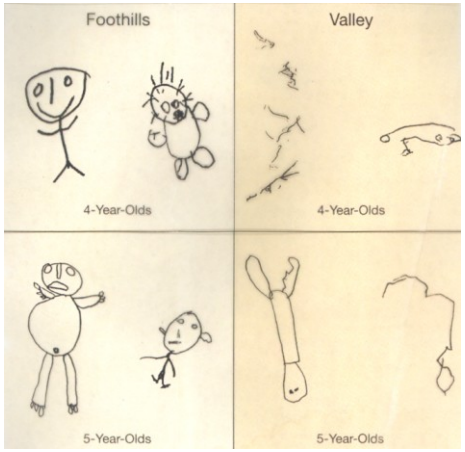
7.11. Consequences of Pesticide Exposures

Neurobehavioral testing of exposed children in Mexico by the University of Arizona showed that exposed children had:

- less stamina,
- poorer gross and fine eye-hand coordination,
- weaker 30-minute recall and
- impaired drawing ability (F7.18).

It was shown that children are born with detectable concentrations of many pesticides in their blood and are exposed further through breast milk.

Many studies[30,31,32] have confirmed that children exposed to high pesticide levels in the womb have lower average IQs than other kids. The most heavily exposed children scored an average of 7 points lower on IQ tests compared with children with the lowest pesticide exposures. Since pesticide exposure after birth wasn't linked to lower intelligence scores, this suggests that the harm caused by the chemicals is greatest during early pregnancy, when the brain is developing. Earlier studies have linked organophosphate pesticides with attention deficit hyperactivity disorder (ADHD).



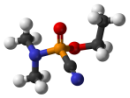
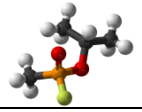
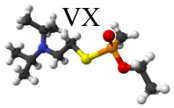
F7.18. Mexican children from "Foothills" (Yaqui Indians) have only one major exposure to pesticides: DDT spraying by the government to control malaria. "Valley" farmers apply pesticides 45 times per crop year and also tend to use household bug sprays daily. These exposures are not significantly different from those in other intensely farmed areas.

7.12. Chemical Warfare Agents

The military are trained to inject themselves with atropine in case of a neurotoxic attack.
Organophosphorus warfare agents as well as insecticides can also be countered by certain drugs such as galantamine, a reversible centrally-acting acetylcholinesterase inhibitor.

Galantamine, otherwise used in Alzheimer's treatment, was effective up to 3 hours before and 5 minutes after exposure to the organophosphate[18].

T7.19. Warfare Neurotoxicants.

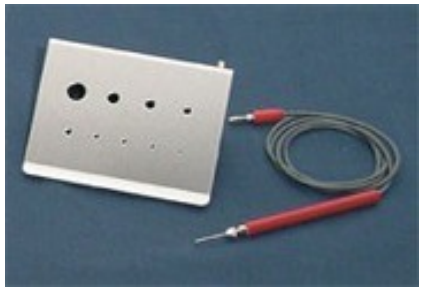
Agent	Appearance & odor	Absorption	LD_{50} (mg/m^3.min or mg/kg)	Symptoms
Tabun 	Colorless to amber-brown liquid; slightly fruity odor	Skin contact and inhalation	400, inhaled 1000, skin	Runny nose, constricted pupils, tightness in chest, blurred vision, nausea, vomiting, confusion, loss of control of body functions, respiratory paralysis.
Sarin 	Colorless liquid; no odor when pure		70, inhaled 1700, skin	
VX 	Colorless to amber liquid; no odor when pure		50, inhaled 10, skin	

7.13. Neurotoxicity Testing

Neurotoxicity testing is complicated by the need to differentiate between CNS and peripheral nerve damage, as well as between reversible and permanent lesions.
Tests in humans can range from sensory-motor (F7.20) to psychological. Batteries of tests give more perspective on neuromorbidity: X-rays, CAT scans, MRI, electromyography, EEG, peripheral nerve conduction velocity and cerebrospinal fluid composition are used. There is some discussion about the definition of the "normal" range appropriate for various results, which is often defined purely by statistics, as opposed to specific thresholds.
In animals models, performance tests such as maze, movement recording and platforms hidden within pools are frequent.

Rats are tested for abnormal posture, closure of eyelids, tremors or convulsions, lacrimation, salivation, gait, mobility, level of arousal, stimulus response, pupil response, righting reflex, forelimb and hindlimb grip strength and foot splay.

Although cells are used to test for neurotoxicity, these models have not made great inroads yet.



F7.20. A device to measure hand tremor.

7.14. Case Study: Chlorinated Hydrocarbon Pesticide

(modified from Williams & Burson)

A physician in Virginia saw a patient who worked in a plant where Kepone (chlordecone) was produced. The patient complained of weakness, nervousness, weight loss, shakiness and difficulty in reading and driving because he could not focus his eyes. The physician thought that the exposure to Kepone might have caused the patient's illness and sent a blood sample to the CDC for a determination of Kepone content. The full chemical name of Kepone is 1,1a,3,3a,4,4a,5b,6-decachlorooctahydro-l,3,4-metheno-2 H-cyclobuta [cd] pentalen-2-one.
The CDC used to have a service for physicians in which, on request, would analyze adipose tissues, blood samples, and other biologic samples for content of chlorinated hydrocarbon pesticides. The chemist at CDC who received the sample, not realizing the emergency, thought that the Virginia physician had previously arranged for such an analysis. He therefore accepted the sample, analyzed it, and found very high Kepone levels. He thought his analysis was wrong, so he repeated it three times before he said anything about it to anyone. In the meantime, the patient visited a neurologist at the Medical College of Virginia. The patient's symptoms and signs now included extreme nervousness, tremor, ataxia, skin rash, and an odd rolling of the eyes called opsoclonus. He also had lost weight. He couldn't concentrate even to read a newspaper, and he still had trouble driving.

Ultimately, the high Kepone blood level determined at CDC was reported to the neurologist at the medical college and also to the requesting physician.
The toxic effects of Kepone observed in animals include excitability, tremor, weight loss, and, in some cases, testicular atrophy. Kepone is a persistent compound. Once absorbed, Kepone will be stored in body tissues for a significant period of time. It is the only representative of the group of persistent halogenated pesticides that is not predominantly stored in adipose tissue. The ratio between blood and adipose tissue is not as large as for other chlorinated aromatic pesticides and industrial chemicals, and a great deal of Kepone is stored in the liver, because of its greater polarity.
After this case was uncovered, we needed some facts about the plant in which the man worked. Several phone calls were made in an attempt to gather information.
The neurologist was asked to question the patient about other employees to determine if a larger problem existed. The patient reported that employees at the plant were unable to even drink much coffee during their breaks because they shook so severely they would spill the coffee, or would be unable to fill their cups, an indication that all probably had Kepone-caused tremor.
This was reported to the Virginia state health department; it became apparent that somebody needed to take direct action. The state epidemiologist inspected the plant and closed it, all within two days.
It was a small facility operating out of an old garage. In making Kepone, the firm used a completely open drying operation. During the drying process, dust was spread everywhere. There were no facilities in which the employees could change clothes or wash, and no separate eating facilities.
It was difficult to understand how people could be affected so profoundly-some people were unable to stand up from a chair-and still continue to work without realizing they were being affected by the chemical. In addition to the rash and nervousness noticed in the index case, many people complained of chest pains, but it was not possible to determine a cause for that complaint. It probably was a neurologic effect.
A divorced worker at the plant even seemed to have Kepone-related problems with alimony pay. He would begin to feel sick after working with Kepone, would stay home with no pay. His ex-wife, thinking this an intentional avoidance of work, went to court, and the man would find himself in jail for failure to support her. While in jail, he would recover; and then he would resume work at the Kepone plant, become sick again, and the whole cycle would repeat. Other workers felt that perhaps they were being affected by Kepone, yet they had been told, "This compound doesn't really do anything. It's probably because you drink too much alcohol."
In addition to the workers, residents in the neighborhood were also examined, as well as employees' family members. Sometimes it is difficult to conduct these types of examinations and evaluations objectively because of publicity about the problem. As mentioned, the workers were unable to change clothes at the plant. Workers often carry dust of the chemicals they are working with -particularly persistent compounds, including chlorinated hydrocarbons, lead, and asbestos- home on their clothes and in their cars, thus exposing their families. Many family members of the employees had Kepone blood levels. They were lower than those of the plant employees, but they were higher than what might be expected in the general population. The Kepone produced at this plant was manufactured for export to other countries. Kepone is not registered for use in the United States, so normally Kepone blood levels are not observed in the general U.S. population.
The plant was closed and never reopened. Many political problems remained after the closure, however. The James River was found to be contaminated with Kepone and was closed to fishing for a while. The state of Virginia is still struggling with the problems resulting from this episode of contamination.

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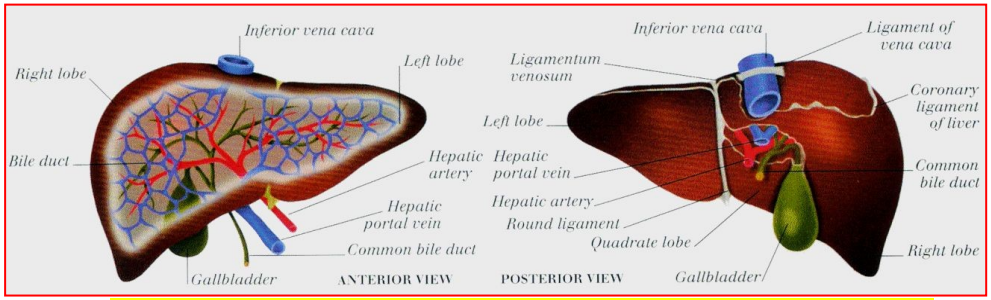
Hepatotoxicity

8· Hepatotoxicity



F8.1. Liver anatomy.

The Roman anatomist Galen (200 AD) made the liver the principal organ of the human body, arguing in his text "On the Usefulness of the Parts of the Body" that it emerged first in the formation of a fetus.

Up to 25% of the hepatocytes are binucleate and another 50% are polyploid, reflecting a *need for redundancy* in this vital organ. The liver is a site where *apoptosis* occurs normally, sometimes obviously associated with toxic exposure◊.

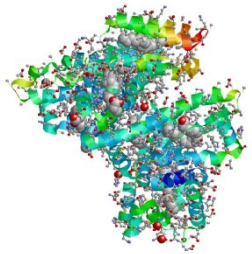
After acute toxic exposure one finds: lipid accumulation in hepatocytes, necrosis or hepatobiliary dysfunction. After chronic exposures, one sees cirrhotic-like or neoplastic changes. Some forms of liver injury are reversible, while others are not.

◊ In rodents, but not in humans, the sedative phenobarbital inihibits apoptosis, leading to considerable liver enlargement.

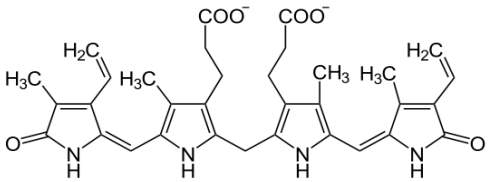
8.1. Roles of the Liver

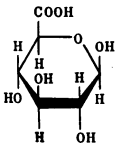
The largest gland, the liver performs over 500 known functions....

1) Metabolism of **foods** (fats, carbohydrates, amino acids and proteins) and most amino acid interconversions and catabolism, bile secretion to enhance the uptake of dietary lipids and vitamins, urea formation (excretion of nitrogen), storing vitamins A and D.
2) It stabilizes the **glucose level** by taking up and storing glucose as glycogen, breaking it down back to glucose when needed, as well as forming glucose from non-carbohydrate sources such as amino acids. Hypoglycemia occurs only late in the course of severe liver disease, because the liver has a large functional reserve, maintaining its function with only 20% of the liver functioning.
3) Synthesis of proteins: secretion of plasma albumin (a carrier for drugs and hydrophobic compounds such as unconjugated bilirubin, shown), most of the globulins (coagulation Factors II, V, VII, VIII, IX, X) other than gamma globulins. Most cholesterol synthesis takes place in the liver.



4) Detoxification (turning fat-soluble poisons into water-soluble wastes that can be eliminated through the kidneys) and excretory bile formation, the major product of cholesterol catabolism.
5) It removes red blood cells and warms the blood.





β-D-glucuronic acid

For example, hemoglobin transformed to bilirubin is conjugated with glucoronic acid (both shown) in the liver, and the xenobiotics Hg and Pb are excreted in the bile…

Glucoronic acid also conjugates excess estrogens, the resulting products exiting unchanged through the kidneys. Cats are vulnerable to many toxicants because of their limited ability to form glucuronide conjugates.

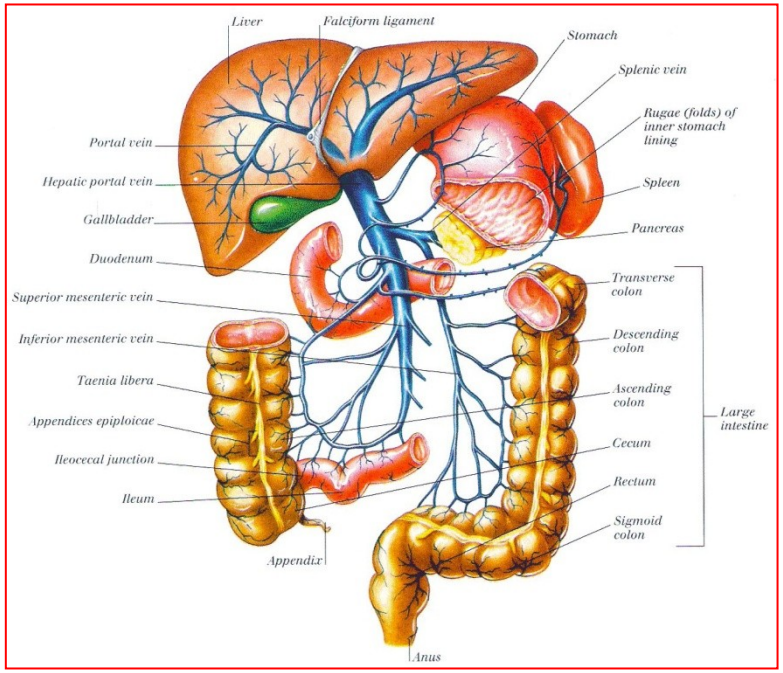
8.2. Anatomy

The liver is the source of the veins and the principal instrument of sanguification, Galen observed. He was not far off the mark, given the organ's position in the circulatory system (F8.2) and its role in intermediary metabolism and biotransformation of xenobiotics.

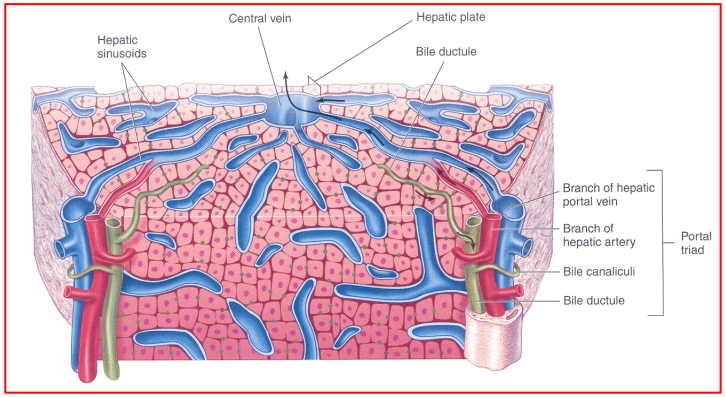
Note the blue portal vein in the center of the diagram, and how blood flows from the various components of the digestive system into the portal vein. Blood output is into the *vena cava*. Some 70% of the liver's blood supply is venous, coming from the mesenteric plexus via the portal vein, while 30% is arterial, from the hepatic artery.

8.2.1. First-Pass

The First Pass effect refers to the influx of toxicants through the portal vein (F8.2), particularly from the digestive system and to the high level of bio-transformation in the liver.

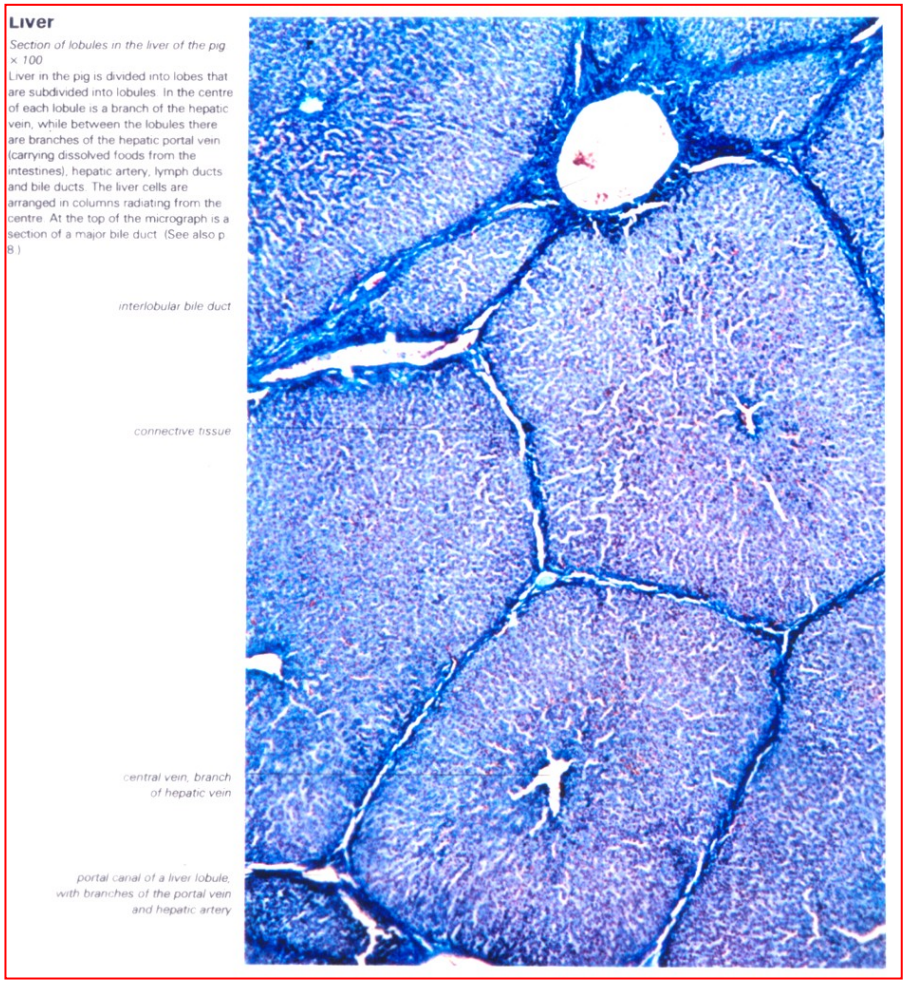


F8.2. Liver's position in the circulation. *Dorling Kindersley, 1998.*



F8.3. Section of a hepatic lobule. Lobules are 1-2 mm in diameter, visible to the naked eye. *Fox, 2004.*

On entering the liver, the blood vessels break up into a dense capillary bed, the blood pressure decreases sharply and there is slow perfusion of the blood through the *sinusoids*, permitting the bordering cells to extract chemicals from the bloodstream by passive diffusion (F8.3). The functional unit of the liver, the hepatic *lobule,* is a radial array centered on the *central vein*. Branches of three conduits converge towards the center: portal vein, hepatic artery and bile duct. Blood flows through *sinusoids*, while *canaliculi* channel bile. The blood of sinusoids flows towards the center, the bile in the canaliculi away from the center, towards a branch of the bile duct.
The sinusoidal lining is fenestrated, allowing chemicals easy access to the hepatocytes, which make up the bulk of the organ. There are at least 6 different types of liver cells: Hepatocyte, Biliairy Epithelial, Endothelial, Kupffer, Ito and Pit.



F8.4. Section of lobules in the pig, x 100.
At this magnification, individual cells are barely discernible.
Biological structures.

Endothelial cells differ from the vascular endothelium elsewhere in the body in that they lack a basement membrane and contain numerous fenestrae that permit hepatocytes to have ready access to nutrients and macromolecules in plasma. Endothelial cells are also responsible for endocytosis of molecules and particles, and play a role in lipoprotein metabolism.

Spindle-shaped *Kupffer's cells* are tissue macrophages. They form an important part of the body's reticuloendothelial system. Their major functions include phagocytosis of foreign particles, removal of endotoxins and other noxious substances, and modulation of the immune response through the release of mediators and cytotoxic agents.
Peri-sinusoidal fat-storing cells (*Ito cells*) store vitamin A. They transform into fibroblasts[⊕] in response to hepatic injury, contributing to hepatic fibrosis.

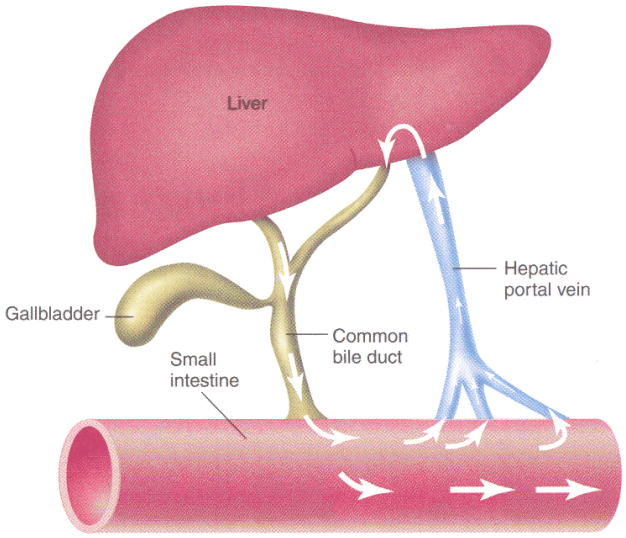
[⊕] Fibroblasts secrete collagen fibers and provide mechanical tension to close wounds.

Pit cells, the least common sinusoidal lining cells, are large, granular lymphocytes, which function as natural killer cells. The extracellular matrix of the liver includes its reticulin framework and several molecular forms of collagen, laminin, fibronectin and other extracellular glycoproteins.

The blood in the liver has a low oxygen content, probably to reduce oxidation reactions. The histologic appearance of the liver is shown in F8.4.

8.3. Physiology

8.3.1. Entero-Hepatic Loop



F8.5. Entero-Hepatic loop.

Compounds that are metabolized (or not) and excreted into the bile duct may be re-absorbed from the small intestine before excretion, resulting in increased toxic exposure.

Phenolphtalein (the active ingredient in Ex-Lax) is an effective laxative because of its persistence through the entero-hepatic loop. After Phase II metabolism in the liver and excretion in the bile, intestinal flora releases it in native form, allowing re-absorption.
Hg is excreted in the bile. If in the form of methyl mercury, it is reabsorbed from the biliary tract, contributing to its long half-life.

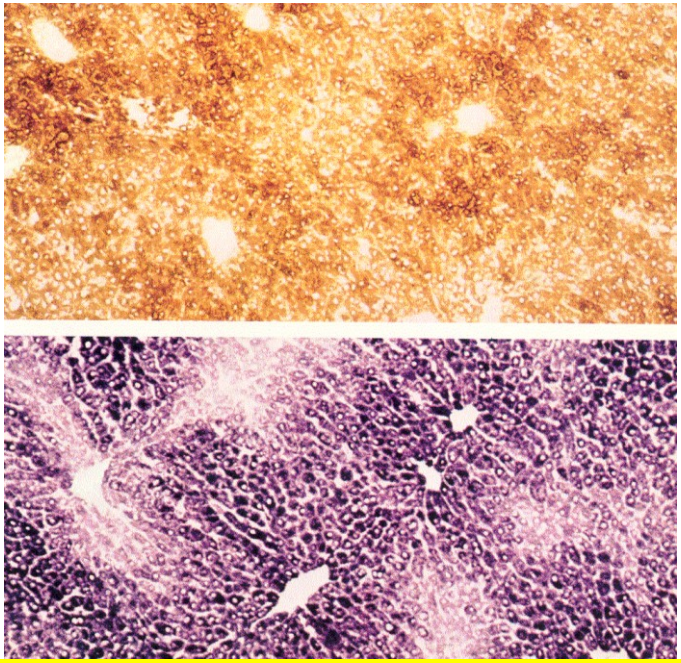
However, the increased exposure to toxicants due to the entero-hepatic loop may be a side-show. Up to 95% of bile is reabsorbed through the entero-hepatic loop, and bile plays a large role in the regenerative ability of the liver. This regeneration is tied to the stimulation by the bile of the FXR gene and protein, which acts as a bile sensor.
Rats fed bile components in their chow have livers 30% larger within a week. The entero-hepatic loop may support a natural cycle that enhances regeneration in the liver[7]. It is the only internal human organ known to naturally regenerate, from just 25% of its tissue.

8.3.2. Liver Enzyme Distribution

The level of enzymes (F8.6) is not constant throughout the lobule. There is a gradation of activity from portal *input* to vein *output*.
Phase I activities *increase* 5-fold from the portal input to the central vein output.
Phase II conjugating enzymes levels (such as glutathione transferase and glucuronyltransferase) *decrease* approximately 4-fold from the portal input to the central vein output.

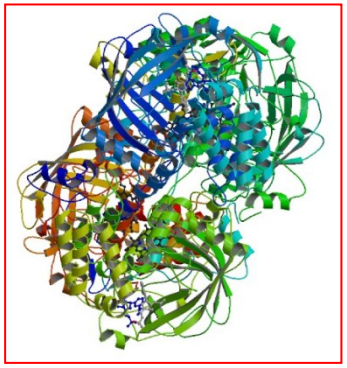
What does this organization signify when considering toxic reactions in the liver, specifically in avoiding irreversible damage to hepatocytes?

It is probably more efficient for liver cells to specialize in a specific aspect of bio-synthesis, thereby creating two cell populations (Phase I and Phase II).
Since Phase I reactive intermediates are particularly toxic, it is also probably better to provide less enzyme at the portal input, where the highest toxicant concentration is expected. This spreads out Phase I biotransformation and toxic stress more uniformly over the lobule.
Phase II enzymes, less likely to increase toxic stress, may be spread according to the increased availability of bio-synthetic



F8.6. Distribution of NADH-oxido-reductase (Phase I, top) and ATP-ase (bottom) in lobules. X 50. *Biological Structures.*

machinery upstream, where Phase I metabolism requirements are less. In a high-level insult, there will be a buildup of reactive intermediates in the central vein region where Phase I metabolism is highest. Once enzyme reserves are depleted, Phase II activity is cast back to a lower basal rate, Phase II being the rate-limiting step in many detoxifications.
These patterns of enzyme expression are not inflexible across species or across time. In male rats, hepatic enzyme distributions alter during aging. Peroxisomes (Phase I) are more active in pericentral than periportal hepatocytes, in contrast to the uniform pattern in young animals. Other age-established lobular gradients were observed in trifunctional enzyme (central > portal) and catalase[ℵ] (portal > central, molecule shown below). Enzyme levels are significantly altered in aged animals, perhaps contributing to disturbances in lipid metabolism.



A chemical toxic to the liver may be more toxic when ingested or injected intra-peritoneally than when absorbed through lungs or skin, because disposition of the toxicant to other body compartments will lower the effective concentration to the liver.

8.3.3. Levels of Cytochrome P450

P450s are heme-containing monooxygenases and are divided into two primary groups: *steroidogenic* and *xenobiotic*.

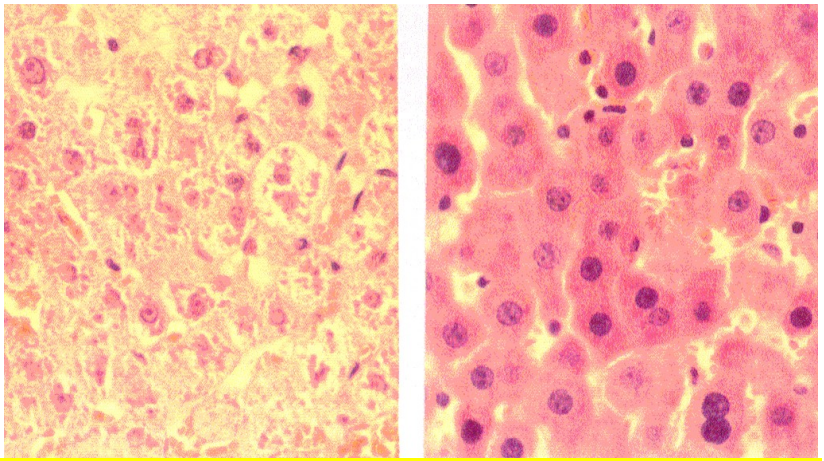
Steroidogenic P450s are found both in prokaryotes and in the mitochondria and smooth endoplasmic reticulum of eukaryotes. They synthesize steroids and other substances

[ℵ] Genetic induction of supplementary catalase in mitochondria has been shown to increase the life-span of rats by 15%.

necessary for the maintenance of cell wall integrity and cellular differentiation.

Xenobiotic P450s are found in the smooth endoplasmic reticulum of eukaryotes and metabolize foreign biological substances. There are about 12,000 members across all forms of life, the human ones being encoded by 57 human genes. This group of P450s appears to have evolved from the steroidogenic P450s during the era of plant-animal differentiation, conveying a survival advantage by allowing animals to detoxify the substances that they consumed. These P450s are responsible for most oxidative drug metabolism: CYP3A (~50%) and CYP2D6[1] (~30%) and CYP1A2, 2C9/10, 2C19, and 2E1 which account for approximately equal percentages of the remaining 20%. Relevant P450s have been established for only ~ 20% of marketed medications.

If hepatocytes encounter a moderate or high level of bio-transformable chemicals (say carbon tetrachloride), the cells induce synthesis of additional Phase I and Phase II enzymes. The synthesis is not selective for individual enzymes, rather, a broad spectrum of enzymatic activities are turned on, with the capability of biotransforming a vast array of exogenous chemicals. This is compatible with adaptation to natural toxins which usually come as a group of related compounds, rather than as a single purified molecule.
This involves the synthesis of messenger RNA (mRNA) which is used by the cellular rough endoplasmic reticulum (RER) to synthesize proteins (Phase I and II enzymes) which are either incorporated into a subcellular membranous organelle - the smooth endoplasmic reticulum (SER) or released into the cytoplasm of the cell as soluble enzymes. These changes are easily visible under the microscope (F8.7).
If the cell copes successfully with this defensive mechanism, the levels of these enzymes will slowly decline back to normal. If it does not, the levels of Phase I and II enzymes will remain elevated and cytotoxicity may result from the accumulating reactive intermediates. A wide range of compounds are able to induce increased levels of cytochrome P450 enzymes.



F8.7. Appearance of rat liver cells at x 100 in the normal state (left) and after 16 hours of intoxication by ketamine-xylazine (right). *Héroux, 1993.*

Drugs: Aminopyrine, Amphetamine, Barbiturates, Chloral hydrate, Chlordiazepoxide (Librium),Chlorpromazine, Diazepam (Valium), Diphenhydramine, Ethanol, Ethanol pyridione, Glutethimide, Halothane, Hypericin and Hyperforin (St. John's wort), Imipramine, Meprobamate, Morphine, Nicotine, Phenylbutazone, Phenytoin, Promazine, Propoxyphene (Darvon), Steroids, Sulfanilamide, Thalidomide, Trimethadione, Urethane, Zoxazolamine.

[1] CYP2D6 has more than 75 known genetic variants, resulting in poor, intermediate, extensive or ultra-rapid activity.

Industrial chemicals: Alcohols, Aldrin/dieldrin, Chlordane, Chloroform, DDT, DDD, DMSO, Heptachlor, Ketones, Lindane, PCB compounds, Piperonyl butoxide, Pyrethrum, Toxaphene.
Polyaromatic hydrocarbons: Benzo(a)pyrene, Dibenzanthracene, 3- Methylcholanthrene.
Some toxicants are *inhibitors* of biotransformation. They achieve the effect through competitive binding, synthesis inhibition of heme or cytochrome P450, inactivation-destruction of cytochrome P450 or of the endoplasmic reticulum. Examples of such chemicals are carbon tetrachloride, bromobenzene and α-naphtyl isocyanate.

Some toxicants are unexpectedly powerful. A rogue tropical seaweed, *Caulerpa taxifolia,* is presently spreading in the Mediterannean. It produces *caulerpenyne*, a toxicant which inhibits fish's P450 enzyme activity to 25% of baseline and alters metabolite composition. The fish do not need to consume the seaweed, just be in the neighborhood !

In hygiene, it is not uncommon to add together the percentages of the TLVs when multiple exposures occur at a single site. The rough nature of such a procedure is clear if we consider that even if the two chemicals have quite identical dose-responses, they are unlikely to have similar half-lives.

8.4. Pathology

Decreased liver function can result in any number of symptoms including fatigue, allergies and chemical sensitivities, acne or broken blood vessels on the skin, intolerance to fatty foods or alcohol, changes in mood or weight, changes in cholesterol or triglyceride levels, nausea and constipation. Overt liver disease presents with more obvious symptoms such as yellowing of the skin, liver enlargement, itchy skin, dark urine, pale stool, easy bleeding, blood sugar problems and gallstones.

Liver enlargement is an effect frequently observed in pesticide toxicity studies. Increases in relative liver weight of <10% are generally considered to be non-adverse, while increases of ≥10% are interpreted as being potentially adverse.
Liver is highly exposed to toxic damage because of its high rate of biotransformation (T8.8).

If the liver did not metabolize benzene, "its half-life would be 100 years" (P450 2E1). Paradoxically, more benzene is metabolized to toxic species at low doses than at high doses ! Liver cells in culture show a progressive fall in expression of xenobiotics metabolism (1-2 days), and although some human B-lymphoblastoid cell lines can be engineered to stably express human cytochromes P450, it has been very difficult to maintain true toxic metabolism in vitro.

T8.8. Metabolism of various microsome fractions.

Organ or Tissue	Metabolic Activity (% of liver microsomes)
Gut	10
Adrenal Cortex	50-75
Testes	10-20
Spleen	5
Heart	3
Muscle	1
Brain	1
Placenta	1
Skin	1

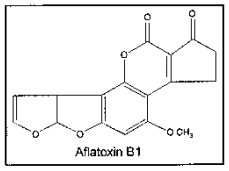
A *cytotoxic agent* may proceed through necrosis or apoptosis. For example, ethanol drives a sequence of cell toxicity, necrosis, fibrosis, resulting in a fatty liver otherwise known as cirrhosis. Cirrhosis often results in jaundice and portal hypertension. The disease may be telomerase-limited.
A *cholestatic agent* triggers inflammation of the biliary tree with a decrease in bile flow and excretion.

8.4.1. Anatomical Classification of Liver Injury

T8.9. The Liver Lobule areas endangered by Hepatotoxins.		
Periportal	**Midzonal**	**Centrilobular**
Acrolein Albitocin Allyl alcohol Arsenic Iron Manganese Phosphorous	Anthrapyrimidine Beryllium Carbon tetrachloride Furosemide Ngaione Paraquat	Acetaminophen Aflatoxin Bromobenzene Carbon tetrachloride Chloroform DDT Dinitrobenzene Trichloroethylene

8.4.2. Cytological Classification of Liver Injury

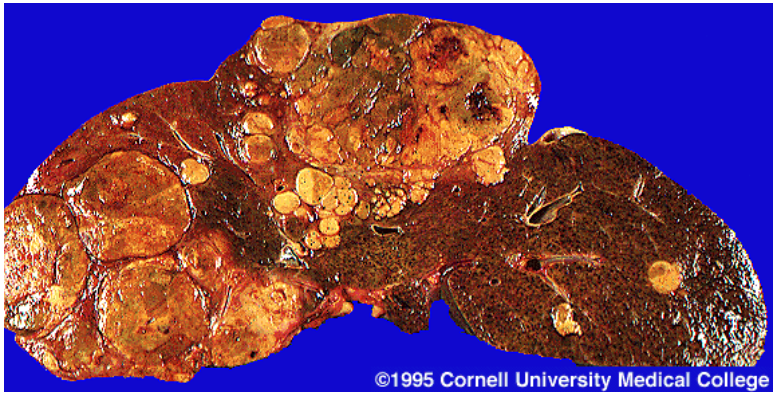
T8.10. Hepato-toxicants classified according to their Target.		
Membrane, Endoplasmic Reticulum	**Mitochondria**	**Nucleus**
Carbon tetrachloride	Hydrazine	Beryllium
Thioacetamide	Ethionine	Aflatoxin
Phallodin	Dichloroethylene	Galactosamine
Dimethylnitrosamine	Carbon tetrachloride	Ethionine
Allyl alcohol	Phosphorous	Nitrosamines



Another important pathology of the liver is *hepatocellular carcinoma*, characterized by large malignant tumors throughout the liver (shown) and hepatomegaly. The most potent hepatocarcinogen known is aflatoxin B1, from the *Aspergillus* mould.

8.4.3. Pathological Classification of Liver Injury

T8.11. Hepato-toxicants classified according to mode of action.	
Cytotoxic Agents	**Cholestatic Agents**
Acetaminophen	Anabolic steroids
Aflatoxin	Arsphenamine
Allyl alcohol	Chlorpromazine
Bromobenzene	Diazepam
Carbon Tetrachloride	Estradial
Dimethylnitrosamine	Mepazine
Phosphorous	Trioridazine
Urethane	



F8.12. Large parts of this liver are invaded by carcinoma nodules (mostly to the left).

8.4.4. Substances promoting Detoxification

Vitamin C is required for Phase 1 detoxification, scavenges free radicals and protects against damage from reactive oxygen species (ROS).

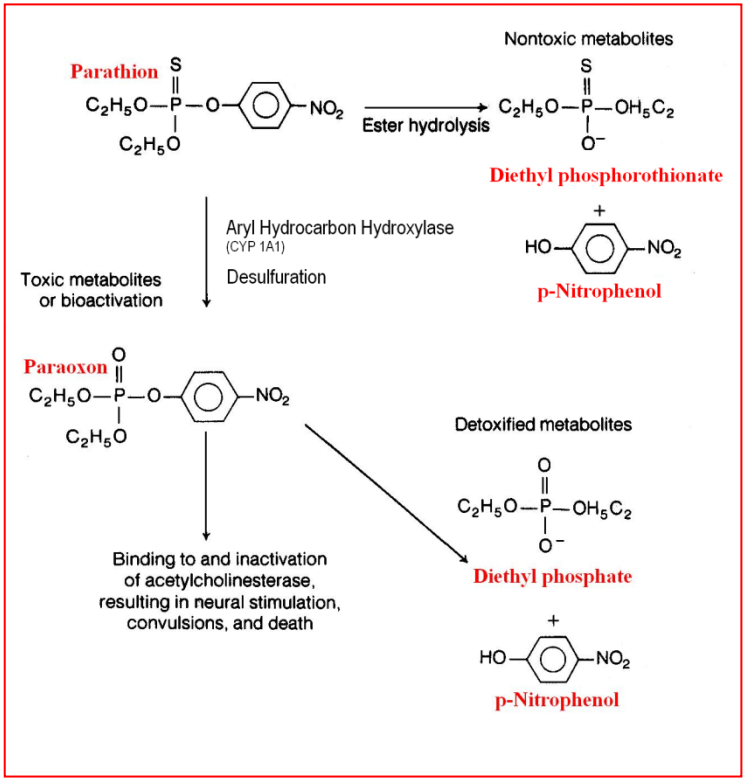
N-acetyl-cysteine (NAC) is a glutathione precursor and protects against ROS damage. Reduced glutathione interacts with toxins such as heavy metals to make them less reactive and thereby less damaging to cells. The oxidized glutathione needs to be replenished. Toxicants such as mercury are removed from the body by direct conjugation with glutathione[11]. Glutathione can be depleted with increased free radical production during Phase 1 detoxification, resulting in decreased glutathione conjugation in Phase 2 detoxification, and the build up of toxins and heavy metals in the liver.
Lipoic acid is a unique antioxidant in that it is both water- and lipid- soluble. Lipoic acid supplementation may be beneficial for the management of liver diseases associated with oxidative stress: alcohol-induced damage, mushroom poisoning, metal toxicity and carbon tetrachloride poisoning[10].

8.5. Action of Toxicants in the Liver

The quest for potency and efficacy in pharmaceuticals leads industry to purify the active ingredients of natural medicines, so that they can then be synthesized in pure form. Pure forms of medicines may reduce toxicity from other ingredients, but may also concentrate metabolic stress on specific pathways. Depending on the toxicity of a therapeutic, it may sometimes be advantageous to use natural extracts, which may induce less toxicity as well as take advantage of an array of molecules with similar action.

8.5.1. Enzymatic Activation of Toxicity

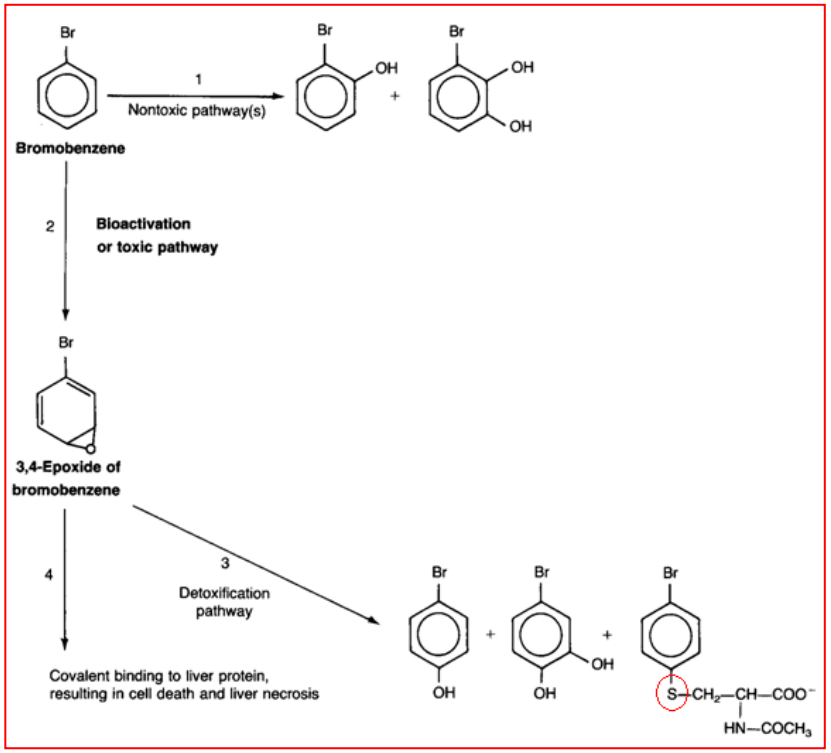
The liver's metabolism can activate toxicity in its attempts at detoxification. We show here three examples in which this occurs.



F8.13. Parathion metabolism. *Williams and Burson.*

8.5.1.1. Parathion

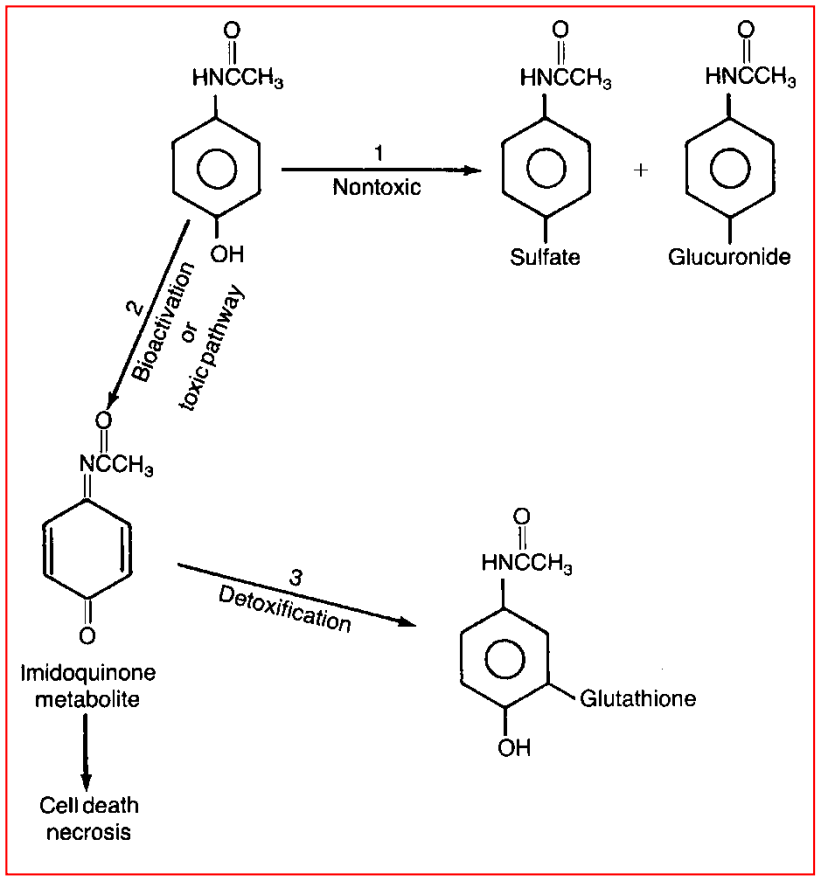
Parathion is converted to the toxic form (F8.13), **paraoxon,** by the enzyme AHH (also known as CYP1A1), resulting in local hepatic toxicity.
Clinically, attempts are made to remove it from the body by dialysis and charcoal ingestion. Atropine would be administered to counter its debilitating effects on the nervous system.



F8.14. Bromobenzene metabolism. *Williams and Burson.*

8.5.1.2. Bromobenzene

Bromobenzene results in centrilobular necrosis and is also a powerful nephrotoxicant. It is metabolically detoxified using glutathione's sulfur bond.



F8.15. Acetaminophen metabolism. *Williams and Burson.*

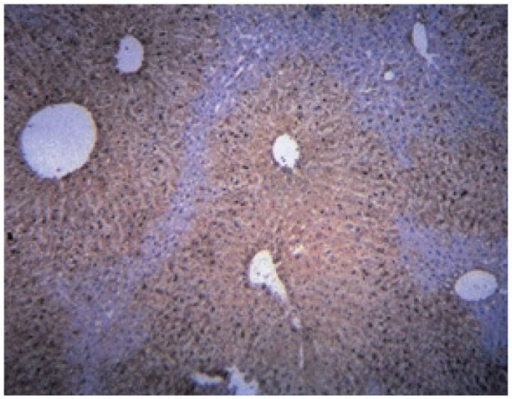
8.5.1.1. Acetaminophen

Approved drugs are frequently recalled from the market, and clinical trials of experimental drugs are often terminated early because of drug-induced liver injury. When 36 inbred strains of mice were given large doses of acetaminophen, large strain-dependent variations in drug-induced liver injury were found

(assessed by histology and by elevated serum levels of the liver enzyme alanine aminotransferase (ALT)[8].

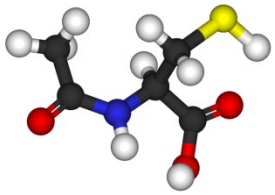
High doses of acetaminophen in humans (15 g) are toxic through centrilobular necrosis.
As can be seen in F8.15A, acetaminophen adducts are formed specifically in this region.

F8.15A. Acetaminophen injury to the liver. Liver section from acetaminophen treated mouse, 300 mg/kg at 4 hours. Stained with brown antibody specific to acetaminophen adducts the centri-lobular regions. Bluish regions are peri-portal. *Josephy and Mannervik, 2006.*



Quantitation of pathways (see F8.15):
Path 1: Sulfate 52%, Glucoronide 42%, Unchanged: 2% .
Path 2: 4% in toxic pathway when 70% of glutathione has been consumed.

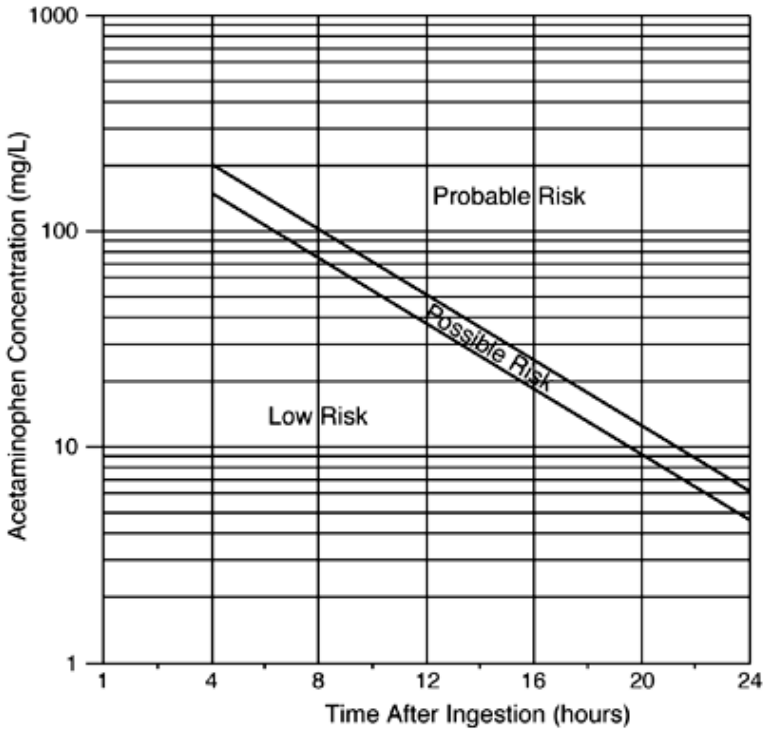
Oral N-acetylcysteine (NAC, shown), a precursor of glutathione, is the standard therapy for acetaminophen overdose. NAC is administered orally



with an initial (loading) dose of 140 mg/kg followed by 17 maintenance doses of 70 mg/kg every 4 hours[3,7]. NAC has a foul smell and tastes like rotten eggs. Not surprisingly, nausea and vomiting commonly occur following administration.
Peak plasma levels of NAC occur approximately one hour after an oral dose and at 12 hours post-dose it is undetectable in plasma. Despite a relatively low bioavailability of only four to ten percent, oral administration of NAC appears to be clinically effective. The biological activity of NAC is attributed to its sulfhydryl group (yellow), while its acetyl substituted amino group affords it protection against oxidative and metabolic processes.

The nomogram below (F.8.16) is a nice application of toxicokinetics, but should not be used to *downgrade* the administration of NAC over time.

NAC is a good example of a compound which can have deleterious or protective effects, depending on its compartimentalization. In F8.17, the number of mutant cells produced as a function of NAC concentration is plotted for two cases: *pretreatment* and *simultaneous* treatment with NAC.
In *pretreatment*, NAC is first metabolized to increased intracellular glutathione, which enhances activation of MNNG near the target molecule, after it enters the cells. This results in more mutants. In *simultaneous* treatment, MNNG is activated by NAC extracellularly, and the reactive intermediate harmlessly reacts with extracellular water.



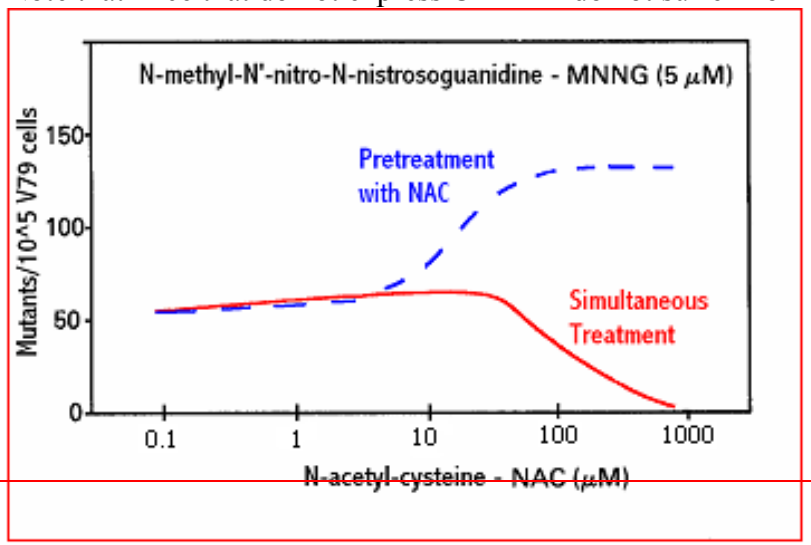
F8.16. Rumack-Matthew nomogram for acetaminophen intoxication from serum values. *Rumack J, Toxicol Clin Toxicol 2002;40(1):3-20.*

Note that mice that do not express CYP2E1 do not suffer from acetaminophen toxicity, while snakes are highly sensitive.

8.5.1.2. Halogenated Alkanes/Alkenes

These hepatotoxic chemicals (T8.18) also involve other target organs (T8.19). They are not toxic by themselves, but are biotransformed into toxic intermediates: radicals and epoxides[3,4].

Carbon tetrachloride and chloroform result *in vivo* in specific cytotoxic intermediates (F8.20). The detoxification of the radicals (carbene, peroxy) and the intermediate (phosgene) involves their conjugation with glutathione (GSH) until stores of GSH are depleted. The isolation and identification of these unstable intermediates has been extremely difficult. Hepatic biotransformation proceeds too rapidly, but biotransformation by renal homogenates or fractions is much slower, and we now know all of the toxic products arising from carbon tetrachloride biotransformation.



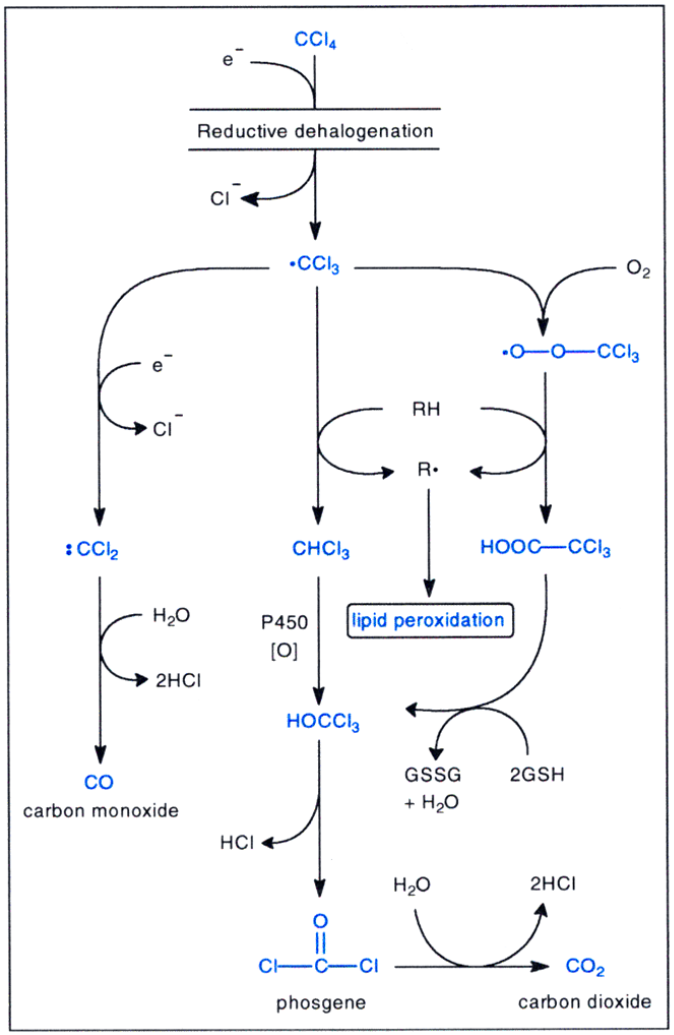
F8.17. Mutagenicity of MNNG depending on the timing and concentration of NAC treatment. *Romert and Jenssen, 1987.*

T8.18. Some halogenated alkanes/alkenes.	
Carbon tetrachloride	CCl_4
Chloroform	$CHCl_3$
Trichloroethane	CH_2Cl-$CHCl_2$
Trichloroethylene	$CHCl$-CCl_2
Halothane	CF_3-$CHClBr$
Freons	CCl_3F CCl_2F_2 $CClF_2$-$CFCl_2$ $CClF_2$-$CClF_2$

Despite the concerns about occupational exposure to such agents as carbon tetrachloride or chloroform (a possible human carcinogen), chloroform was used as a "safe" anesthetic (it did not explode like diethyl ether) until the hepatotoxicity was

T8.19. Toxicity of haloforms. *on acute exposure when catecholamines are administered concomitantly. Other haloforms show the same property.			
Compound	**Hepatotoxic**	**Nephrotoxic**	**Cardiotoxic**
CCl_4	X	X	
$CHCl_3$	X	X	
Halothane	X		
Methoxylfurane		X	
Freons			X*
Vinyl Chloride	X		
Perchloroethylene	X		

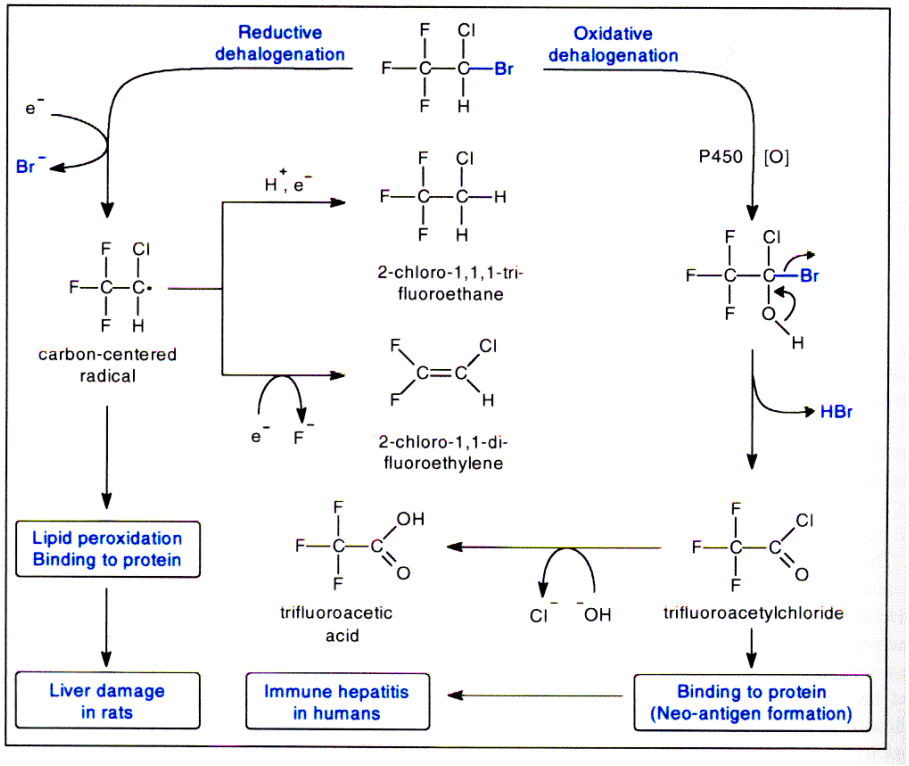
recognized. Carbon tetrachloride is still used as an antiparasitic agent (vermifuge) in third-world nations because it is cheap and effective, and can be administered orally in capsules. Understandably, you should not give it too often (once a year), for fear of hepatic toxicity.
The signs and symptoms of halogenated alkane hepatotoxicity



F8.20. Reductive dehalogenation of CCl_4 to a trichloromethyl free radical that initiates lipid peroxidation.

include protracted nausea and vomiting, severe prostration, jaundice, enlarged palpable liver (right side below rib cage or below sternum) and elevated serum transferases.

Via a needle biopsy, one can see swollen cells and fatty degeneration, predominantly in the centrilobular area but radiating out toward the portal triads (the mid-zonal regions of the lobule), as coagulative necrosis.
At necropsy, one sees an enlarged liver (hepatomegaly) with accentuated lobulation having a pale appearance or the classical "pearly" liver - surface cells outlined with a ring of lipid around each. Cells around the central vein have a pale appearance upon staining and contain large fat globules.
A gradient in necrotic damage is seen from the central vein out into the mid-zonal regions, most severe in the central vein region.
It is apparent that the Kupffer cells play some role in carbon tetrachloride induced hepatotoxicity[5]. Carbon tetrachloride appears to activate Kupffer cells by increasing their intra-cellular levels of calcium ions which, in turn, trigger the release of cytokines and eicosanoids (leukotrienes). Those are chemotactic factors for neutrophils which become activated, are attracted to the region, and secrete superoxide anion and other toxic mediators which amplify the inflammatory response leading to cell injury and death. It is important to appreciate that the above mechanism of hepatotoxicity is not unique to carbon tetrachloride but applies to all haloalkanes and alkenes and, perhaps, to other hepatotoxicants as well.
Back in the 1960s, there was considerable concern about the severe hepatotoxicity witnessed when halothane was first used as an anesthetic. Halothane was almost "finished" in the marketplace before it was realized that the anesthesia machines through which it was administered had copper kettles in which the anesthetic was placed before air was passed over it. The high cost of this anesthetic (250 $ for 100 ml) meant that the residual anesthetic was kept, frequently left in the copper chamber. Of course, halothane is covered with halogens (F, Cl, and Br) which react in the presence of copper to produce a toxic intermediate which the next patient receives when anesthetized. When the copper chamber was replaced by glass, hepatotoxicity dropped sharply. As yet, not all of the possible biotransformation products of halothane have been identified (F8.21).



F8.21. Activation of halothane by reductive and oxidative dehalogenation. Their role in rat and human liver toxicity.
Casarett & Doull.

8.5.2. Natural Hepatotoxicants

Cyanobacterial blooms often take place in lakes during sustained warm spells in autumn. Cyanobacterial hepatotoxins can kill a drinker within 24 hours. The toxins attack a protein in the membrane of liver cells, and the cell membrane breaks down. Subjects die from internal bleeding.

8.5.3. Persistence of Hepatotoxicity

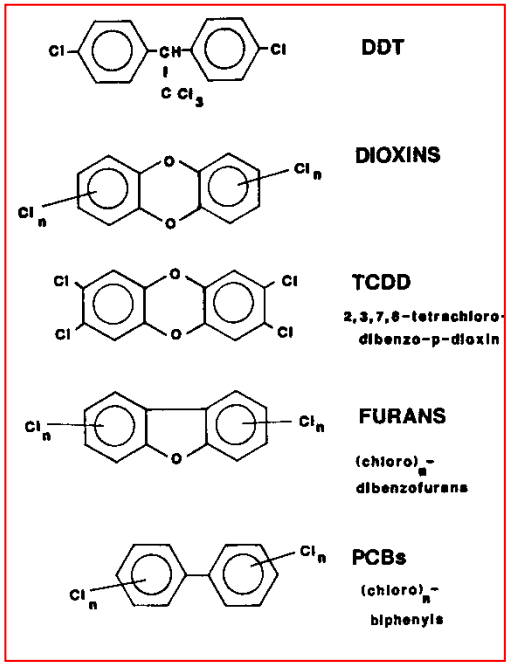
Many chemicals can exert subtle effects on the liver, specifically those that the liver finds difficult to biotransform, or the liposoluble ones that penetrate cell membranes and are stored in the body for exceptionally long periods of time. Detectable blood levels of brominated and chlorinated dioxins can be demonstrated 35 years after exposure[3]. The effects of these agents during that period are uncertain. We all possess low adipose tissue stores of DDT and PCBs that are slowly released.

The oldest example of such agents is the insecticide DDT, not very toxic, difficult to biotransform, highly liposoluble, persistent for decades both in environmental food chains and in the human body. Much of the body burden is stored in the adipose tissue, with a small amount circulating in the bloodstream.

Polychlorinated biphenyls, dioxins, and furans (F8.22) are not terribly toxic to the liver but are very liposoluble and persistent *in vivo*.

TCDD is the most toxic of the congeners to animals.

The liver treats such agents as if they were a high level exposure situation, even though the level may be quite modest. If the liver cannot biotransform the agent, enzymes are induced in the hope of eliminating them. This is the liver's only response to a situation that is *perplexing* to it. However, the resistant chemicals remain and the elevated enzymatic activity enhances the biotransformation of other chemicals to which the subject may be exposed.



F8.22. Structures of persistent halogenated aromatic hydrocarbons capable of long-term induction of tissue enzymes.

The Phase I and II enzymes will not decline back to normal, since the agent persists in the bloodstream, maintaining an *alert state*.

8.6. Tests of Liver Function

Tests of hepatic injury are serum enzyme tests (below), hepatic excretion tests (sulfobromophthalein, indocyanine green), tests on alteration of the liver's chemical constituents and histological analysis of liver injury.

Current hepatic tests in the clinic are blood tests that detect the presence of damage or inflammation, rather than how well the liver functions.

Severe hepatic damage results in leakage of a number of cytosolic (soluble) enzymes (SGOT, SGPT, LDH, Sorbitol DeHydrogenase, etc.) through the hepatocellular membrane, with sharp elevations of these species in serum where only low

levels are usually found. This may persist for prolonged periods of time. Particularly with PCBs, one will find elevated serum triglycerides and sometimes cholesterol, as well as serum enzymes and dermatological acne for months and even years[1,2].

An example of carbon tetrachloride-induced hepatic damage in rats (F8.23) shows which enzymes are diagnostic for toxicity. The same can be seen in humans. However, hepatocellular damage occurs at different rates with different chemicals, and one blood sample may not yield the desired diagnostic results. For example, benzene will produce damage (and enzyme release) in approximately 8 hr whereas, with carbon tetrachloride, it takes more than 24 hr before significant cellular damage and enzyme leakage occurs.

Fasting Serum Bile Acid (SBA) determination can be used clinically in the diagnosis and prognosis of liver disease in conjunction with standard liver function tests. Because of the sensitivity of SBA determination as compared to standard liver function tests, SBA offers significant additional diagnostic information concerning liver function, especially in minor hepatic derangements. It is of particular benefit in the determination of hepatic dysfunction as a result of chemical environmental injury.

Liver injury as a result of occupational or environmental exposure to a wide variety of chemical substances can be determined to a much finer degree by SBA than by standard liver enzymes, especially when the liver has been only slightly damaged. In one study of individuals exposed to organic solvents, 73% of the exposed cases had elevated SBA levels,

whereas increased levels of SGGT, alanine aminotransferase ALT), aspartate aminotransferase (AST) and bilirubin were observed respectively in 8, 3, 2, and 1% of exposed workers.

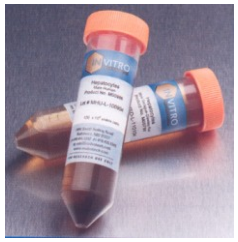
These results and others support the hypothesis that standard liver function tests are not sensitive enough to determine hepatic dysfunction caused by such organic solvents as toluene, xylene, acetone, styrene, n-butlyacetate, n-butanol, ethylacetate, other aromatic hydrocarbons and ketones[6].

There are a number of useful clinical tests beyond serum enzymes, such as:

- Functional test: clearance of bilirubin or dyes (bromo-sulfo-phthalein, indocyanine green).
- Albumin is a major protective blood protein formed in the normal liver. Levels below 3.5 mg/dL are abnormal.
- Prothrombin Time: clot formation is indicative of liver function. Blood from the patient is oxalated to prevent coagulation. Later, large amounts of calcium and tissue extract are mixed in. Prothrombin starts transforming to thrombin, leading to coagulation. Normal is about 12 seconds. Deviations of more than 2 seconds are abnormal.
- Liver scan using radio-opaque chemical.

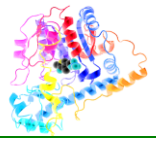
8.7. In Vitro Liver Experimentation

A number of products are available on the market to experiment *in vitro* on liver function. Fresh animal hepatocytes, cryopreserved hepatocytes from various species (shown), individual and pooled microsomes or S9 fraction.



Cryopreserved hepatocytes should be stored at less than -150ºC to assure long-term viability. Microsomes and S9 should be stored at less than -70ºC to preserve P450 enzymes.

Primary hepatocytes, when cultured, begin to lose liver-specific gene expression within just a few days, unless co-cultured with non-parenchymal cells (non-liver) such as fibroblasts or endothelial cells[9].

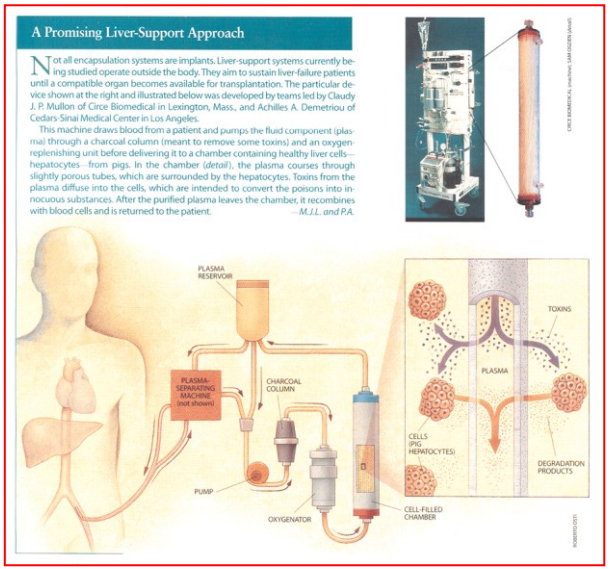
T8.23. Hepatic damage in male rats with 0.1 ml/kg of CCl_4. Serum enzyme activity, 3 days post treatment.			
Enzyme name Target Tissue	**Control**	**Treated**	**Increase**
Alanine Aminotransferase (ALT, formerly SGPT) Liver & heart	42 ± 4	440	10x
Aspartate Aminotransferase (AST, formerly SGOT) Liver & muscle 	91.2 ± 11	720	8x
Alkaline Phosphatase (ALP) Bones & liver 	917.2 ± 51	2560	3x
Acid phosphatase	20.9 ± 2	43.8	2x
Creatine (phospho)kinase (CPK) Cardiac/skeletal muscle	218 ± 61	284	
Gamma Glutamyl Transaminase (SGGT) Cholestasis			After alcohol intake
Lactate Dehydrogenase (LDH) Muscle, Erythrocyte, liver 	108 ± 31	1019	9x

8.8. Temporary Liver Support

Liver regeneration may actually occur from bone marrow cells, as was recently found. First, bone marrow stem cells may serve as a source for the replacement of endothelial cells. Second, hematopoietic cells, including lymphocytes, neutrophils, macrophages, and platelets, may provide crucial factors required for efficient healing of damaged liver.

When regeneration fails, temporary liver support (F8.24) can be very important. In acetaminophen intoxication, for example, if the patient survives, complete liver recovery *can* occur.

ALP and SGGT reflect cholestatic injury more effectively than necrotic injury. LDH, AST and ALT are more sensitive to cytotoxic hepatic injury. These enzyme determinations are less sensitive than histological pathology.



F8.24. Research in Temporary Liver Support.

8.9. Case Study: Acute Dimethyl Nitrosamine Poisoning

(modified from Williams & Burson)

In Omaha, Nebraska, the county health department became concerned because there seemed to be an outbreak of a new disease; five people had suddenly become ill. The sick patients experienced vomiting, severe abdominal cramps, diarrhea, and two of them developed bruising. The first person had become ill on a Sunday morning while painting his house. Later in the day, his 2½ year-old daughter became sick. During the afternoon, his wife's sister, her husband, and another child visited them. They stayed for a little while, drank some lemonade while there, and left. At six o'clock that night, they had also started feeling ill.

The man who had become ill early Sunday morning went to emergency rooms and then to a number of physicians, but he was repeatedly told he had the flu and should go home, rest, and take aspirin. Later, he developed severe nosebleeds; his nose was packed twice in emergency rooms early in the week. After each episode he was sent home again. He persisted in seeing physicians and, finally, on Thursday of that week, he walked into a physician's office and collapsed. At that point he was admitted to a hospital. On admission he showed abnormal liver function tests. The fact that his bilirubin was elevated also indicated a liver problem. The reason for the bleeding problems was that his platelets were severely decreased; there were about 6,000/mm^3 instead of the 150,000-300,000/mm^3 or more that would be normal.

Because he became comatose, the child of the family who had visited the home on Sunday afternoon had already been admitted to another hospital. It took the hospitals a while to recognize that there might be a connection between the two cases. Once that was established, the county health department was called. The county health department then started looking at the other people who had visited the home and eventually established that of this group-a total of ten people who had been involved-five were ill and all of them had abnormal liver function tests. They all had low platelet counts. Not all of the people had platelet counts as low as those of the man or of the child who had visited the home.

After interviewing all who had visited the home on the Sunday when the illnesses were first noticed, the health department investigators concluded that the vehicle for the illness-causing agent must have been food or drink. The only food item all the ill people had consumed was lemonade. The parents of the man who had first become ill had visited the home, but while they were there, they did not consume any food or drink; they did not become ill. A sister living with the first family did not drink lemonade and did not become ill; and a baby did not eat any foods because it was being breastfed. The fact that the baby did not become ill eliminated to some extent the possibility of a highly contagious illness (except possibly a food-borne organism).

The lemonade therefore seemed implicated. By this time, of course, the family had discarded any remaining lemonade (always a problem in these types of cases).

The child died. The next day the man died. After that, the CDC was called. The county health department wanted to know what they should do, what kind of tests they should run, and what kind of samples they should take from the man. The CDC suggested that, in addition to taking tissues as is normally done for microscopic examination, they should also freeze tissue so that chemical analysis could be performed on the tissue if it became necessary to do so.

The autopsies of the father and child produced two common findings. One was that severe liver damage was the cause of death. In addition, there was extensive bleeding owing to the decrease in platelets. There were no other significant findings. No other organs were specifically affected. Review of the liver sections and other organs did not suggest an infection, because there was no inflammatory reaction in the tissues.

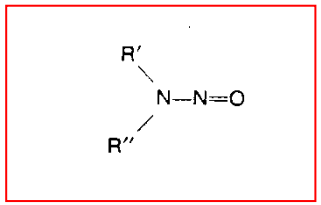
The question was, what type of chemical would produce these toxic effects? Quite a number of chemicals damage the liver, but they usually affect other organs as well. Because the investigation implicated the lemonade and the people had not complained about the lemonade's tasting peculiar, the chemical had to be something that did not taste strange and something that was water soluble. These observations somewhat limited the number of compounds. The substance also had to be relatively toxic so that a person would ingest a lethal dose by drinking a single or a half glass of lemonade in which this material was dissolved.

At the CDC we examined a number of the more common compounds that cause liver necrosis. We did not include in the list of compounds substances that, like carbon tetrachloride, severely affect the liver but also affect the sensorium Carbon tetrachloride also has some effect on the kidneys but would be detected by people drinking lemonade. Portions of the liver tissues were examined for arsenic and yellow phosphorus.

None of this made very much sense. We searched further. Some people are sensitive to acetaminophen, a pain medicine. It can damage the liver, but typically not everyone in a family suddenly becomes ill. Usually there is a history of the patient's having taken this medication, but these patients had not taken acetaminophen. Aflatoxin, a mycotoxin and another hepatotoxin was also a suspect, but it would be very difficult to have access to this type of compound. Furthermore, the microscopic appearance of the liver did not suggest aflatoxin. However, certain types of alkylating agents that are either used in cancer chemotherapy or cancer research might selectively damage the liver and could cause the severe centrilobular liver necrosis.

In talking to the county health department, I asked for additional information about the family to determine whether they would have had any contact with people in a cancer research institute or a hospital. The county health department thought this was a peculiar idea, but passed the request on to the police. A police officer in Omaha went to his files and found that this family did indeed know a man who was now working at the Eppley Institute in Omaha, which is a cancer research institute.

Five years earlier this man had had a love affair with the wife of the man who later died, and had confronted the family with a gun; shots had been fired. He had then been sentenced to prison and was free on parole. Because he held a degree in biology, he was able to obtain a job at the Eppley Institute. His job was to mix diets for cancer research studies in animals, and one of the compounds he was working with was the alkylating agent dimethyl nitrosamine. The essential chemical structure of dimethyl nitrosamine is



Generalized structure of N-nitroso compounds

Dimethyl nitrosamine was eventually established as the compound that caused the illness and death in the family. While the family was away from their house, the suspect had climbed into a back window and added the poison to a pitcher of lemonade found in the refrigerator. His intention was to cause the people to have cancer; he wanted to watch them die. However, he picked a chemical that was also very acutely toxic. The oral LD_{50} in rats for dimethyl nitrosamine ranges between 27 and 41 mg/kg of body weight. The calculated lethal amount required for a child is less than one gram and for an adult roughly three grams.

Dimethyl nitrosamine is a yellow oil, so it mixes very well with lemonade. It is water soluble. It is somewhat more stable in a slightly acid environment. Other nitrosamines were examined to determine whether some other compound could have produced the effects evident in this case. They were ruled out for a number of reasons, and they were also not available at the institute. Many are not nearly as toxic, and the other more toxic nitrosamines do not specifically cause the selective centrilobular liver necrosis.

Dimethyl nitrosamine is very rapidly metabolized and excreted; therefore, five days after poisoning (the time that elapsed before investigation of this case began), all of the material would be metabolized and excreted and would not be found in the body. Nitrosamines and other alkylating agents do one thing by which they can be traced, however. They methylate nucleic acids. such as, for example, guanine.

There is now a test that has been developed, using high pressure liquid chromatography, by which methylation of guanine can actually be measured. Early in our investigation liver tissue from one of our patients was frozen. A total of eight tissue samples were then blindly submitted for analysis: seven controls and our sample. The chemist analyzed the eight samples and identified methylated guanine in the liver specimen that turned out to be our sample. Products like methylated guanine are also excreted in urine and would also probably be measurable in urine specimens. It is presently not possible to demonstrate methylation with acute yet nontoxic doses because the method is presently not sensitive enough. The amount of the concentration found in the liver of the adult male was as high as those demonstrated in rats that have been acutely poisoned with dimethyl nitrosamine, however. We were able to show this in court and the man who poisoned the family was convicted.

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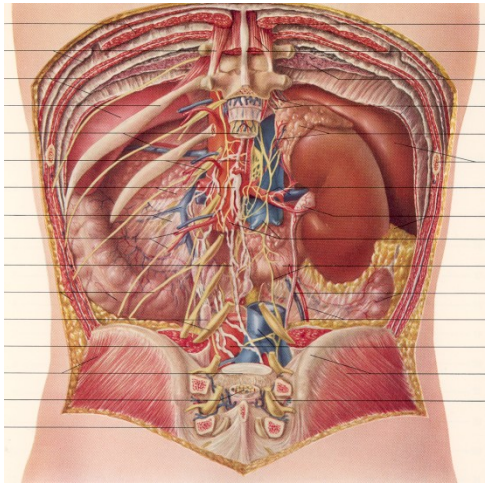
Nephrotoxicity

9· Nephrotoxicity

9.1. Roles of the Kidney



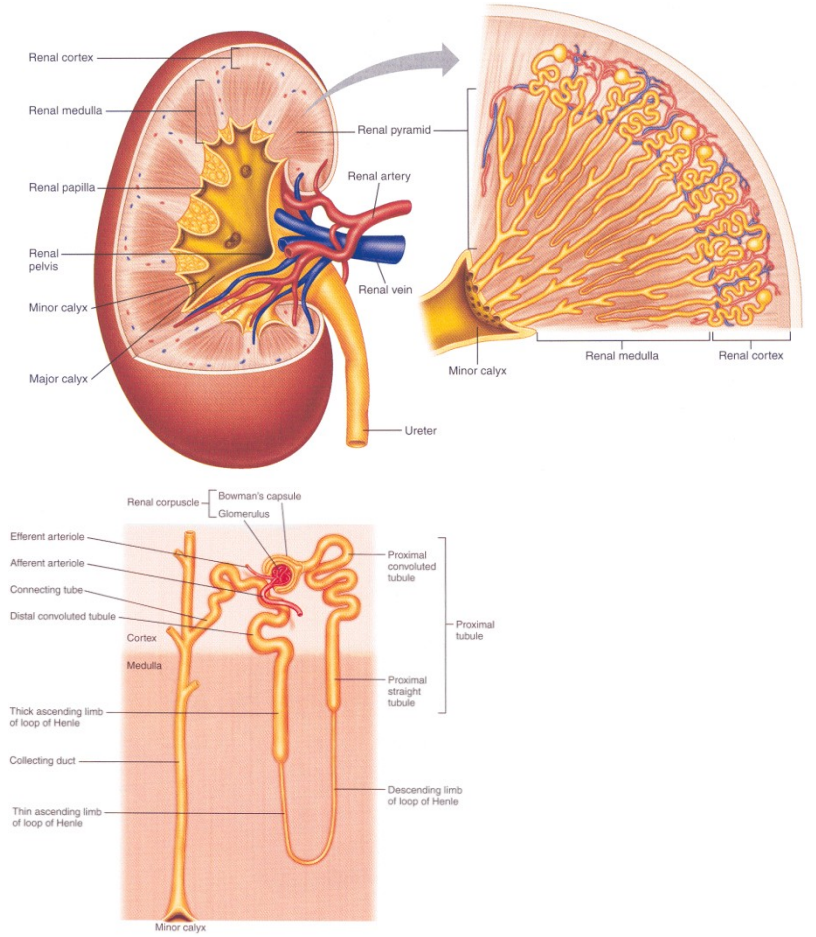
The kidney is the main organ responsible for the expulsion of endogenous metabolites and xenobiotics.

F9.1. Gross anatomy of the kidney. *Atlas of Normal Anatomy.*

Its functions are:

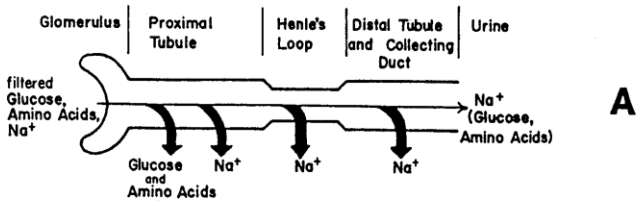
- Glomerular filtration, (a passive process),
- Tubular reabsorption,
- Tubular secretion (an active process),
- Regulation of extracellular volume,
- Regulation of electrolyte balance (a familiar example of imbalance results in muscle cramps),
- Acid-base regulation,
- Calcium metabolism.

There are approximately 10^6 nephrons per kidney, with a total length of approximately 120 km. There are indications that mothers restricted in protein give rise to offspring with fewer nephrons. The remaining overworked nephrons release hormones and retain sodium. The offspring is then vulnerable to hypertension[3].

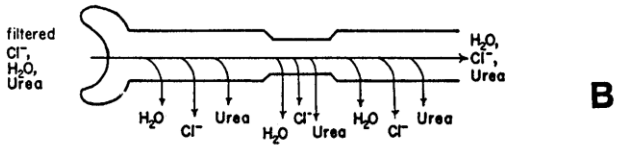


F9.2. Kidney anatomy. *Germann & Stanfield, 2002.*

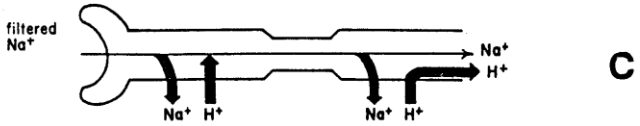
The length, the large surface area and the thin barrier between the lumen of the tubule and the extensive capillary blood supply (F9.2) insures a rapid transport of many substances. Approximately 20 to 25% of the blood pumped out of the heart each minute is routed through the kidneys. They filter the entire blood volume every 4 to 5 minutes.



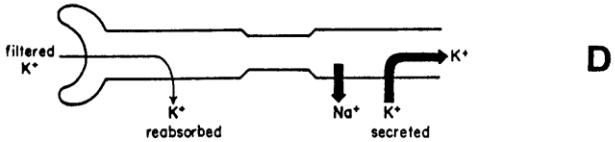
Glucose and amino acids are excreted only when the amount filtered exceeds saturation of active transport. Na is excreted in direct proportion to intake.
The heavy arrows indicate active transport, the light arrows diffusion.



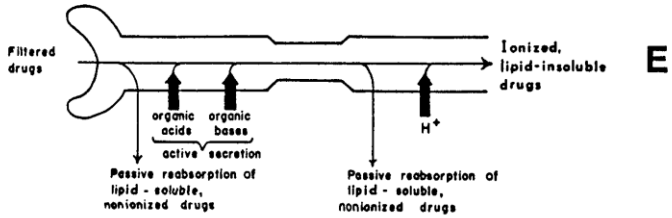
Diffusion of water, chlorine ions and urea along osmotic grdients established by active transport, particularly of sodium. With ADH, water is excreted in direct proportion to intake. Without ADH, water is not reabsorbed in the distal tubule and collecting duct. 55 % of the urea is reabsorbed. Chlorine and sodium are excreted equally to maintain electroneutrality.



Acidification by active reabsorption of sodium in exchange for protons secreted into urine.



Potassium is almost completely reabsorbed in the proximal tubule.
It is excreted in the distal tubule, in exchange for sodium



Lipid-soluble and non-ionized drugs are passively reabsorbed. In distal segments, the secretion of protons favors reabsorption of weak acids (less ionized) and excretion of weak bases (more ionized), compatible with the Henderson-Hasselbalch equation. Active secretion of organic acids and bases occurs only in the proximal segment.

< F9.3. Functions of the Kidney Tubule.

The kidney loses 20% of the plasma volume through the glomerulus, which acts like a sieve, and recuperates the needed components later, glucose at a rate of 99.96%, using the *proximal* and *distal* tubule. Therefore, the overall personality of the kidney is that it is *a **recycling organ***.
In F9.2, note that the medulla consists only of tubular structures for blood and urine and that the glomeruli are located in the cortex.
The nephron is a very active tissue, as glomerular filtration occurs on-goingly with tubular secretion and reabsorption.
The kidney has the same enzymes (Phase I, II) as the liver, but with only 20% of the activity. While low exposure levels of toxicants probably pose no problems for the kidney, moderate and high levels might.

9.2. Physiology

Xenobiotics biotransformed into water soluble products will most likely be excreted by passive filtration through the glomerulus and into the urine. By contrast, lipid-soluble, nonionized chemicals will be passively re-absorbed into the blood from the lumen.

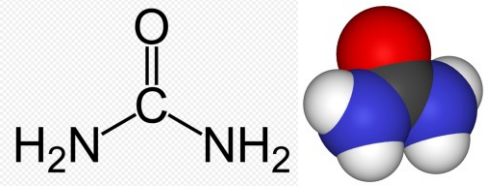
The various segments (A-E) of F9.3 describe components of tubule functions.

A – All glucose and amino acids are recuperated, unless transport mechanisms are overwhelmed. Normally, only 0.04% of glucose is lost.
Sodium is actively reabsorbed in the tubule (A and C) and hydrogen ions are actively secreted as a substitute (C), so the pH of the urine becomes acidic (from 7.4 to 6.5-6.0).

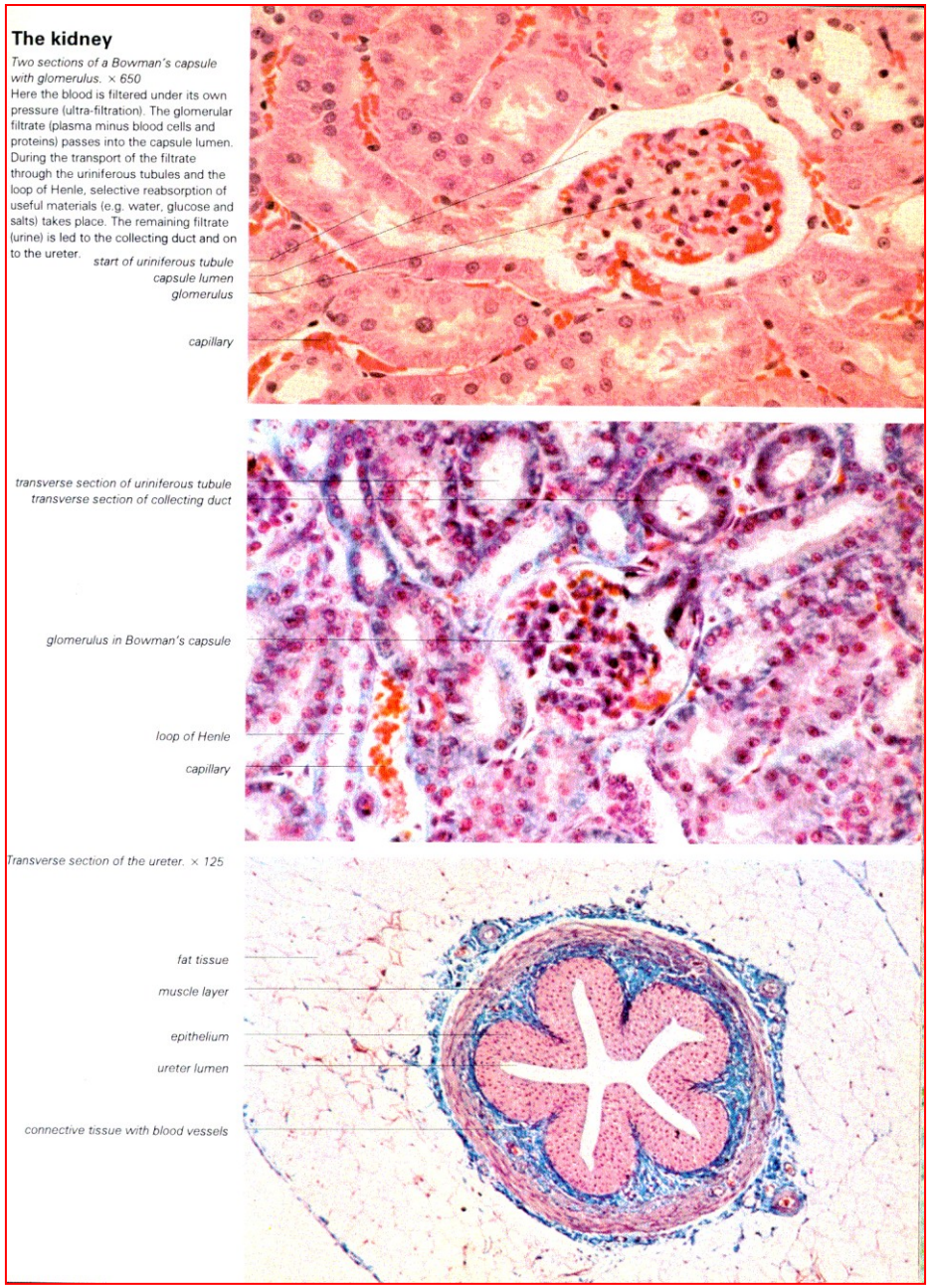
Cl and water follow Na (B), itself excreted in proportion to intake.
pH values in the urine range from 4.7 to 8, with a normal of 6.
The control results from the combined effects of the various buffer systems, particularly the carbonate, phosphate and ammonia buffer systems.
For organic acid excretion, a basic urine is desirable; for organic base excretion, acidic urine is desirable.
For example, the glucuronide of the xenobiotic phenol is more polar and more water soluble at pH 6.5 than at pH 7.4 (the pH of the blood), and so can be more easily eliminated.
For organic bases, the acid pH of the urine enhances the polarity of the excreted product and insures that it remains in the fluid, and is not re-absorbed.

F9.4. Structures of the Kidney. *Biological Structures.* >

B - Urea, formed in the liver from metabolism of proteins, has

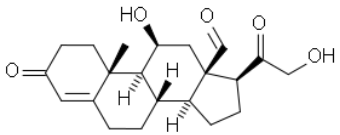


a pKa of 0.1. According to the Henderson-Hasselbalch equation, a miniscule amount is non-ionized in the plasma, but lowering the urine pH allows for somewhat increased passive reabsorption. Ultimately, about 55% of urea is reabsorbed. Its usefulness may be that it acts as an anti-oxidant in the blood.
E – Many toxicants are secreted in the proximal tubule, but can be reabsorbed, if not ionized, all along the tubule.
Some detoxification products, particularly those conjugated to glutathione, may be partially reabsorbed and may be toxic at the point of entry. Thioethers (R-C-S-C-R) are particularly nephrotoxic.



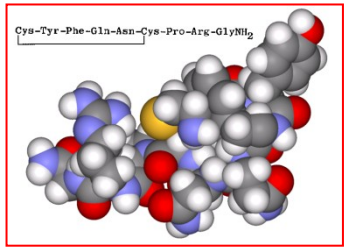
The tubular processes of secretion are controlled by a number of endogenous substances.

9.2.1. Aldosterone



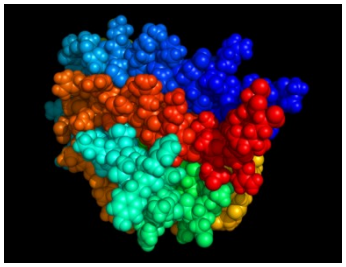
It controls blood volume and therefore maintains blood pressure, by enhancing salt reabsorption in the tubule, which is followed by a corresponding water intake. Renin, from the juxtaglomerular cells, changes angiotensinogen to angiotensin I, to angiotensin II and finally to aldosterone, which is issued from the adrenal cortex.

9.2.2. Anti Diuretic Hormone (ADH)



Also known as vasopressin, controls urine <u>concentration</u> by increasing *reabsorption of water* in the distal tubule.

9.2.3. Renal Erythropoietin Factor

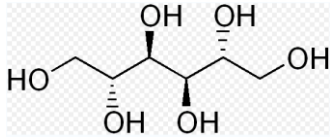


This factor increases RBC production and explains why chronic kidney failure often leads to anemia. Erythropoietin is produced in the proximal tubule cells, the same area where lead is known to accumulate in the kidney[8].

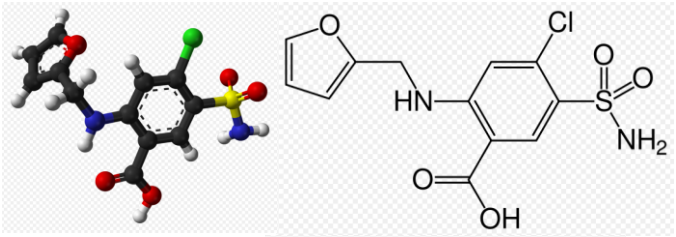
9.2.4. Metabolism of Calcium

Calcium concentration in the blood is controlled by parathyroid hormone, which prevents it from falling too low, and by calcitonin, which prevents it from going too high. But there is only 1 g of Ca in extracellular fluid. In diarrhea, several grams of Ca can be passed in the feces each day. Therefore, bone acts as a Ca buffer. Parathyroid hormone secretion allows complete reabsorption of Ca in the kidneys.

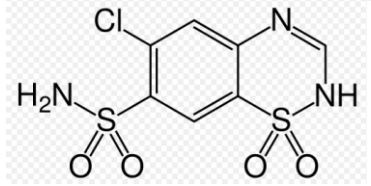
Diuretics are medical agents used to treat edema (compartment syndrome, brain swelling) and hypertension (which can lead to kidney disease). There are a number of categories of diuretics: osmotic agents (mannitol),



loop agents (furosemide),



thiazides (chlorothiazide shown),



aldosterone antagonists or ADH antagonists, that all act at various points along the tubules.

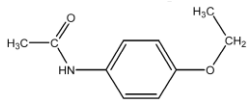
9.3. Pathology

Change in kidney function is often described using *creatinine clearance* as an index, and the Cruikshank formula:

$$Creatinine\ Clearance = \frac{(140 - Age) \times Body\ Weight}{72 \times Serum\ Creatinine}$$

Creatinine clearance in ml/minute, age in years, body weight in kg, serum creatinine in mg/dl. For women, multiply the figure by 0.85.
From a peak of kidney function at about 20 years of age, the creatinine clearance decays (because fewer nephrons are functional) such that by 60 years of age about a quarter of kidneys are considered defective, and the organs would cease functioning entirely at age 140.
The glomerulus is most vulnerable to immune complexes. The proximal tubule is vulnerable to halogenated hydrocarbons, heavy metals, antibiotics, antineoplastics and mycotoxins. The Loop of Henle is specifically vulnerable to fluoride ions.
Clinically, two entities are often distinguished.

- *Nephr **o** tic syndrome*: characterized by proteinuria. Inducible by exposure to lead or heroin, as well as prolonged standing and strenuous exercise. Typical presenting features of nephrotic syndrome are marked edema, proteinuria, hypoalbuminemia, and usually hyperlipidemia.
- *Nephr **i** tic syndrome*: characterized by hematuria. It can be induced by mercury and phenacetin (shown).



9.3.1. Glomerulus Pathology

Pores of the normal glomerulus are about 0.01 μm, 100 times larger than those of capillaries in skeletal muscle. They limit molecule penetration to about 70,000 Daltons.
The filtration of albumin, a negatively charged molecule, is more restricted than neutral dextran of equivalent size. Loss of the fixed negative charge of the glomerular capillary wall results in enhanced filtration of anions. With loss of charge discrimination in *glomerulonephritis*, the capillary wall becomes size-selective only.
Proteinuria is often the first indication that disease is present. Normally there is a net protein excretion of 40 to 80 mg/day. Abnormal proteinuria is a protein excretion in excess of 150 mg/day. The classic intoxicator specific to the glomerulus is the immune complex. Toxic action in the glomerulus leads to decreased filtration rates, for example, with some drugs (gentamycin, kanamycin) or increased albuminemia (puromycin).

9.3.2. Tubule Pathology

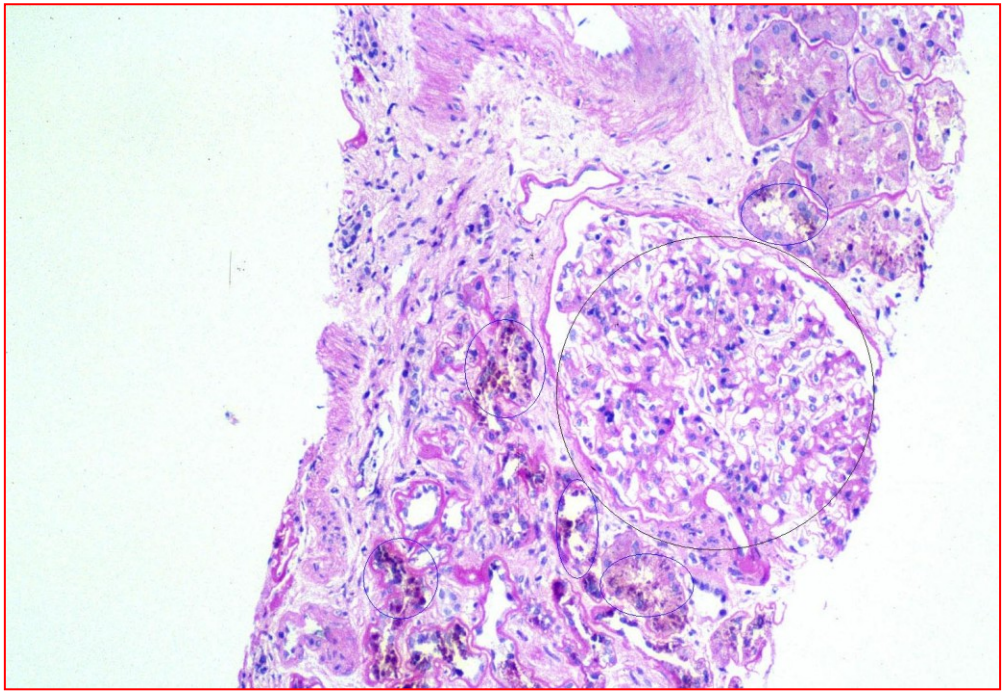
The tubule cells are subjected to high toxicant concentrations, even to possible crystallization.

In *shock*, reduced blood flow to the kidney does not allow the high tubule metabolism to be sustained, and tubular cells go into apoptosis. Normally, neighboring cells will stretch over the missing ones within a couple of hours, but with more apoptosis, serious lesions occur.

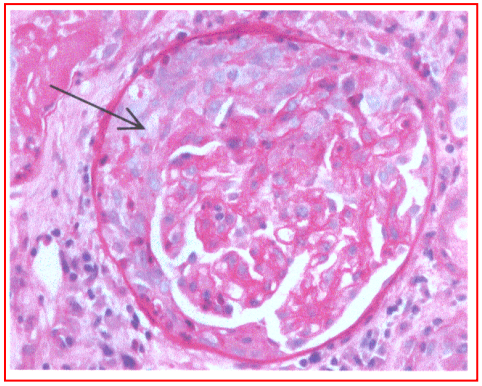
Toxicants (heavy metals, antibiotics, barbiturates, organic solvents) also can produce this effect, called *acute tubular necrosis*. Reduced urine flow causes buildup of toxic wastes in the body, leading to death.

Reabsorption of glucose and amino acids can be impaired by Cd, Pb, Hg, leading to glycosuria and aminoaciduria. Secretion of H^+, K^+ and xenobiotics can also be impaired.

One major site of nephrotoxicity is the proximal convoluted tubule (F9.2), regardless of the nature of the toxicant. There are three reasons for this…



F9.5 In patients with sickle cell disease, who present with chronic renal disease and proteinuria, marked glomerular enlargement is present, often with mesangial expansion and segmental sclerosis. This glomerulus (thin black circle) shows glomerulomegaly and mesangial expansion. There is abundant brown pigment, representing broken down hemoglobin, in surrounding tubules (thin blue ovals), and interstitial fibrosis and vascular sclerosis.
Hematoxylin and eosin, x100. Atlas of Renal Pathology, National Kidney Foundation.

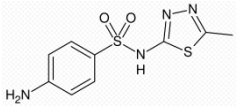


F9.6. Epithelial crescents (arrow) are found in a majority of the glomeruli in kidneys affected by rapidly progressing glomerulonephritis. *Scientific American Medicine.*

(1) the content of the tubules becomes concentrated, as water is recycled (97.5% of the water is recovered). Cells lining the tubules are irritated by toxicant concentration, and the formation of crystalline structures from marginally soluble toxicants,

(2) detoxification and unmetabolized products are drawn into the cells of the proximal tubule along with water, and remain there while water moves on into the bloodstream,

(3) de-conjugation biotransformation, e.g. de-glucuronidation, de-sulphation yield uncovered reactive intermediates which cause cell damage.



A good example is the drug sulfamethizole, introduced many years ago. Its advantage over sulfanilamide is that it is not extensively metabolized *in vivo,* and exerts a prolonged action. However, one has to "push fluids", because sulphamethizole can precipitate in the tubule, causing laceration to the border cells, and bloody urine. This problem can be avoided by drinking a lot of water to keep the solution diluted, so that precipitation does not occur.
There are also active secretory mechanisms located in the proximal tubule, one for organic bases and one for organic

acids (F9.3E). As with any active process, carrier protein and enzymes are involved, energy is required, and these secretory pathways can be saturated. Any damage to the proximal tubular cells by agents in the lumen can affect the active secretion process.

Effects of chemical exposure on renal function can be very insidious and subtle, evading detection for a long time. This is partly due to the redundant capacity in the kidney, where only about 25% of nephrons are used at any one time. An increased fluid load will bring additional nephrons into play, but after the "crisis", the extra nephrons reduce their activity. Considerable damage can occur in the kidney, with complete loss of many nephrons, before no reserve capacity remains. By that time, the damage may not be reversible.

9.4. Toxicity

9.4.1. Heavy Metals

Nephrotoxic Metals: **Cadmium, Lead, Mercury,**
Arsenic, Bismuth, Chromium, Platinum, Thallium, Uranium.

The kidney is the main route of heavy metal excretion from the body, and most heavy metals are nephrotoxic.
At relatively low levels, a variety of signs and symptoms appear: glycosuria, aminoaciduria and polyuria, pointing to problems in efficient reabsorption in the proximal tubule.
At higher levels and at later stages in chronic exposure, elevated Blood Urea Nitrogen, renal necrosis, and anuria will precede death.
The histological picture is one of necrotic proximal tubules with the adjacent lumen filled with proteinaceous material.

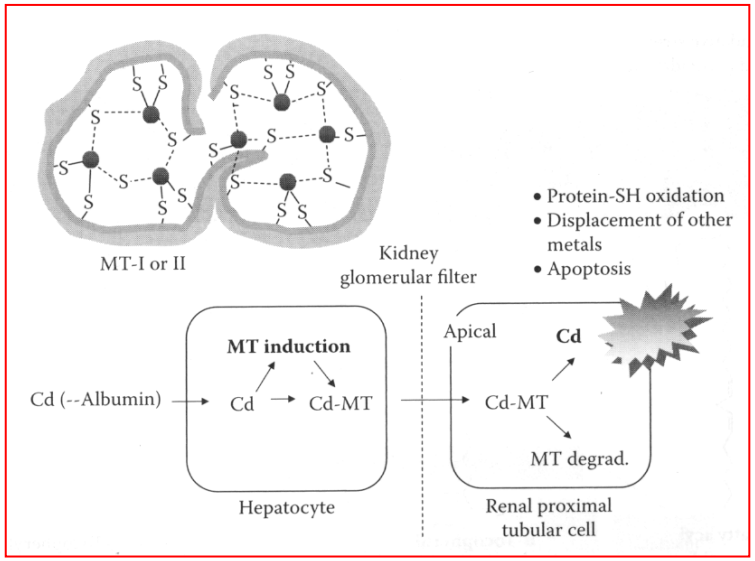
Before toxicity is observed, the kidney will retain significant amounts of heavy metals, some (Hg, Pb) bound to various intra-cellular proteins, e.g. lysosomal acidic lipoproteins followed by a lysosomal endocytosis of these metal-protein complexes. The mitochondria are particularly susceptible to damage, and one sees autophagy of damaged subcellular organelles and extrusion of denatured proteins, all possibly protective reactions.

9.4.1.1. Cd

The kidney is the most sensitive organ to cadmium. About 5% of the US population has enough exposure to cadmium to injure the kidneys. The liver is the main synthesizer of a small (6,500 Dalton, 61 amino acids, 20 of which are cysteine) specific protein, metallothionein (MT), rich in free -SH groups, which binds (chelates) up to seven atoms of Cd, as well as Zn and Cu. The Cd-MT complex is stored in soft tissues of the body such as liver and kidney (which contains 10 times more than the liver). Metallothioneins are a surprisingly diverse group of molecules, but they are spread in a vast range to taxonomic groups, from prokaryotes and up. Metallothioneins have diverse metal-binding preferences. The rat's MT1 prefers binding divalent metal ions (Zn(II), Cd(II),...), while yeast CUP1 prefers binding monovalent metal ions (Cu(I), Ag(I),...).

When exposure to Cd exceeds the ability of tissues to synthesize MT and to store the Cd-MT complex, toxicity occurs above 300 µg Cd/g of cortical tissue. The complex is excreted via glomerular filtration, but can be reabsorbed in the proximal tubule. It was once thought that free Cd in tubular cells was the toxicant, but more recent evidence shows that the Cd-MT complex is toxic.

Cd normally accumulated in kidneys until age 50. It s half-life may be as long as 30 years. It is recommended that Cd intake not exceed 70 µg/day.
Progressively, low molecular weight proteinuria appears and cells concentrating Cd are lost, the Cd then evacuated in the urine. Chronic intoxication produces irreversible renal failure.



F9.7. Cadmium toxicity. *Boelsterli, 2007.*

Cd also produces brittleness as it replaces Ca in bones.

Cadmium has worrisome estrogenic effects in cell culture. Data from rats show that cadmium can act as an estrogen mimic in the whole animal, inducing conditions ranging from uterine hyperplasia to early onset of puberty[5].
Cd is important in electroplating and color pigments. The greatest concentrations are found in the liver and kidneys of animals. Shellfish often have high concentrations.
There has recently been concern in Sweden over body burdens of Cd from consumption of vegetables. Gastrointestinal absorption is 6%. Smoking doubles the daily Cd dose.
Prolonged exposure to moderate levels of Cd in rice (0.3-1.0 µg/g) resulted during and after WW II in a strange muscular condition known in Japan as Itai Itai Byo (or ouch-ouch disease). It is characterized by deep muscle and bone pain of considerable severity, and bone deformation. Renal effects include proteinuria (particularly low molecular weight proteins), aminoaciduria (with elevated proline and hydroxyproline, suggestive of problems in bone metabolism), glucosuria and reduced absorption of phosphate and enhanced excretion of calcium (hypercalcuria)[1]. The proximal tubule is the target site.
The effects were seen predominantly in older women, multiparous, calcium, and protein deficient. Cadmium absorption in the gastrointestinal tract can be enhanced by dietary deficiency of calcium, iron, and protein. Being older, and having had a number of children, these women would have been calcium deficient. Dietary problems during the war would have insured an enhanced absorption of Cd. Estimates exceeded 150 µg/day from food (mainly rice). The source of this Cd was paddy irrigation water taken from a river downstream from a metals mine, which allowed tailings and washings to run uncontrolled into the river for many years.

9.4.1.2. Pb

Lead causes two types of kidney toxicity in humans:
(1) proximal tubular damage reflected in glucosuria, aminoaciduria, hyperphosphaturia and problems of reabsorption,
(2) glomerular atrophy, interstitial fibrosis and sclerosis of vessels seen at prolonged, high level exposure, resulting in chronic nephritis.
Only the proximal tubular effects can be reversed with chelation therapy.

9.4.1.2.1. EDTA Therapy

Short term lead intoxication is usually reversible with EDTA (ethylene diamine tetra-acetic acid) therapy. In the early 1950s several deaths occurred from kidney toxicity after EDTA treatment. At that time, the dosage used was around 10 grams of EDTA per infusion, while it is now 3 grams.
EDTA kidney toxicity is related to the dose and the rate of infusion, which must be adjusted so that the infusion will not harm the kidney. If the patient is very elderly, has low parathyroid activity or is suffering from heavy metal toxicity, treatment should be modified to use less EDTA less frequently (once per week). The heavy metals that damage the kidneys during excessive infusion therapy are lead, aluminum, cadmium, mercury, nickel, copper and arsenic.
Tests of renal function should be performed before chelation therapy is started. In cases of renal impairment, lower EDTA dosage is indicated, with sufficient periods of rest between the infusions.
Long term exposure to lead may cause irreversible kidney dysfunction. “Saturnism” is lead induced gout because of increased uric acid levels.

9.4.1.3. Hg

Mercury is a classical nephrotoxin, used in experimental models of kidney failure.
The damage is primarily in the *pars recta* of the proximal tubule, with proliferation of the smooth endoplasmic reticulum (SER) and extrusion of SER "packets". Organic acid secretion (p-aminohippuric acid, PAH), predominantly in the pars recta, is reduced by Hg exposure. The basic toxic action of Hg is inhibition of the free sulphydryl groups (-SH) of enzymes.

Acute poisoning: brief polyuria may be followed by anuria. If subject survives, polyuria follows, lasting many months.

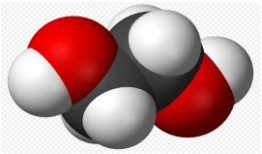
Chronic poisoning: proteinuria, glomerular and tubular damage.
Morphologically, one sees a selective necrosis in the pars recta region, loss of brush border, (villi) from cells, with the formation of clumps of membranes in the cellular cytoplasm, vacuoles, distinct mitochondrial changes characteristic of impending necrosis with eventual necrotic destruction.

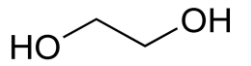
9.4.1.4. Cr

Chromium produces proximal tubular damage with glucosuria, but lower down than does Hg. Ischemic patches on the kidney surface are suggestive of a vascular effect.

9.4.2. Ethylene Glycol

Ethylene glycol (car anti-freeze) is biotransformed both in the liver and in the kidneys to oxalic acid, but the kidney is particularly susceptible. Oxalic acid crystallizes in the lumen and in the cells of the tubule into calcium oxalate salt, leading to laceration of the tubules. Leaves of rhubarb contain large amounts of oxalate. Ethylene glycol proceeds through a number of oxidative reactions leading to acidosis (F9.8). The final product, glyoxylic acid, can be converted either into formic acid or oxalic acid. Oxalic acid may react with calcium ions to produce insoluble crystals of calcium oxalate which precipitate in the renal tubules, eventually producing kidney "stones" and tubular epithelial necrosis with lipid inclusions. Also visible, microscopically, is a thickening of the basement membrane in the glomerulus along with granular deposits.

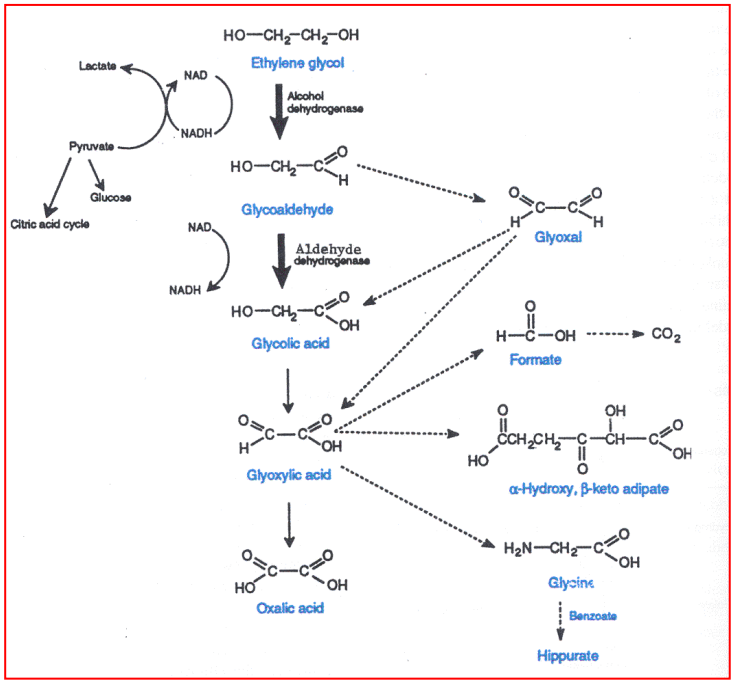




100 deaths occurred in 1937 when ethylene glycol was used as a solvent for an oral sulphanilamide pharmaceutical preparation. Within two days of consumption, nausea, vomiting, intestinal cramps, diarrhea, and back pain in the region of the kidneys were experienced. These initial symptoms led to progressive hepatic

necrosis, renal tubular degeneration and death. Substitution of ethylene glycol by propylene glycol avoids all renal toxicity.

Lavage and **activated charcoal** can be used in the upper digestive tract to minimize absorption. The principal goal in such poisoning is to correct the acidosis (administration of bicarbonate), eliminate the unmetabolized ethylene glycol (by hemodialysis), and to block further tissue biotransformation by administering **ethanol** (20%) which will compete successfully for the enzyme alcohol dehydrogenase to block the formation of glycoaldehyde. The ethanol should be administered within 8 hr of exposure in acute cases (ingestion) and be continued for at least 5 days.



F9.8. Ethylene glycol toxicity. *Casarett & Doull.*

In addition to the renal effects, ethylene glycol has other target organs: CNS (depression, initially), muscle paralysis (CNS related), decreased tendon reflexes, convulsions and tetany (due to hypocalcemia), pulmonary edema and congestive heart failure.

Ability to concentrate urine is often the last property to be regained by the kidney after recovery from intoxication.

9.4.3. Organic Solvents

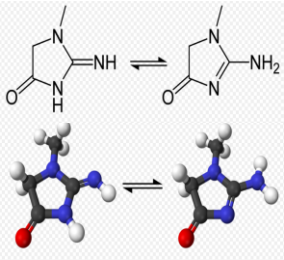
Carbon tetrachloride and chloroform act predominantly in the proximal tubule. Carbon tetrachloride causes severe hepatic

necrosis, but the cause of death is kidney failure.
Renal tubular disease can be caused by a variety of volatile organic solvents, most likely as a consequence of reabsorption into the proximal tubule and biotransformation there into reactive and toxic intermediates. Glomerular damage can also be produced by such agents, a slow progressive disease with an insidious onset seen in adults[2,3].

9.5. Tests of Kidney Function

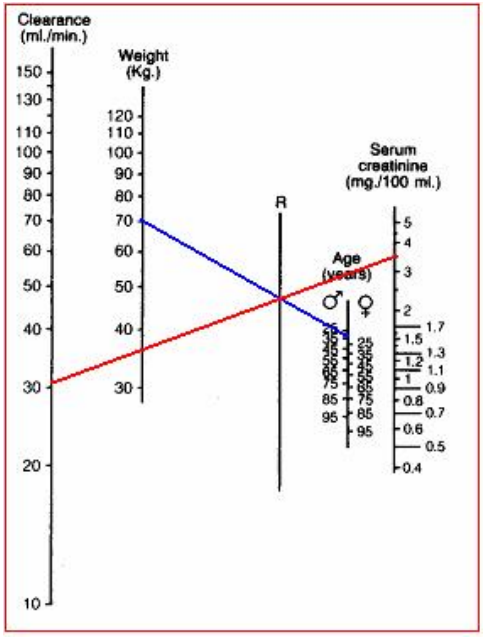
The general medical indicators of kidney function are *Blood Urea Nitrogen* (catabolism of proteins, normal 2.9-7.1 mmol/l) and *serum creatinine levels* (from muscle metabolism, 60-130 mol/l). Elevated levels in the serum are indicative of kidney dysfunction, specifically low Glomerular Filtration Rate.
The basic test of kidney function is *clearance*. Clearance can be higher or lower than the glomerular filtration rate if a substance is actively secreted or reabsorbed, and is routinely assessed by physicians to predict the rate of drug elimination in patients. BUN and serum creatinine are related to GFR (F9.9).

9.5.1. Creatinine Clearance



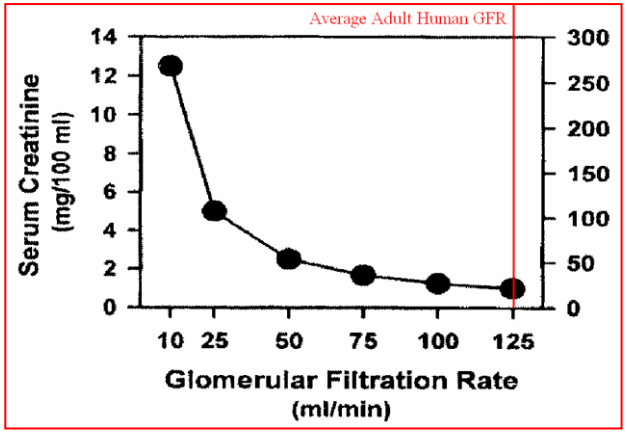
The rate at which a drug is eliminated by the kidneys is commonly proportional to the clearance of creatinine. It is not always convenient before starting treatment to wait for a creatinine (shown) clearance measurement, but it can be estimated by using the Siersbaek-Nielsen nomogram to estimate creatinine clearance from weight, age, and serum creatinine concentration (F9.9).

F9.9. Nomogram for rapid estimation of endogenous creatinine clearance. *Lilly.*



With a straightedge, join weight to age. Keep straightedge at crossing point of line marked "R." Then move the right-hand side of the straightedge to the appropriate serum creatinine value and read the patient's clearance from the left side of the nomogram.

When kidney function is stable, this gives a valid estimate. But in the presence of shock, severe cardiac failure, or oliguria, the estimate is too high.



F9.10. The relation between the most frequently used medical kidney parameters and the glomerular filtration rate. *Casarett & Doull.*

9.5.2. Glomerular Filtration Rate

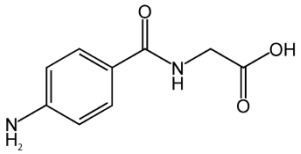
The Glomerular Filtration Rate, in ml/min, evaluates the volume of plasma being passed into Bowman's capsule.
It is measured by administering a single injection if Inulin, a fructose polymer with molecular weight of 5200 Da. This sugar dissolves in the blood, but is not bound to any plasma component, is not metabolized or stored, is not reabsorbed or secreted. It becomes a permanent component of urine.

$$GFR\ (ml/\min) \approx \frac{[I_{Urine}](mg/dL)}{[I_{Plasma}](mg/dL)} \times Urine\ Flow\ Rate\ (ml/\min)$$

The numerator product is "mg excreted per minute", while the denominator is the concentration found in plasma from the injection. A normal GFR is about 125 ml/min.
If this decreases, there is a possibility of longer half-lives for toxicants being eliminated, and consequently more toxicity.
Any significant renal disease or toxicity will affect the GFR, as will hypotension or shock.

9.5.3. Tubular Secretion Rate

To evaluate the volume of plasma being passed through the kidneys as a whole, one can use, in the same way, PAH clearance.



P-Amino-Hippuric Acid is so well secreted into the urine by the tubules that about 91% of it is eliminated from the whole plasma present in the kidney in a single pass.
Normal values, compensated by 0.91, are 650 ml/min.

9.5.4. Artificial kidneys

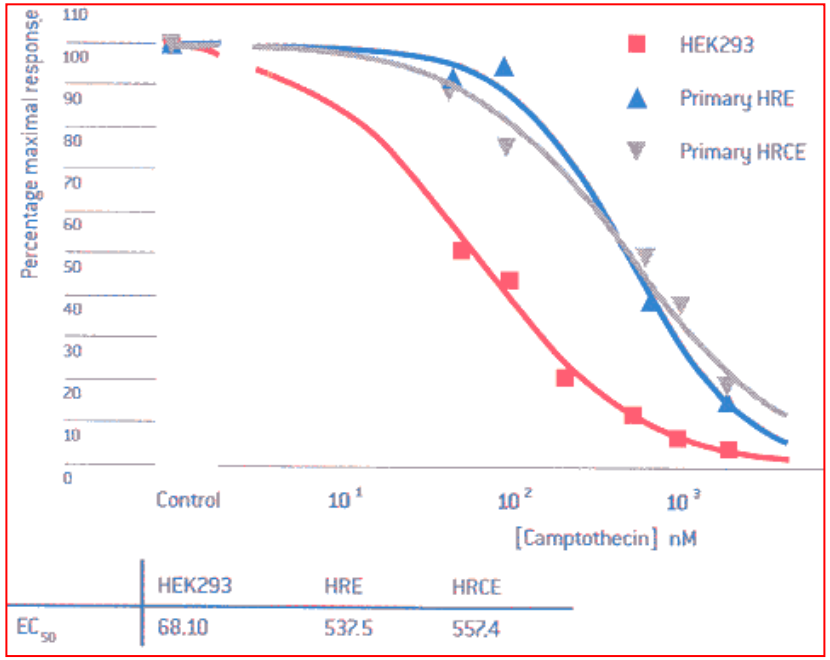
Dialysis machines remove toxins from the blood through diffusion, but are not specific and do not produce hormones and enzymes.
Membranes made of cellulose readily pass low molecular weight molecules (urea, creatinine, uric acid, electrolytes). Amino acids, vitamins, glucose and water are also lost, and so must be replenished.
A patient requires 3 sessions/week at 3-6 hours per session, during which 120 liters/week are filtered at a yearly cost of about 25,000 $. By contrast, kidneys cleanse 1200 l/week.
As a result, urea levels in artificial dialysis patients are 14-200 mg/100 ml blood compared to 20-40 mg/100 ml of blood for normal individuals. Life expectance in middle-age is only 5 years on artificial kidneys.
In the future, a chamber could be used to grow kidney cells from pigs, and a patient's blood made to interface with the chamber. This system should provide improved control of blood ions, particularly potassium, in dialysis patients.

9.5.5. In Vitro

Initial research on the efficacy and toxicity of drugs is often conducted on cell lines that have been transformed, so that they may proliferate indefinitely, insuring repeatability of the test.

Specific cell lines are used to simulate the proximal tubule (LLC-PK_1, pig), and the distal tubule (MDCK, dog). Most of the current cell models cannot be used universally. Two problems are loss of renal characteristics and limited lifespan.

Renal continuous cell lines such as Human Embryonic Kidney 293 cells (HEK293) have been popular in pharmacology screening. The cells, assumed to be from a kidney fibroblast, endothelium or epithelium, were *transformed* in culture with adenovirus in the early 1970s. But a 2002 study suggested that HEK293 cells have many properties of immature neurons. The suitability of HEK293 as an *in vitro* model for kidney cell function was in question. When HEK293 was compared to Human Renal Cortical Epithelial Cells and Human Renal Epithelial Cells of more recent fabrication in their response to a drug, Camptothecin, responses were very different. Camptothecin induces apoptosis in a dose-dependent manner in vitro. As can be seen in the figure below, the effective concentration (EC_{50}) of Camptothecin on HEK293, compared to primary kidney cells in triggering apoptosis varies by a factor of almost 10. This probably led in the past to the use of lower and probably ineffective concentrations of drugs being used in tests of efficacy and toxicity.



F9.11. Effect of Camptothecin on viability of primary (HRE & HRCE) and continuous (HEK293) renal cells.

9.6. Short Kidney Cases

Modified from Sherman.

9.6.1. Kidney Case #1

James smoked cigarettes, beginning at age 17. He operated an antique car restoring business, handling a number of plastics, paints, and solvents: toluol, xylol, acetone, isopropyl alcohol, methylene chloride, ethylene glycol monoethyl ether, methoxyethyl acetate, acrylic paint systems, and a number of chemicals identified only as petroleum distillates, aromatic hydrocarbons, and aliphatic hydrocarbons.

In the MSDSs, many of the ingredients listed did not add up to 100%, others were not specific as to their identity, and others indicated only as solid acrylic, proprietary alcohol, bodying agent. Many of these chemicals are taken into the body not only via inhalation but through the intact skin. Many of them are eliminated in the urine, contacting kidneys and bladder cells.
James was 47 years old when he noticed blood in his urine. Test showed cancer of his right kidney, and it was removed in 1982. He stopping smoked cigarettes at the time his kidney cancer was diagnosed.

9.6.2. Kidney Case #2

Albert worked 24 years as a laborer at a company where lead was handled on a regular basis, for over 40% of his total lifetime. Lead is concentrated in the bones and excreted by the kidneys. Kidney cells are damaged, resulting in decreased ability to eliminate fluid and solute loads. While lead is stored in the bones, it interferes with the body's ability to make blood cells, producing anemia. Albert was admitted to the hospital because of kidney failure and hypertension when he was 58 years old. He had been admitted twice before because of lead poisoning, and within the previous month because of severe anemia. Six days later he was dead, his final diagnosis being chronic glomerulonephritis and uremia.

9.6.3. Kidney Case #3

David, a non-smoking police officer, was medically evaluated in January 1982. An Intravenous Pyelogram showed no abnormalities of his kidneys or bladder. In March 1984, David crashed into a utility pole in the automobile chase of a suspect. His patrol car and an electrical transformer exploded and burned. He was heavily exposed to the fumes and smoke. PCBs used as coolants in many older electrical transformers may be contaminated with dioxins (TCDD), potentially causing

malignancies. Even brief exposures have consequences, as PCBs have a long half-life within the body. By September 1984, he noticed blood in his urine. In January 1985 he had bladder cancer at 39 years old, and underwent surgical removal of the malignancy. Bladder cancer is rare at 39 years, and police duty does not involve exposure to toxic chemicals. With a baseline of no disease in 1982, exposure in 1984, and the development of a malignancy ten months later, a cause-and-effect relationship is highly likely. This sort of documentation is rare, in that few people have a body system evaluated twice within such a short time, with the appearance of cancer during the interval.

9.6.4. Kidney Case #4

Harry worked for 32 years buffing chrome-plated bumpers. He used a dry buffing compound and said that although it was extremely dusty work, he did not wear any sort of mask until after 1978. He said the bumpers were plated in an area about 100 m away from his workstation, and that the fumes and the acids “cut his wind”, especially when equipment broke down. Exposure to chromium is associated with lung disease and kidney damage. Chromium compounds are taken into the body via inhalation, by ingestion with the saliva, and through the skin, causing damage at a number of sites. Chromium is excreted in the urine, and causes damage as it passes through the kidneys.
He first learned of high blood pressure and diabetes in 1967, and he took medication for each.
Harry is now 60 years old. His blood pressure readings are 250/130 on the right, and 240/142 on the left, and he has significant protein in his urine.

9.6.5. Kidney Case #5

Charles worked as a welder after finishing high school. He did aluminum welding, and then silver brazing for the next 16 years, followed by electric welding for the last 2 years.
The brazing material's MSDS indicated: silver 50%, copper 15.5%, zinc oxide 16.5%, and cadmium oxide 18%. The product was "dangerous mostly from cadmium fumes. Inhalation of dusts or fumes of cadmium affects the respiratory tract and may affect the kidneys. Cadmium is listed by the National Toxicology Program as anticipated to be a carcinogen. Studies indicate that there is an increased incidence of prostatic cancer, and possibly kidney and respiratory cancer in cadmium workers."
The MSDS recommends respiratory protection. At 37 years old, Charles applied for life insurance. A routine examination found protein in his urine and kidney dysfunction. Biopsy revealed a focal sclerosing glomerulosclerosis, labeled as *nonspecific*. As an incidental finding, he had reported that he had essentially lost his sense of smell ten years previously.

9.7. Case Study: Acute Arsine Poisoning

(modified from Williams & Burson)

A patient was admitted with what seemed to be hemolysis. The hospital also reported that a fellow employee of the patient had become ill at about the same time and had been admitted to another hospital. A visit to the plant where both employees worked was organized. The concern was that other workers might have been exposed as well.
It was learned at the plant that the two ill employees had worked at cleaning a room on the preceding day. There had been a clogged floor drain in this room, and the workers used a drain cleaner that was one of the commercial products manufactured by the company. Once in the drain, the cleaner had bubbled and given off a gas with a pungent odor. Even though the employees developed headaches, they continued at their work for about four hours.
At home that night, the employees had become ill. They were nauseated, and vomited. One went to a hospital emergency room, was examined, and was sent home. The other went to another emergency room; his urine was sampled and it was bloody looking; he was admitted to the hospital. The first worker became

progressively worse and was admitted to a hospital the next day. In the meantime it was determined in the hospitals that both workers had severe hemolysis, which caused the bloody-looking urine. The only gas that will cause hemolysis is arsine. One of the necessary ingredients for the production of arsine is arsenic. The plant was therefore inspected for potential sources of arsenic, and samples from the floor drain were collected and analyzed. All the samples were analyzed by neutron activation analysis, which is somewhat expensive but is a relatively easy way to analyze multiple matrices. In addition, urine and blood from the two patients, the drain cleaner, and air samples were also submitted for analysis.

The plant consisted of a large work area and several offices. There were two loading docks, one small and the other large. One room was used for mixing the different products made there. In another area workers bagged products. The stopped-up floor drain from which the gases had bubbled was in the center of this area. The loading dock outside this room was higher than the floor of the room; when it rained, water on the loading dock would run into the building. The manager of the plant had worked there for only a few years and was unaware of any arsenic ever having been used in the plant. However, one of the maintenance men told investigators that before its present operation, the company had made arsenical herbicides, primarily arsenic trioxide; in fact, a tank located outside on the loading dock had previously been used for mixing arsenical herbicides. When the employees were conducting their general cleanup, they thought only water was in this tank and had drained it; the contents drained into the stopped-up drain. When the employees added their company's drain-cleaning product, primarily a mixture of sodium hydroxide, aluminum chips, and sodium, the gas had been emitted from the drain. Clearly the water and arsenic trioxide from the forgotten mixing tank had reacted to produce the gas arsine, as follows:

$$6\ NaOH + 6\ Al + 6\ H_20 + As_2O_3 > 6\ NaAlO_2 + 3\ H_2O + 2\ AsH_3$$

Arsine is extremely toxic. Arsine poisoning does not reveal itself in elevated arsenic levels in those patients who have been poisoned by arsine; it does not produce the same type of symptomatology as arsenic poisoning. Indeed, arsenic poisoning is entirely different from arsine poisoning.

The most important consideration was to demonstrate that arsenic was present in the drain, that an arsenic-to-arsine reaction could have taken place, and that there was arsine in the gas the employees had inhaled. Drain samples from the herbicide tank, samples from the drain cleaner, and the aluminum chips were all tested for arsenic. As shown in the Table, there was a great deal of arsenic, comparatively speaking, in the drain and in the herbicide tank. There was also some antimony. If antimony were substituted for arsenic in the reaction shown above, the result would be formation of stibene. However, stibene is not as stable as arsine, and it is highly unlikely that it would have caused the illness in the patients. The drain cleaner and the aluminum chips had no arsenic. Analysis of the urine from a number of other workers demonstrated that the two people who were ill had higher arsenic levels in their urine than some of their fellow workers who had been in the vicinity of the drain but who had not gotten sick. A supervisor, who had never gone into the area where the drain was, showed no arsenic in his urine. It was concluded that the presence of arsenic in the drain resulted in the formation of arsine when the drain cleaner was added. The arsine gas caused the hemolysis in the patients.

The two patients, after a very stormy medical course including renal failure and other problems, finally recovered, partly because such cases can now be treated with dialysis units. Before dialysis units were available patients with severe arsine poisoning usually died of renal failure. There are two effects of arsine poisoning on the kidneys. One is the breakdown of the red blood cells with excretion of hemoglobin, which will damage the kidneys; in addition, arsine itself may directly damage the kidneys. Both employees had to be dialyzed for several weeks.

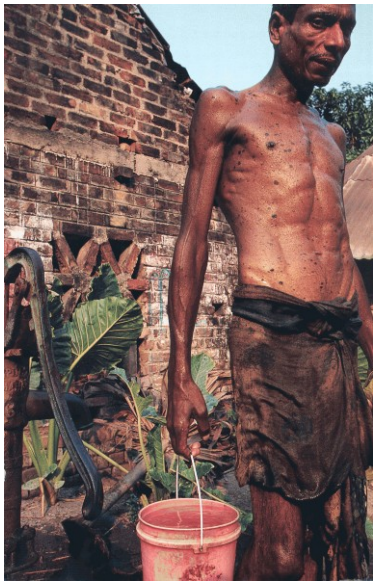
T9.12. Arsenic and Antimony Levels in Environmental Samples Gathered at the Chemical Plant Where Arsine Poisoning Occurred

	Concentration (ppm)	
Source	**Arsenic**	**Antimony**
Drain		
Upper fraction		
Liquid	440	44
Solid	970	55
Lower fraction		
Liquid	410	43
Solid	4,460	170
Air sample	l3µg	
Abandoned herbicide tank		
With agitation		
Before	7,490	393
After	11,400	698
Drain cleaner		
NaOH and $NaNO_3$ solid	0.26	1.0
Aluminum chips	0.15	40.0

*Forty liters obtained on charcoal tubes minus background from unused tube

9.8. Bangladesh and Arsenic (National Geographic, 2002)

Because surface water had bacterial contamination, 12 million wells were dug in Bangladesh to minimize infection in the water supply.



Unfortunately, high levels of arsenic from the deeper wells has caused 7000 deaths since the early 1990s.
After 8-14 years, white or black spots (melanosis, see at left) appear on the skin (palms and soles), leading to gangrenous ulcers. Finally, renal diseases and cancers of lungs and bladder appear. It appears that arsenic attached to sedimentary grains is released in deep groundwater in great part by bacteria[4].
More than 10 micrograms per liter of the poisonous element were associated with an elevated risk of all-cause mortality[6,7].
The Environmental Protection Agency (EPA) signaled on October 31, 2001 that it will be reducing the amount of arsenic allowed in U.S. drinking water from a maximum of 50 micrograms per liter to the tighter 10 micrograms per liter.

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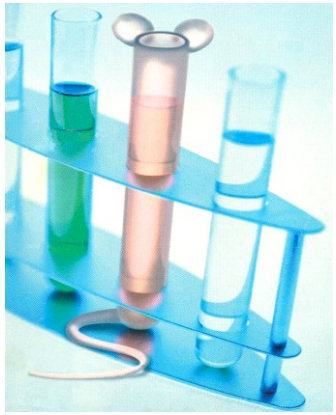
Techniques in vivo & in vitro

10· Techniques in vivo & in vitro

Toxicity determinations are influenced by our choice of toxicity models, and by our choice of variables used to assess these models.

10.1. Selection of Experimental Technique



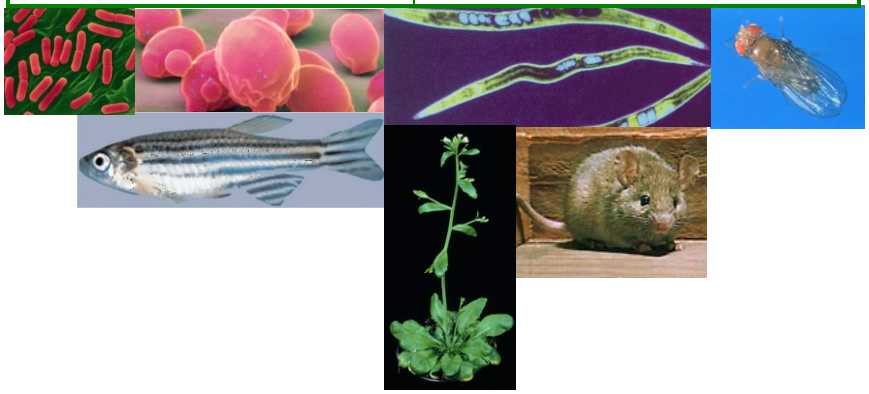
To experimentally determine toxicity, a model as well as investigation methods must be chosen. These specific choices have a critical influence on outcomes. When general toxicity is the intended determination of the test, these choices, which by necessity use a specific models and investigation techniques, are powerful determinants of toxicity, essentially by possibly excluding other aspects of toxicity from consideration.

10.1.1. Choice of a test system

Test systems are chosen to facilitate investigations, and make them as productive and economical as possible. For example, biologists use a set of models (T10.1) somewhat different from those of toxicologists, because their interests are directed towards basic life mechanisms, rather than toxicity.
Intestinal bacteria, yeast and mice are familiar and accessible. The *C. Elegans* worm was convenient for microscopy, developed rapidly and had a short generation time.

T10.1. The Classic Models used by Biologists

Formal Name	Description
Escherichia coli	Intestinal bacterium
Saccharomyces cerevisiae	Yeasts, single nucleated cells
Schizosaccharomyces pombe	
Caenorhabditis elegans	Small soil-dwelling worm
Drosophila melanogaster	Fruit fly
Danio rerio	Zebrafish
Arabidopsis thaliana	Mustard plant family
Mus musculus	Mouse



Drosophila rose to prominence in the early 1900s because of its short generation time and large chromosomes. Although the fruit fly encodes about 14,000 proteins and humans almost twice that number, many proteins accomplish the same tasks in both organisms. When human disease genes are put in flies, they *often* cause similar symptoms. Addition of a human gene in a fly can compensate for the deletion of the same gene from the fly.
The zebra fish is also also an old environmental toxicology model for organ toxicity, developmental toxicity and acute toxicity (LC_{50}). You can see through zebra fish, so necrosis and changes in organ morphology can be seen under a dissecting microscope. Embryos are transparent enough for observation of

the heartbeat (cardiotoxicity). Neurotoxicity has been studied using staining antibodies. It has been used to study fetal alcoohol syndrome and teratogenicity.

Arabidopsis thaliana has a short life cycle (6 weeks), seed production is prolific and the plant is easily cultivated in restricted space. The use of frogs (Xenopus laevis) in pregnancy testsfor led to their recruitment as a model for developmental research.

Models used in toxicology include an even larger range of organisms than in biology, partly because many organ diseases must be represented: hematotoxicity, cardiotoxicity, gastrointestinal toxicity, hepatotoxicity, nephrotoxicity, ototoxicity, bladder toxicity, neurotoxicity, pulmonary toxicity, endocrine toxicity and reproductive toxicity. Each of these organ systems is easiest to observe in specific models. Therefore, bacteria, algae, cell lines, tissue culture, mice, rats, rabbits and primates are all part of the experimental models used in toxicology. Worms can be used instead of rodents for lethality (LD_{50}) testing. The digestive system is very similar in structure to worms.

In a collection of critical studies on the toxicity of benzene applicable to humans, the following models were used: mouse (15), rat (10), human (8), rabbit (5), dog (2) and one study each in cat, guinea pig and frog. Rats are a preferred model for studies of the heart, kidneys, immune system, reproductive system and nervous system.
In the future, *in vitro* proteomics and genomics may become popular, as cellular or molecular endpoints appropriate for high throughput toxicity screening are developed.

10.1.1.1. Model Problems

It is difficult to determine that a model properly represents what it is intended to, and the foldback position is to use a traditional model that is heavily represented in the literature. Funding agencies dominated by sceptical colleagues support work on a shortlist of favoured species, and as a consequence, research on life revolves around a handful of species, under the assumption that model organisms offer universal insights. This undue focus comes with a much reduced field of view.

The lack of developmental plasticity in Drosophila and of genetic variability in inbred rats limit what these models can tell us[19]. Although the mouse and human genome are about 85% identical, the remaining 15% difference can introduce substantial variations in toxicity reactions, because so many elements of livings systems are functionally bound together. Rats replicate high blood pressure and atherosclerosis more readily than mice do. Rabbits are closer to humans, physiologically, than either mice or rats. Genetically, dogs are much closer to humans than rats. However, dogs are more susceptible than humans to certain foods, for example, alcohol, coffee, chocolate, macadamia nuts, garlic, onions and grapes. Although the dog split off first from the common ancestor, the rat experienced a much higher mutation rate.

In animal models, factors such as gender, age, nutritional and disease state, time of exposure, daily rhythms of cortisol and melatonin, and the general environment may all affect test results.
Researchers generally tend to gloss over the weakness of their models.

Thalidomide, a sedative drug tested with good results in the late 50s to treat morning sickness associated with pregnancy, unexpectedly produced amelia and phocomelia in humans. Mice and rats are resistant to it; hamsters and rabbits show variable effects.

10.1.1.2. Model Cost

The choice of test system strongly influences the price of the test. Prices for relatively inexpensive biocompatibility tests are compiled in T10.2, while prices ($) for more ambitious studies in the rat are shown below.

A Balb/c mouse is less that 20 $, but a cat, which makes a good models for allergy studies, costs 800 $. A beagle is around 700 $, but if the animal has a defined antibody profile, it might cost 1500 $. A monkey that is *specific pathogen free* might go for 8000 $.

Acute inhalation toxicity test	10,000
Repeated dose test: 14-day exposure	50,000
Reproductive toxicity test	100,000
Repeated dose test: 90-day exposure	120,000
Repeated dose test: 1-year exposure (dietary intake)	250,000
Repeated dose test: 1-year exposure (forced feeding)	1,000,000

In view of the costs associated with experiments, it is often relevant to consider the minimum testing required, as opposed to what would ideally be needed.

10.1.2. Choice of a Response

The end-point of the study should be easily observable and quantifiable. Cell counting, bio-chemical product determination, cell morphology, tumor count, changes in weight gain...can all be considered, as long as they can be done reliably. The selection of the *response variable* is probably the most under-played aspect of toxicity testing.

10.1.3. Duration of the test

Eye irritation may only take a few seconds to apply and as little as a day to interpret. Reproductive and carcinogenicity tests take years. The most basic classification of toxicity tests refers to duration (acute vs chronic).

10.1.4. Dosages to be tested

To anticipate the appropriate dosages, something must be known about the toxicity of the agent tested. The dosage is usually in mg/kg, as opposed to the more exact scaling according to $\text{Weight}^{0.66\text{-}0.75}$. The dosages are displayed on a log scale, usually in multiples of 3, 5, or 10. Attempt is made to cover the dosage range with a reasonable number of groups that will include effective or lethal doses, as well as the *threshold* dose.

10.1.5. Test Control

10.1.5.1. Reproducibility vs Representativity

Within the test procedure specification lies a decision between the value of Reproducibility vs Representativity. The more heavily controlled a test is in terms of the organism used, husbandry and environmental variables, the more restricted it is in the conditions that it represents. In general, investigators are more concerned about Reproducibility of results than about Representativity because Reproducibility is easier to prove.

For example, it is easier to purchase rats with identical genetic makeup and control completely their food and environmental conditions than it is to insure that this particular test will properly represent human safety, which should involve a rich array of possible conditions (age, sex, social, environmental, medical), all of which are costly to simulate.

T10.2. Cost in US $ of Biocompatibility Tests (Nelson Labs).

CATEGORY	TESTING OPTIONS	COST	TIME	SAMPLE
Characterization (non-GLP)	Differential Scanning Colorimetry	$115 w/o GLP	10 days	1 gram
	Infrared Spectroscopy (FTIR)	$95 w/o GLP	7 days	
Cytotoxicity	MEM Elution (USP)	$360	15 days	120 sq cm or 4 grams
	Agar Overlay (USP)	$360	15 days	
Sensitization	Magnusson-Kligman Method (ISO) with 2 extracts	$4635-6725	6-8 weeks	600 sq cm or 32 grams (min. 6 samples)
	Buehler Method	$3600-4225	6-8 weeks	125, 1"X1" pieces or 45 grams
Irritation	Intracutaneous Reactivity (ISO) with 2 extracts	$750-870	3-4 weeks	240 sq cm or 8 grams
	Primary Skin Irritation (ISO)	$540-775	3-4 weeks	120 sq cm or 4 grams
	Primary Eye Irritation (ISO)	$640-810	3-4 weeks	
Systemic Toxicity	USP/ISO Systemic Injection with 2 extracts	$350-545	3-4 weeks	240 sq cm or 8 grams
	Material Mediated Pyrogen	$425-635	2-4 weeks	600 sq cm or 30 grams
Sub-chronic Toxicity	Intraperitoneal Toxicity w/Histopathology	$5625-9645	6-8 weeks	1900 sq cm or 100 grams
	Intravenous Toxicity w/Histopathology	$5625-9600	6-8 weeks	
Genotoxicity	Ames Test w/ 2 extracts	$2250	5 weeks	240 sq cm or 8 grams
	Mouse Micronucleus (ISO)	$8700-10890	8-10 weeks	
	Chromosomal Aberration (ISO)	$8800-10850	8-10 weeks	240 sq cm or 12 grams
Implantation w/ Histopathology	7 Day Observation (ISO)	$1350-1875	5-6 weeks	14, 1 x 10mm strips
	14 Day Observation (ISO)	$1525-1925	6-8 weeks	
	30 Day Observation (ISO)	$1600-2100	8-10 weeks	
	60 Day Observation (ISO)	$2155-2575	17 weeks	
	90 Day Observation (ISO)	$2750-2975	19 weeks	
Hemo compatibility	Dog Thrombogenicity (ISO)	$3800-3965	6-8 weeks	6, 2 inch long & < 3mm diam.
	Hemolysis	$565	2 weeks	
	Partial Thromboplastin Time (PTT Non-Activated)	$550	3 weeks	150 sq cm or 5 grams
	Prothrombin Time (PT)	$550	3 weeks	120 sq cm or 4 grams
	Immunology/Complement (C3a, SCb-9)	$2450	4 weeks	

It would require many independent experiments performed on a number of different species and under many different conditions to raise confidence in Representativity, which drives the costs much higher.

Therefore, toxicologists attempt to achieve Reproducibility in individual tests, and to attain Representativity by compiling a number of diverse experiments. It should be realized that for many important biological variables, there is an inherent instability (because of complexity) in any living system. Our knowledge base and our illustrations of living systems tend to under-estimate true natural complexity. As a consequence, experiments intended to be carefully controlled sometimes yield diffuse results.

For example, genetically identical mice raised as far as possible in the same environment show significant variability in life spans[6]. A common fear of experimenters is to end up with ambiguous data that is difficult or impossible to interpret. Any "realistic" toxicological representation includes an array of conditions so complex that the smearing out effect on the data risks making the results unintelligible. Therefore, such simulations are rarely attempted and are left for epidemiologists to extract from human populations.

T10.3. The US Good Laboratory Practices provide guidelines for conducting nonclinical laboratory studies. (21 CFR Part 58, effective April 1st 1997) http://www.nal.usda.gov/awic/legislat/21cfr97.htm

1. General Provisions
2. Organization and Personnel
3. Facilities
4. Equipment
5. Testing Facilities Operation
6. Test and Control Articles
7. Protocol and Conduct of a Non-Clinical Laboratory Study
8. Records and Reports
9. Disqualification of Testing Facilities

Sections within each of these parts identify procedures to which laboratories must comply. Compliance is monitored by the FDA with audits every 2 years, verifying **Standard Operating Procedures**, study data (in-life data, necropsy, clinical pathology data, histology/pathology worksheets, tables, statistics, final reports, computer validation, training records, etc.).

10.1.5.2. Standardization of Methods

Over the past three decades, toxicological assessment protocols have evolved into complex tests of a variety of endpoints of toxicity. Considering toxicity methods, one can wonder about the value of *protocol diversity* as opposed to *protocol standardization*. Advantages can stem from both, but they are to a point incompatible. Diversity leads to discoveries, while standardization leads to comparability.

With standardization comes bureaucracy. The US FDA and other national and international bodies have led protocols to become highly formalized, and consequently expensive.

If all the research done in the past had been done according to Good Laboratory Practices (F10.3), this text might be written with a quill, rather than a computer…

GLP keeps track of *everything* through reporting, even the trivial, absorbing precious resources. GLP also leads to an effective monopoly on research for rich corporations or governments by disabling small laboratories, or at least taking funding away from them.

Contrasting study styles can influence conclusions. For example, in assessing the bioeffects of bisphenol A, the US FDA relied mostly on industry GLP studies, while the US National Toxicology Program relied on studies performed by university scientists. The two procedures yielded different conclusions ("little or no risk" vs "some concern").

The Organization for Economic Cooperation and Development has also published "Good Laboratory Practices" but in this case specifying which toxicology tests should be run on any given chemical (cosmetic, pesticide, solvent). Note that in practice, this acts to *restrict* as much as it does to *require* testing.

There are also Good Clinical Practices (GCP) for clinical studies. Generally, this bureaucracy seriously increases the price of science.

10.1.6. Ethics

Using surrogates species for testing is based first on the principle that humans are more *valuable* than other species. We wish to protect ourselves from injury even if it means injury to other species, because we have less in common with them than with other humans.

> Criticism: All species are "equal". Genetic programs differ, but none is inherently more valuable than another. Our anthropomorphic view is an abuse of other ("lower") life forms.

The quest for knowledge is a second principle supporting testing. Experimentation that leads to improved knowledge, whether done on animals or humans, will benefit countless other humans in the future.

> Criticism: We injure the few to save the mass, which is contrary to individual rights. This happens to some extent in clinical trials.

But in the end, we go ahead with testing mostly because we have acquired through technology a position of power on our planet…a power that we should not abuse.

F10.4. A PETA poster depicting the sadistic scientist.

A naïve suggestion to "use computers" for toxicity testing is

irrelevant. If we could program computers with an accurate description of living systems useful for toxicity testing, we would have such knowledge that there would be no need to test in the first place.
Short of testing on humans, which is done in clinical trials, we use the most primitive and inexpensive surrogate that will do the job.
Contrary to what some extremists would like you to think (F10.4), sadists are not common in science laboratories. But there are tired, resource-strapped people who may cut corners. Ethics committees are active to insure that unnecessary pain is not inflicted on experimental animals.

Lay people assessing scientific research often harbor misconceptions, particularly under-estimating the effort required to achieve discoveries. The labs presented in popular films are lavishly equipped, with lots of redundant staff.

10.2. In Vivo Models

Animals models are useful to unravel mechanisms of action and uncover potential effects, but are weak when attempting to predict impacts on humans.
The correspondence between carcinogenicity determinations in humans and animals can be evaluated using databases that list carcinogenicity to humans and animals for a given chemical.

T10.5. Evidence of *Animal* vs *Human* Carcinogenicity for 597 chemicals (IARC database), in%

	Sufficient	Limited	Inadequate	No data	Animal Total
Sufficient	3.5	2	7.9	20.6	34
Limited	1.3	0.2	4.7	20.8	27
Inadequate	1.0	0.2	5.5	27.5	34.2
No data	0.2	0	0.2	4.0	4.4
Human Total	6	2.4	18.3	72.0	~100

From the first column, evidence in humans is sufficient to establish carcinogenicity in 6% (total) of chemicals. But 42% of those (2.5 / 6) did not provide corresponding Sufficient animal evidence. From the first line, 34% of the 597 chemicals show sufficient evidence in animals. Only 10.3% (3.5/34) of those agents also proved to be human carcinogens. This is due in great part to lack of human toxicological data. The conclusion is that animal experiments are modestly predictive of human carcinogenicity, but that lack of adequate human data is an even more serious problem.

10.2.1. Species Selection

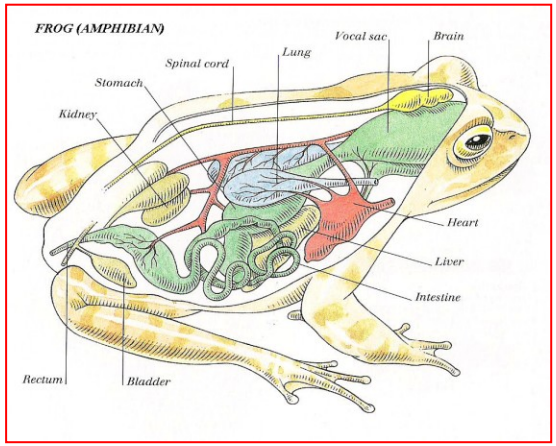
Since toxic responses frequently differ between species, multi-species testing has been included in many official protocols (T10.6) after the 1940s. Present requirement for rodent *and* non-rodent might suggest the existence of two large categories of animals in toxicity responses, but there is no special similarity between rodents as opposed to non-rodents.
The requirement for *non-rodents* prevents undue concentration on rodents, just because they are inexpensive. Not long ago, if the toxicology results from two species diverged, a third species was recommended, the monkey. This is now abandoned in favor of fundamental studies: specific receptors, toxicokinetics, and metabolic studies with *in vitro* models. Once the cause of the divergence is known, directed investigations are conducted in humans. In practice, studies in a second species are most useful to *stop* the development of a drug, since a repeat problem implies a small chance that humans would be somehow be spared.

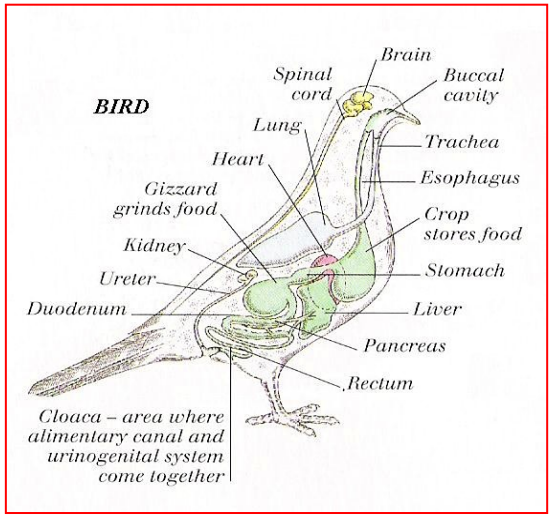
Even a superficial examination of anatomical differences between possible *in vivo* models (next 2 pages, from *Ultimate Visual Dictionary of Science*) should alert us to many differences these models have with humans, making the choice of a particular animal model a very strategic decision.

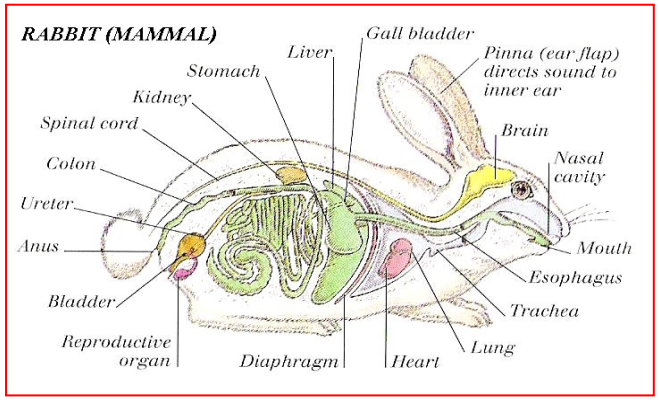
The changes in use of various animal species for research and toxicology testing is affected by availability of research funds and ethical concerns (T10.7). There has been a substantial move towards *fish*, which humans identify less with.

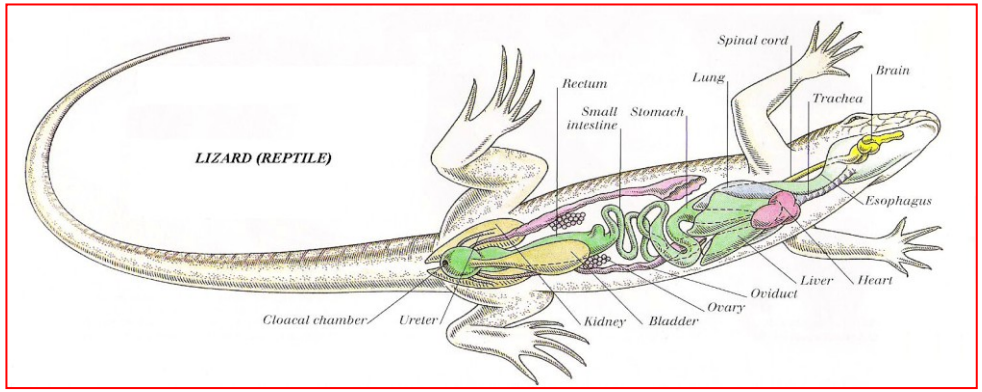
It would be rational to investigate interspecies differences questions and interpret divergent findings *before* larger studies are performed, but for practical reasons, this is rarely done.

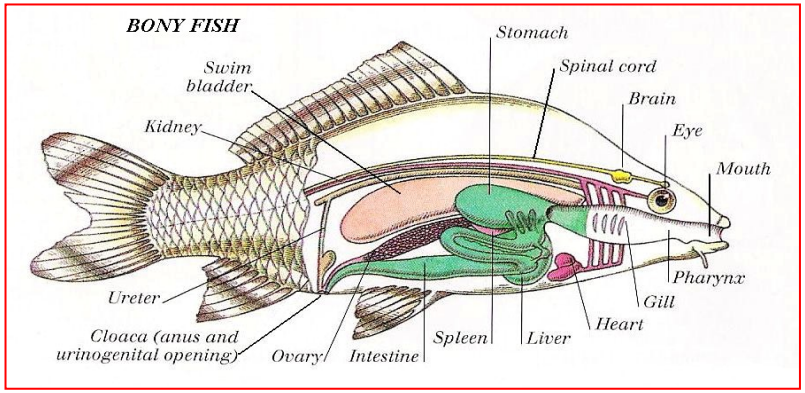
Many large corporations have their own closed rodent colonies, leading to some uncertainty as to whether all tests on "rats", for example, are truly comparable. In one view, chemicals should be tested in the same strain, age, and sex of animals, so that comparisons can be made. Companies like ICI, Eastman

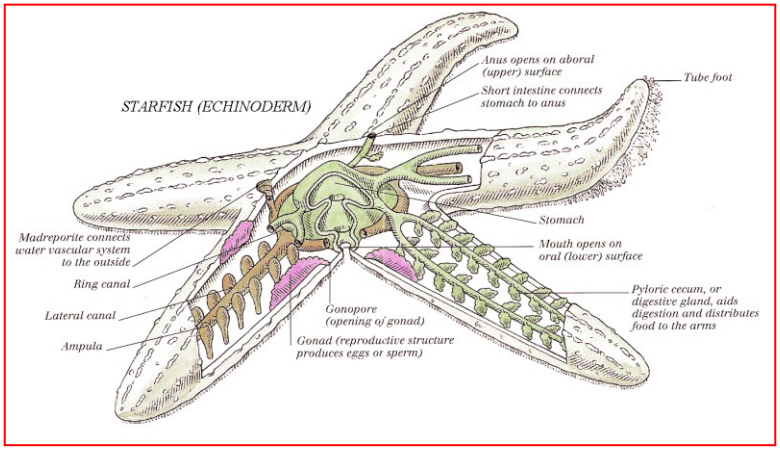


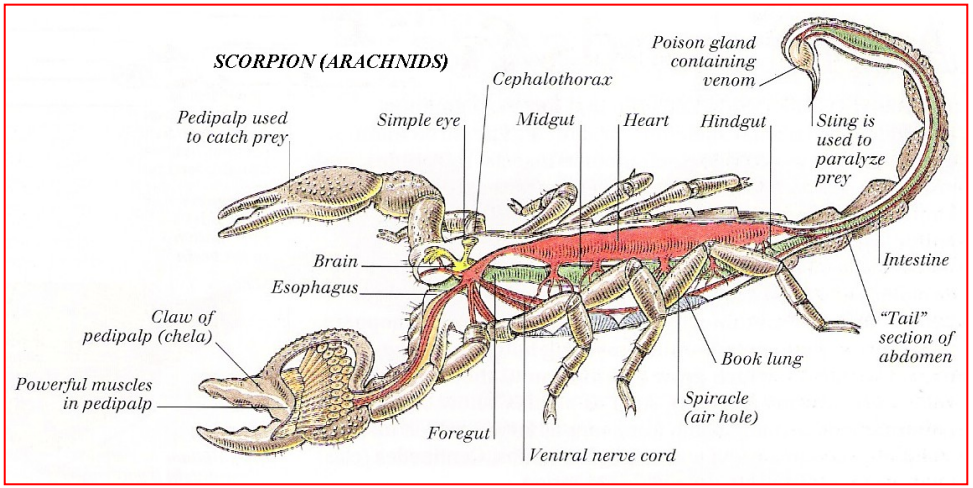


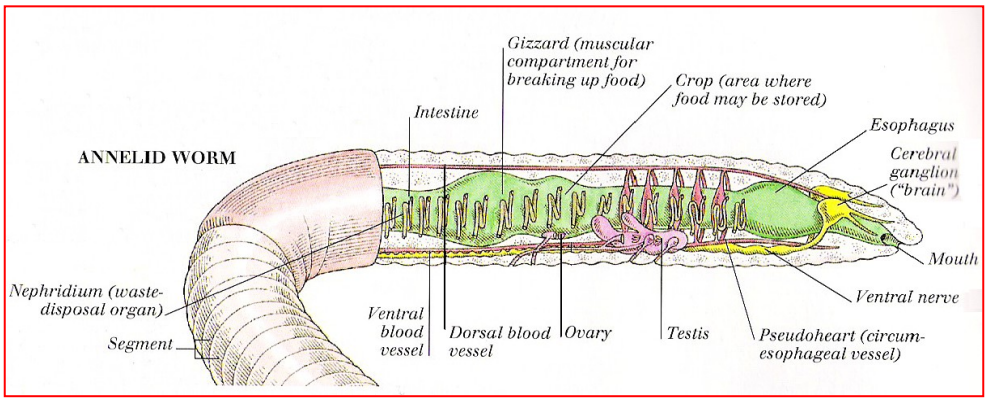


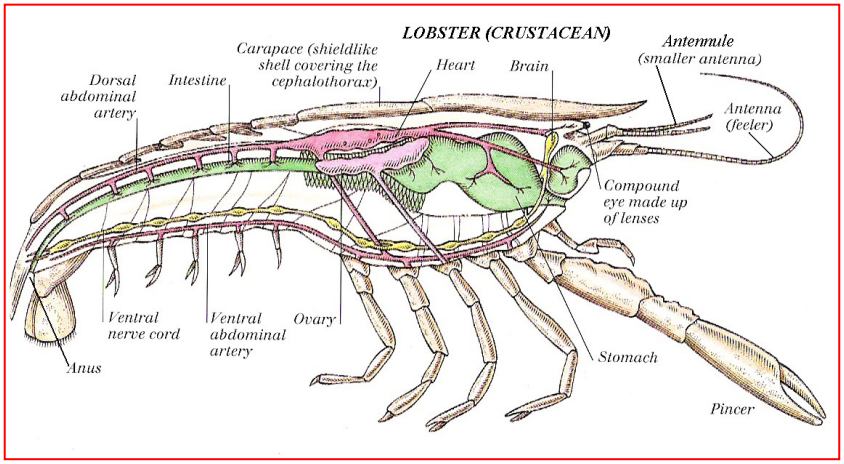


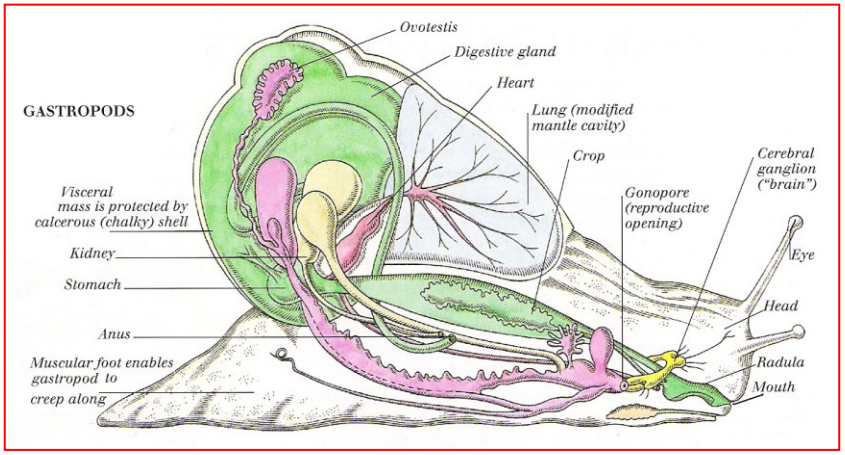


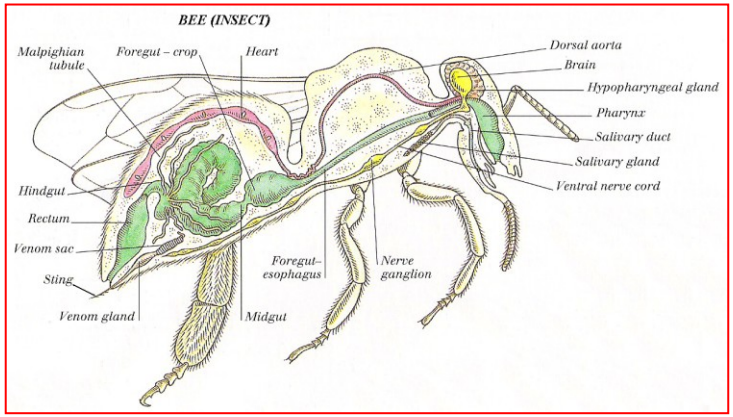

STARFISH (ECHINODERM)
Anus opens on aboral (upper) surface
Short intestine connects stomach to anus
Tube foot
Stomach
Mouth opens on oral (lower) surface
Madreporite connects water vascular system to the outside
Ring canal
Lateral canal
Ampula
Gonopore (opening of gonad)
Gonad (reproductive structure produces eggs or sperm)
Pyloric cecum, or digestive gland, aids digestion and distributes food to the arms


SCORPION (ARACHNIDS)
Poison gland containing venom
Cephalothorax
Pedipalp used to catch prey
Simple eye
Midgut
Heart
Hindgut
Sting is used to paralyze prey
Brain
Esophagus
Intestine
Claw of pedipalp (chela)
"Tail" section of abdomen
Book lung
Powerful muscles in pedipalp
Spiracle (air hole)
Foregut
Ventral nerve cord


ANNELID WORM
Gizzard (muscular compartment for breaking up food)
Crop (area where food may be stored)
Intestine
Esophagus
Cerebral ganglion ("brain")
Mouth
Nephridium (waste-disposal organ)
Ventral nerve
Segment
Ventral blood vessel
Dorsal blood vessel
Ovary
Testis
Pseudoheart (circum-esophageal vessel)


LOBSTER (CRUSTACEAN)
Antennule (smaller antenna)
Carapace (shieldlike shell covering the cephalothorax)
Heart
Brain
Dorsal abdominal artery
Intestine
Antenna (feeler)
Compound eye made up of lenses
Ventral nerve cord
Ventral abdominal artery
Ovary
Stomach
Anus
Pincer


GASTROPODS
Ovotestis
Digestive gland
Heart
Lung (modified mantle cavity)
Crop
Cerebral ganglion ("brain")
Gonopore (reproductive opening)
Visceral mass is protected by calcerous (chalky) shell
Eye
Kidney
Stomach
Head
Anus
Radula
Muscular foot enables gastropod to creep along
Mouth


BEE (INSECT)
Malpighian tubule
Foregut – crop
Heart
Dorsal aorta
Brain
Hypopharyngeal gland
Pharynx
Salivary duct
Salivary gland
Ventral nerve cord
Hindgut
Rectum
Venom sac
Sting
Venom gland
Foregut–esophagus
Nerve ganglion
Midgut

Organic Chemicals, Dow Chemical and Dupont have their own in-house testing facilities and closed colonies of test animals. However, there are many other companies providing data for their products via contracted research establishments using "white laboratory" mice or rats of various origins.

T10.6. Historic Evolution of animal species required for toxicity testing.

	Acute Toxicity	Sub-Acute Toxicity	Chronic Toxicity
FDA/AMA 1943	Three or more	One or more	Three or more
FDA 1963	Four, one non-rodent	Two, one non-rodent	Two, one non-rodent
ESSDT 1965	Three or more, including rat & dog	Two, usually rat & dog	Two, usually rat & dog
PMA 1977	Three or more, one non-rodent	Two or more, one non-rodent, usually rat & beagle dog	Two or more, one non-rodent, usually rat & beagle dog
EEC 1983	Two or more. If no sex diff. in first, 1 sex only in 2nd	Two or more, rodent & non-rodent, or selected on similarity to man	Two or more, rodent & non-rodent, or selected on similarity to man
FDA-Level 1 2000		1 rodent	Unspecified
FDA-Level 2 2000	1 rodent	rodent & non-rodent	1 rodent
FDA-Level 3 2000	1 rodent	1 non-rodent	rodent & non-rodent

European Society for the Study of Drug Toxicity / Pharmaceutical Manufacturer's Association / European Economic Community / Food and Drug Administration

Number of Animals Used for Scientific Purposes in 1975 and 2002

(% of Total Animal Use)

SPECIES	1975	2002	% change
Mice	1,174,350 (43,51%)	759,790 (36.1%)	-35%
Rats	594,678 (22,03%)	332,065 (15.78%)	-44%
Birds	293,563 (10,88%)	117,958 (5.60%)	-60%
Fish	130,984 (4,85%)	607,367 (28.87%)	+463%
(Sub-total)	**2,193,575 (81,27%)**	**1,817,180 (86.35%)**	**-17%**
Guinea Pigs	69,268 (2,57%)	28,659 (1.36%)	-59%
Rabbits	114,822 (4,25%)	14,374 (0.68%)	-87%
Hamsters	13,830 (0,51%)	5,100 (0.24%)	-63%
Dogs	33,431 (1,24%)	9,518(0.45%)	-72%
Cats	10,087 (0,37%)	3,561 (0.16%)	-65%
NHP	4,728< (0,18%)	2,109 (0.10%)	-55%
(Sub-total)	**246,166 (9,12%)**	**63,321 (2.99%)**	**-74%**
Others	259,259 (9,61%)	222,634 (10.66)	-14%
Total	**2,699,000 (100 %)**	**2,103,135 (100%)**	**-22%**

T10.7. Changes in species used in toxicity testing.

The reproducibility of results from the single animal type is then lost. As a result, the LD_{50} is often presented as a range, e.g. 335-1050 mg/kg (mouse). This usually indicates that two or more experimentally determined values using different strains of either mice or rats were conducted, making it more difficult to define toxicity precisely, but easier to appreciate its range.

The LD_{50} reported typically have no notation concerning strain, age, and sex of the species used.

F10.8. 2000 Great Britain Scientific Procedures on Living Animals.		
Categories	**Numbers**	**Change Since 1999**
Total number of procedures on animals	2,714,726	UP 2%
Total number of individual animals used	2,642,993	UP 3%
Procedures on cats	1,813	UP 12%
-in respiratory & cardiovascular research	1	DOWN 95%
-in nervous system or special senses research	294	SAME
-in toxicity tests	195	DOWN 29%
Number of individual cats	613	DOWN 10%
Procedures on primates	3,690	DOWN 8%
-in toxicity tests	2,563	DOWN 6%
-in nervous or special senses research	514	DOWN 27%
-in respiratory or cardiovascular research	224	DOWN 36%
Number of individual primates	2,951	DOWN 7%
Procedures on dogs (beagles: 6,872; others including crossbreds:760)	7,632	DOWN 7%
-in respiratory & cardiovascular research	1,300	UP 1%
-in toxicity tests	3,956	DOWN 24%
Number of individual dogs	4,745	DOWN 20%
Procedures on mice (59% of total)	1,606,962	DOWN 2%
Procedures on amphibians	15,574	UP 7%
Procedures on rabbits	39,683	DOWN 4%
Procedures on birds	124,209	UP 17%
Procedures on horses, donkeys or crossbreds	9,272	DOWN 1%
Procedures on fish	243,019	UP 98%
Procedures using transgenic animals	581,740	UP 14%
Procedures using animals with a harmful genetic defect	256,937	UP 2%

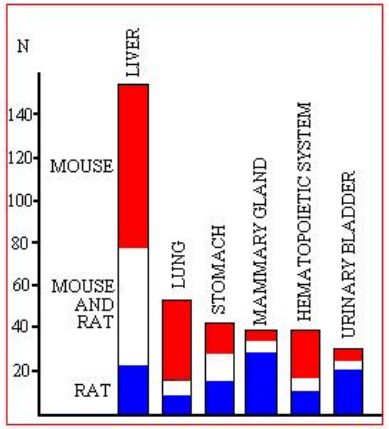
In the pharmaceutical industry, drug-target validation and preclinical drug development are performed almost exclusively using inbred mice and rats. This affords substantial savings by increasing data *reproducibility* because of the small genetic heterogeneity of the animals. However, these savings may be offset by failed clinical trials, sometimes reflecting the inadequacy of inbred animals to model genetic variations in humans. The solution could include using mouse strains closely related to wild mice and, in the future, comparative genomics of the drug targets in humans and mice.

But fundamental interspecies differences do occur occasionally (TF10.9), leading to false positives or negatives from animal models: alpha 2u-globulin, for example, is specific to male rats. Tubular hypertrophy and regeneration is specific to alpha 2u-globulin and is not relevant to humans.

AGENTS	CANCER	SPECIFICITY
Ethylene bisdithio-carbamate Fungicides Amitrol Goitrogens Sulfamethazine	Thyroid gland tumors	**Rats** show decreased thyroid hormone half-life, increased thyroid stimulating hormone level. **Humans** have thyroid-binding protein, and so develop no tumors.
Unleaded gasoline 1,4-Dichlorobenzene D-Limonene Isophorons Dimethyl-methylphosphonate Perchloroethylene Pentachloroethane Hexachloroethane	Renal tumors	**Male Rats**: chemicals bind to a_{2u}-globulin, which accumulates in kidney cells, leading to necrosis, regenerative hyperplasia, renal tubular calcification, neoplasia.
		Female Rats and **Humans**: do not have a_{2u}-globulin, and so develop no tumors.

T10.9. Rat tests can be irrelevant to human health. *Modified from Casarett & Doull*

F10.10. Positive Response for Carcinogenicity in Rats and Mice for Selected Target Sites. *Zbinden.*



The choice of animal model can be critical: there is only 70% correlation in general carcinogenicity between rat and mouse.
By target site, the situation is even

worse (F10.10).
Observation implies that differences between species are mostly *quantitatively based* on toxicokinetics in uptake, distribution, metabolism and clearance, rather than on fundamental differences.

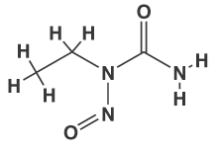
10.2.2. Sex Bias

Sex biases are based on practical concerns.
Males are larger, (easier to attach and implant things into) and do not have estrous cycles (that complicate pharmacology).
Females are easier to handle, smaller (drug saving) and cheaper. They are preferred in dermatology because they fight less, simplifying skin observations (wounds and scars).

Concentrating on one sex can be viewed as an advantage because it builds a larger database on a single target.
A stronger bias than the sex bias is the bias against children. It is not seen as ethical to experiment on them. So children are viewed as small adults, although their metabolism is different.

10.2.3. Mouse Strains

90% of research mammals are mice, because of their small size, quick breeding and easily discernible variations.
Mouse research started in the early 1900s as a model for Mendel's genetic theories. Later, breeding of brothers and sisters for 20 generations led to a homogenous breed, the DBA mouse, that would not reject tumor homografts, a useful trait for cancer research.
Although some warned that the inbreeding reduced their value as models, many other inbred strains became popular: C57BL, BALB/c, C57BR, "A" and 129. By the 1950s, the mapping of genes in mice had developed, and the nuclear era brought fine structure mapping of the genome because of the systematic observation of large deletions and translocations.
Ethylnitrosourea (shown), ENU, was used to produce single point mutations on the mouse genome. In 1962, the *nude* mouse, without a thymus and lacking T- and B-cells, was used to study human tumor xenografts (implantation of human tumors into mice). The late 70s and 80s brought recombinant DNA techniques to create *transgenic mice*, and later more than 1000 types of knockout mice (where a specific gene is silenced).



In some cases, mouse models do not sustain close scrutiny. A favorite model in which to study *ras*-associated cancers has been the mouse. However, it has recently been found that *ras*-associated cancers are triggered by different pathways in mice and humans, rendering two decades of work useless[5].

Specific animal models can be examined using genetic techniques to reveal the molecular details of, for example, cancer. However, for breast cancer alone, there are at least 100 animal tumor models in use. Investigating them in detail will take considerable time.

10.2.4. Standardized Rats

20 or more generations of brother-sister matings among rats leads to low phenotypic variance with more consistent experimental results. Inbred rats tend to become too susceptible to specific cancers (for example, F344/N rat and B6C3F1/N mouse). Genetic drift causes body weights to increase and survival rates to decline in rat colonies. This is deleterious in particular to 2-year carcinogenicity studies.

- Inbred rats are better in experiments aimed at clarifying mechanistic principles.
- Outbred rats present a genetic variability similar to human populations, but larger numbers are then required for statistically appropriate data.

The corporate Global Alliance for Laboratory Animal Standardization (F10.11) maintains the genetic profile of its rats by examining polymorphism frequency for 17 loci on 9 chromosomes. Each colony is resampled and monitored annually to verify genetic consistency. Mating of closely related animals is minimized by a rotational breeding system. Each of the 4 breeding colonies in the world are restarted every 5 years from the original source colony or from cryo-preserved embryos.

The genetics of the rat or mouse itself is not the only concern. The large intestine of mammals is populated with hundreds of types of bacteria. Bacterial diversity increases from carnivory to omnivory to herbivory. Conventional wisdom is that they help the organism absorb nutrients that would otherwise be indigestible, manufacture vitamins, kill germs, neutralize bacterial toxins and modulate the immune system.

By contrast, farmers have known for a long time that some amount of antibiotics in the feed improves weight gain by limiting bacterial biomass.

But bacteria may have more complex interactions with the body by turning on specific intestinal genes, promoting the growth of blood vessels in the gut and triggering production of chemicals that control bacterial populations.

It is possible to create germ-free rodents using antibiotics, thereby providing a *minimalist* bio-system.

However, these rodents must consume 30% more calories to maintain normal body weight, and are unusually susceptible to infections. They also have a poorly formed intestinal network of capillaries. The need for some bacterial flora, which is difficult to control, renders the goal of a monotypical test animal even more difficult to reach and maintain.

10.2.5. Genetically Modified Animals

Over three decades, the conventional rodent bioassay has been the standard for identifying carcinogens. But mice can be mutated by gene insertion or inactivation to more closely resemble human models. This has been achieved for human diseases such as colorectal cancer and sickle cell anemia. Roughly 7500 mutant mouse strains have been created.

10.2.5.1. Transgenic Mice

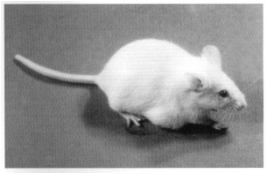
Transgenic mice may improve accuracy and efficacy because they respond to carcinogens *more quickly* than conventional rodent strains, and they may provide more *mechanistic information*. These animals have specific genes added to their normal genome by microinjecting DNA into the pro-nuclei of one-celled embryos. Typically, “transgenic” mouse models carry activated oncogenes or inactivated tumor suppressor genes (F10.13) involved in neoplastic processes. After exposure to chemicals in the animal, the added gene can be extracted from the DNA of various tissues of the animal (retrievable mutation shuttle vectors) and tested for integrity (absence or presence of mutation). Transgenic rodents (F10.14) can substantially **speed** *in vivo* toxicology testing, but have been found to lack sensitivity, detecting only a few of the known carcinogens.

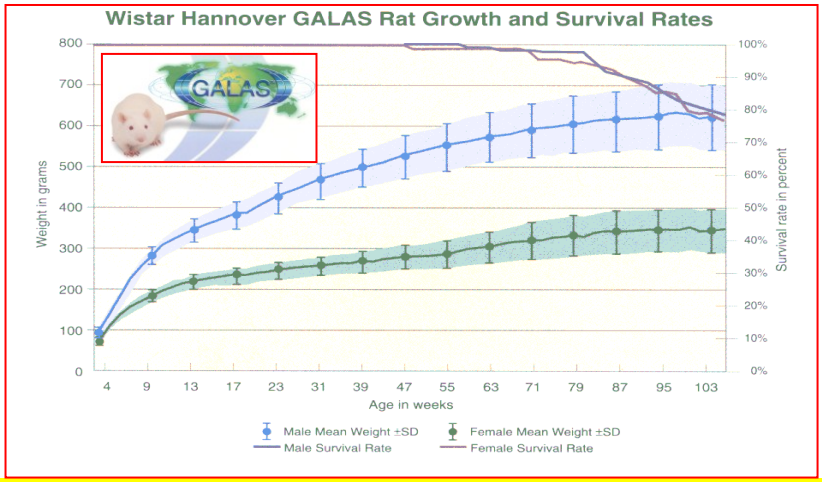
Stratagene introduced the Big Blue Transgenic Mouse to assess mutagenic potential of test compounds. The assay is an alternative to tests such as the two-year animal bioassay proscribed by the National Toxicology Program (NTP). The NTP bioassay involves the dosing of a large number of animals with an endpoint of tumor formation. In contrast, the endpoint in the Big Blue assay is *mutation of a test gene*, and it only takes *three to four months*. Integrated into the chromosomes of the Big Blue mice is a retrievable lambda phage shuttle vector containing the bacterial lacI gene. The lacI gene serves as a reporter gene for mutation that can be retrieved from any organ of the animal to assess mutation frequency in that tissue.

After a few weeks of exposure to different doses of the test compound, the transgene is shuttled into bacteria, where

mutations in lacI can result in a blue plaque phenotype when plated on bacterial lawns.

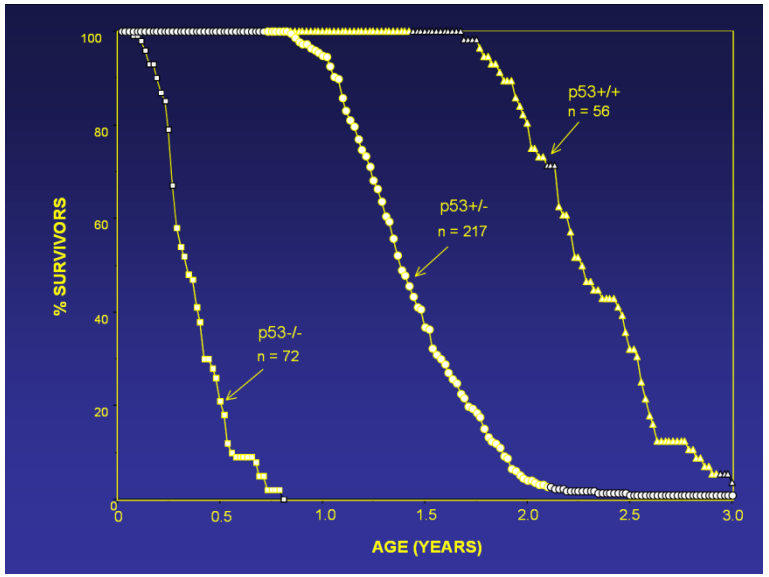
F10.11. Harvard Medical School's Oncomouse, looking for patent protection. The various flavors of the mouse have a single transgene (ras, c-myc, c-neu, SV40) and promoter inserted into an normal genome for the study of various cancers.





F10.12. Creating and marketing genetically controlled rats: Wistar Hannover GALAS rat.

A typical transgenic model is the Tg.AC mouse, which carries the *v-Ha-ras* transgene, one of a class of oncogenes that plays a key role in cell proliferation in the early stages of tumor induction. The Tg.AC mice would allow detection of both genotoxic and non-genotoxic agents.
These models will probably seem naive in a few decades, because the methods probably underestimate the complexity of toxicity processes. The problem is that genetic systems are themselves complicated, and that toxic reactions are difficult to predict purely from genetic knowledge.



F10.13. The effect of loss of p53 genes on mouse survival.
Donehower LA, 2003.

10.2.5.2. Knockout Animals

Knockout animals (we now have over 1000 knockout rodents available) are transgenic animals in which specific genes have been disabled in order to assess separately the impact of their removal.
Almost all genetic models are designed to detect cancer or mutations. F10.13 shows survival data from three C57B6 mice experiments. In animals with two p53 genes (p53 +/+ or "wild type"), 50% of the mice die of cancer. When one (p53 +/-) or two (p53 -/-) copies of p53 are removed, survival is considerable shortened, and almost all mice die of cancer.
It is believed that the p53+/- mouse would be more sensitive to genotoxic agents. Note that mice that express excess p53 have low incidence of cancer, but short life-spans[14].

These new techniques adds to the richness, but also to the complexity of the dataset. But actually, the genetically

manipulated mice may contribute the most knowledge in relation to their own mutation. Not one has yet contributed to produce a drug that cures a disease. Furthermore, knocking out a gene can have one effect in one strain of mice, but a different one in another strain[7]. The availability of genetic information is an irresistible draw, and the pharmaceutical industry is gearing up to use bioinformatics to find drug targets (F10.16).

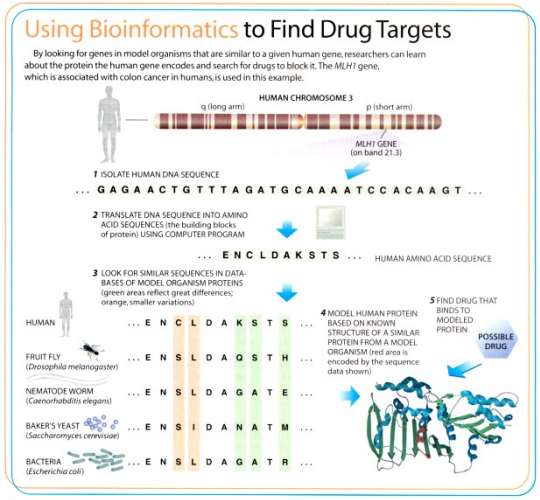
F10.14. Studying Mutations with Transgenic Rodents.

MOUSE	GENE INSERT	DETECTION OF MUTATION
BigBlue	LacI (bacteriophage λ)	Phage particles are plated on a bacterial host in the presence of Xgal. Wild type lacI gene will produce a clear bacteriophage plaque, while those that have sustained a mutation in the lacI gene will produce a blue plaque.
MutaMouse	LacZ (bacteriophage λ)	Phage particles are plated on a lacZ- E.coli strain in the presence of X-gal. Wild type lacZ will produce blue plaques and lacZ mutant clear plaques.
lambda cII	CII (bacteriophage λ)	The cII protein induces the integration of the lambda DNA into the bacterial genome, and this results in lysis. A mutation in the cII gene results in growth of lambda particles and lysis of the cell.
gpt-delta	gpt with the plasmid pYG144 (bacteriophage λ)	*gpt* gene is sensitive to point mutations. *rad* and *gam* are sensitive to deletion mutation. Phage particles are plated on E. coli YG6020. This results in excision of the plasmid from the bacteriophage DNA, and creation of a plasmid. Plating on plates with 6- TG selects for mutants in the *gpt* gene. When the phages are used to infect XLI Blue MRA (P2) cells, only those particles that are missing the *rad* and *gam* genes will be Spi-, which means that they form plaques on the P2 lysogen (Spi = Sensitive to P2 Interference).
PhiX –174	X174 am3 (bacteriophage ϕ)	Reversion assay at the A:T base pair, am3 site.
SupF	SupF (bacteriophage λ)	supF mutants do not suppress amber mutations in the lacZ gene in the host cell.
RpsL	rpsL, confers streptomycin sensitivity (from E. coli)	Recover plasmid from animal cells by electroporation. Plasmid with a mutated rpsL gene will result in colony formation on streptomycin plates.

F10.15 . Studying Carcinogenicity with Transgenic Rodents.

TRANSGENIC MODEL	GENETIC CHANGE	APPLICATIONS
TSG-p53	deficient in p53	Tumor biology, potential carcinogens
v-Ha-ras	v-Ha-ras fused to fetal zeta globin promoter	Initiated skin for non-genotoxic carcinogens
K6-ODC	Expresses ornithine decarboxylase	Low-dose chemical risks
rasH2	C-Hras with promoter	Rapid onset, higher incidence of tumors (agiosarcomas, lung adenocarcinomas)
Mdr1a	Disrupted Mdrla	Blood-brain barrier deficiency
Mdrla/b	Disrupted Mdrla/b	Penetration of drugs in brain, testis, ovaries, adrenal glands
pim-l	More pim-l oncogenes	Lymphomas by chemicals
TSG-p53/BigBlue	lacI, p53 knockout heterozygous	Screening of mutagens and tumorigens
Xpc	Xpc knockout	Skin carcinoma after UV

F10.16. Using Bio-Informatics to find Drug Targets.



People directing the *human genome project* foresee that 100 years will be needed to take advantage of the information gathered. Genetically modified animals are still restricted from the point of view of general toxicology.

10.2.6. Classic Types of Animal Tests

F10.17. Classical Animal Toxicity Study types.				
STUDY	**DURATION Recuperation**	**DOSE**	**TESTS**	**ANIMAL**
Acute	0.5 month/ recuperation	Single dose & day	LD_{50}	Mouse, rat/M&F
			Draize/Skin & Eye	Rabbit
Sub-Acute	0.5-1 month/r	Repeated	Many	Rat
Sub-Chronic	1-3 month/r	Repeated	Many	Rat & Dog
Chronic	3-18 month/r	Repeated	Many	Rodent & Non-R?
Carcinogenicity	24 month	Repeated	Focused	Rodent

Each type of animal study prepares the next level, to insure that large sums are not invested in studies using the wrong doses, for example.
Some important aspects of animal testing have been very poorly controlled until now. In an effort to minimize test costs, mouse chow has traditionally been obtained from low cost sources (Purina LRD5001). Unfortunately, such food contains 36 times more arsenic than the EPA recommends for drinking water (10 ppb)[16]. Since these foods also contain cadmium, chromium, copper, lead and zinc, the unfortunate toxicologist looking for low-level effects will be completely and unwittingly blinded: how can he detect the effect of 10 ppb of arsenic if the animals are subjected to a change from 400 to 410 ppb?

10.2.7. Acute Tests

Objectives:
1. Quantitative determinations of LD_{50}, skin & eye irritation thresholds.
2. Identification of target organs and clinical manifestations.
3. Establish the reversibility of toxic responses.
4. Provide dose ranges for further (longer) studies.

Acute studies administer single, large doses of an agent to an animal. The route of entry should be as representative of likely human exposure as possible.
Acute skin irritation studies are often performed in rabbits for 4 hours of contact on a shaved back.
Acute inhalation studies are often also performed over 4 hours.

The effects are often dramatic, but frequently, the jumble of signs and symptoms are not useful in determining mechanisms of action or of assistance in determining possible therapies. Acute toxicity experiments can be a frightening experience, with effects ranging from death to severe, transient incapacity and illness to very mild signs and symptoms. There are situations where an acute, high level, exposure might result in the delayed appearance of some form of toxicity (hepatic or renal function, neurotoxicity, etc).

Given the diversity of responses to chemicals, what endpoint would be common to all? Death is the only clear choice. This determines the risk to human handlers of the chemical and allows comparison of the potency of one chemical with that of another (T1.9). Physicians need to know the LD_{50} as they cope with symptoms in a patient.

> *LD_{50} (median lethal dose) for acute oral toxicity* is the statistically derived single dose of a substance that can be expected to cause death within 14 days in 50 per cent of young adult albino rats when administered by the oral route. The LD_{50} value is expressed in terms of mass of test substance per mass of test animal (mg/kg).

The safe handling of chemicals in an industrial setting depends on adequate information on lethal dosages, as does transport of such agents and even environmental exposures. We have all seen media reports showing derailments of tank cars containing chemicals that threaten (real or imaginary) the safety of a community. LD_{50} is also essential to police and firefighters engaged in controlling accidents, in patrolling or cleaning up a contaminated area or in combating industrial fires.

Today, the move is toward obtaining an estimate of the relative potency using, essentially, a range-finding study with *only a few animals*. This is hardly a new concept, an extensive study published in 1948 showing that most values obtained in this manner were within 30% of the LD_{50} obtained by the more classical study approach.
Is this sufficiently accurate for most purposes? Canadian, U.S.A., and EC regulatory agencies say so, and no longer require that full LD_{50} studies be done.

10.2.8. Acute Toxicity Test Techniques

10.2.8.1. Classic LD_{50} (Trevan, 1927)

The Classic LD_{50} test determines the lethal dose (LD_{50}) of a substance that kills 50% of test animals. It typically uses 100 or more animals.
The substance is administered in increasing doses, usually 5 or more, to groups of 10 male and 10 female animals. Mortalities are recorded within a given period, and the LD_{50} is determined with statistical calculations.

10.2.8.2. Organization for Economic Co-operation and Development (OECD) Test Guideline 401 (1987)

Acute oral toxicity is estimated with three dose groups of five rats of one sex, with confirmation in the other sex using one group of five rats. This reduced the minimum number of animals used for acute oral toxicity to about 20.

10.2.8.3. Up & Down

In the Up and Down Procedure, one animal is orally given an appropriate dose (175 mg/kg is the default starting dose) and observed for up to 14 days.
If the animal is alive at 48 hours after treatment, a second animal is orally given a preset higher dose (0.5 log spacing or 3.2 times more, by default).
If the first animal dies, then the second animal is dosed at a preset lower dose (0.5 log spacing by default). Dosing stops when one of three stopping criteria is satisfied, with as few as six, but not more than 15 animals used per test.

10.2.8.4. Limit Test

The limit test is used to determine if the toxicity of a test substance is above or below a specified dose. Five to ten animals of each sex or 10 animals of the susceptible sex are administered a dose specified by regulations. Toxic responses occurring within a given period are recorded. Based on the results, a regulatory action or additional testing may be required.

What is sacrificed by not conducting the Classic LD_{50} study? More precision on the LD_{50} value and a more solid knowledge of the morbidity associated with the agent (because of the increased number of "cases" observed).

In the classical LD_{50} determination (5 treatment dose levels, 10 animals per sex per treatment), there are usually 30 to 40% of the animals surviving. Those surviving at high dosage are more valuable than those dying, since it gives the investigator the opportunity to record:

(1) the persistence of toxic effects,
(2) the development of late toxicities.

10.2.9. Subchronic Tests

The goals are:

- to establish a NOAEL and a dose-response and
- to detail organ alterations at various levels of toxicity.

Workers are typically exposed to potential toxicants repeatedly, at levels far below those of acute toxicity. Is there any cumulative effect from repeated exposure?
There is a need to examine short-term (subchronic) and long-term (chronic) toxicity of the agents at exposures much lower than those causing acute toxicity.
In acute toxicity, there is rarely opportunity to determine mechanisms of action, since the observed effects arise from a general overwhelming of the animals' body systems. General failure is not the ideal situation to identify mechanisms. Logically, at lower dosages, one should be able to correlate more moderate signs and symptoms of toxicity with particular target organs.

The longer toxicity studies last, the more complicated t**oxicokinetics** (absorption, distribution, metabolism, excretion) and **physiological adaptations** become: changes in membrane permeability, specialized membrane transport, synthesis of binding molecules, up- and down- regulation of receptors in excitable cells, cytokine transduction, gene expression and transcription, cell populations.

The **duration** of a subchronic study may vary from agent to agent, but is usually 21-90 days. Shorter times may be counteracted by higher dosages, e.g. a high level might show the adverse effect more rapidly than a low level.

The **exposure** is always via the route by which the human might acquire the agent. A minimum of three dosages (highest dose not more than 10% of the LD_{50}) plus the control group should be used, based on the results of acute toxicity data. The investigator hopes to obtain a graduation in effect with dose. The highest level of exposure must produce overt toxicity, without altering normal physiology unduly.
If sub-chronic tests have already been performed, perhaps the **Maximum Tolerated Dose** is already known. This is the dose that leads to a 10% decrease in animal weight, compared to controls, at 3 months.

What **endpoints** of toxicity should be examined in sub-chronic studies? Although one target may dominate your observations, you should cover multiple organs and rely on a broad range of toxic endpoints. The biomarkers used by the toxicologist range from simple parameters as growth and development (sensitive yardsticks of general wellbeing in small animals) through hematological and biochemical measurements, to microscopic examination of tissue morphology at termination of the animals' lives.

- Dead are necropsied.
- Animals examined daily: body weight, diet, fur, respiratory or cardiovascular distress, motor, behavioral problems, masses.
- Gross and microscopic condition of all ±12 major organs of all animals.
- 3 instances of complete hematology. Hb, hematocrit, erythrocytes, total & differential leukocytes, platelets, clotting time, prothrombin time and blood chemistry (glucose, Ca, K, Urea nitrogen, alanine aminotransferase, serum aspartate aminotransferase, transpeptidases, dehydrogenase, phosphatase,

creatinine, bilirubin, triglicerides, cholesterol, albumin, globulin, total protein).
- Urinalysis: specific gravity, pH, glucose, ketones, bilirubin, urobilinogen.

From such a study, a NOAEL may be obtained leading with the proper safety factors to establishment of the Reference Dose which may establish regulatory limits (EPA) of human exposure.

Seemingly straightforward biological markers can prove misleading. Some drugs developed to fight osteoporosis increased bone density, but also increased the risk of fractures. Former heart drugs encainide, flecainide and morcizine normalize irregular heartbeats, and were once prescribed to hundreds of thousands of patients. Long-term trials later proved that death rates for drug users were 2.5 higher than for controls.
It is very difficult to tie specific biomarkers firmly to clinical outcomes.

The **reversibility** of the observed toxicity is an important aspect frequently overlooked in both animal and human studies. Patients and physicians want to know how long this illness will last, e.g. the prognosis of recovery. Animal studies are designed so that, at the end of the treatment period, sufficient animals remain to be allocated to a recovery study, to see whether the effects of the toxicant are completely reversible, partially so, or irreversible. While this is useful information to obtain, few toxicological studies provide this type of information.

Little will be seen in **MSDS sheets** (T1.24) on the subchronic and chronic toxicity of many chemicals, because such studies have not been conducted.

10.2.10. Chronic Tests

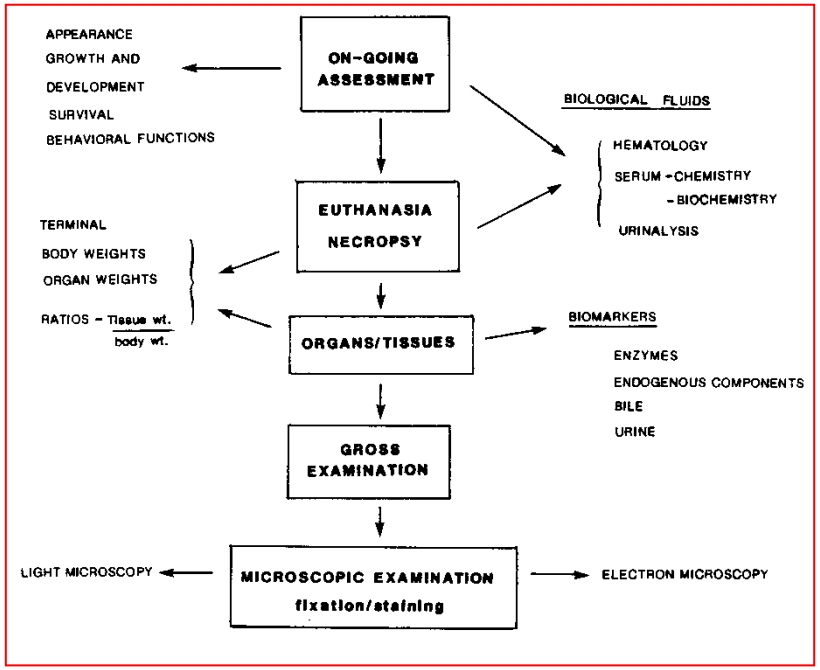
The goal is a complete assessment of the long-term toxicity of a chemical. In this context, feeding practices are important.

Larger lab animals are fed at meals, while rodents are commonly fed *ad libitum* (constant access to food in unlimited quantity) which gives them higher blood fat, higher, cholesterol, and greater incidence of heart damage and cancer, a diabetic-type model, compared to the meal-fed rodents, which have enhanced insulin sensitivity[18]. Meal-feeding is "an important synchronizer of many behaviors and biological rhythms," and appears to alter the expression of genes that code for proteins involved in the rate and amplitude of biotransformation.

The public and the workforce are concerned with effects of long-term low level exposures to toxicants in food, air and water. Chronic toxicity studies conducted in rodents are nothing other than extensions of the subchronic studies.
The same endpoints of toxicity are monitored in chronic studies as in subchronic studies, but there are specific aims to such studies:

- administer the toxicant for a **major portion of the lifespan** of the animals,
- demonstrate a **dose-effect relationship**,
- provide data that can be used to **extrapolate safe humans doses.**

While a two-year rodent study will represent somewhere between 80 to 99% of the lifespan of the laboratory rat, a study of the same duration in higher species is only a fraction of the lifespan. A 24-month study in the dog covers 20% of its lifespan. The exorbitant costs associated with prolonged studies has led industry to press the regulatory agencies to reduce the duration, first to 12 months and, more recently, to 6 months for rodents. Two-year studies are still mandatory for dogs, the usual non-rodent species. The case is made that, by adjusting dosages upward, one can attain, in 6 to 12 months, the levels of exposure encountered in 24 months, and that toxicity produced in the 6-month study is the same as that found in a much longer study.



F10.18. Variables measured in subchronic and chronic studies to assess the general health of the test animals and to monitor the appearance of adverse health effects.

You can certainly spot the weakness in such an argument: excessively high dosages, problems in the normal biotransformation and elimination of the high levels of the toxicant and any reactive intermediates, the young age of the animals (when most chemical-related toxicity appears in mature humans), etc.

However, the regulatory agencies have accepted the shorter studies. In spite of this, one still sees classic two-year studies in the literature.

As with the subchronic studies, the **dose-effect** relationship should be developed by proper selection of doses. Furthermore, euthanizing representative subgroups of animals at selected time intervals throughout the treatment period will help in documenting the initial stages and the development of the toxic lesion. This allows in depth exploration of possible mechanisms of action.

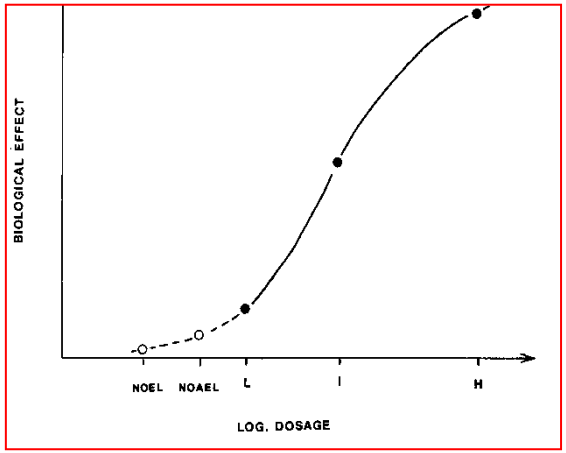
As in the subchronic study, sufficient animals should remain at the end of the treatment period in order to study the **recovery** from the toxic insult, to answer questions about the reversibility or permanence of the lesion.

The NOAEL (No Observable Adverse Effect Level) and the NOEL (No Observable Effect Level) are desirable endpoints of chronic studies. Occasionally, the lowest dosage may be close to provide one of these values (F10.19).

Few MSDSs contain information about chronic toxicity. In most cases, any evidence of chronic toxicity has been obtained from epidemiological studies of occupationally-exposed individuals. Relating specific endpoints of toxicity to many years of estimated exposures often involving a mix of agents is difficult.

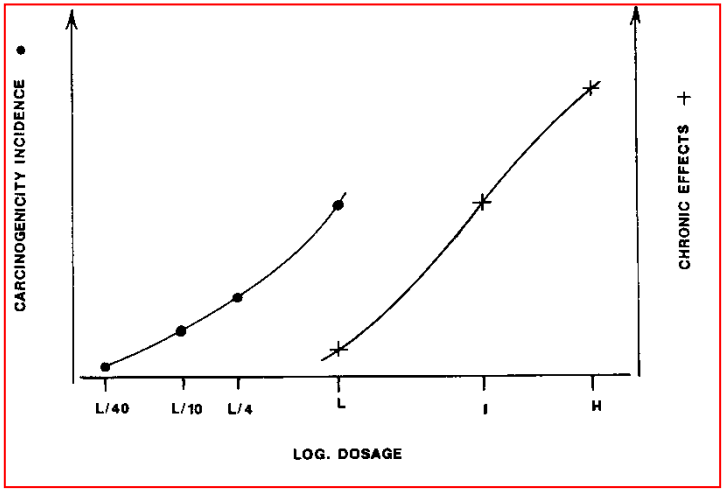
10.2.11. Carcinogenicity Tests

Cancer-causing agents are usually effective only after prolonged exposure. Since 24-months of exposure is necessary to allow the development of some tumors in rats, shortening chronic studies to 6 months would likely miss the carcinogenic potential of a chemical. Very few chemicals produce tumors in young animals, bis-chloromethyl ether being one exception, capable of causing malignant tumors within approximately 90 days. Regulatory agencies demand 24-month or lifespan carcinogenicity studies in rodents (mice, rats, hamsters) to support submissions for the registration of chemicals for importation, exportation, and sale. This means that the chemical industry (pharmaceuticals, pesticides, solvents) is committed to providing study data on the carcinogenicity of their agent and to bear the cost of such studies.



F10.19. Dose-effect relation based on a 3-level exposure chronic study, with approximate derivation of the No Observed Effect and No Observed Adverse Effect Levels.

The endpoint is tumor (benign, malignant) formation. Since carcinogenesis may occur at much lower levels of exposure than those required to demonstrate other types of chronic toxicity, such studies typically use low levels of exposure (F10.20). Given the large costs involved, the carcinogenicity study and chronic toxicity study are often incorporated together, with an extended dosage range (5 or 6 dosages rather than 3). Those animals on the higher dosages will be terminated at the 6-month time interval (except those for the recovery study), while those at the lower dosages will be continued for the required 24-month period, examined only for tumor incidence (site, number, benign or malignant). It is possible, though unlikely, that tumors might be seen in some of the "chronic toxicity" animals exposed to higher levels of the toxicant.



F10.20. Theoretical results for a combined chronic toxicity/carcinogenicity study where additional groups of animals were exposed at 25% of the low dose (L/4), at 10% of the low dose (L/10) and 2.5% (L/40).

However, for many chemical exposures, the potential carcinogenicity is not clear-cut, the results of the various studies being equivocal, some being positive, some negative, and some inconclusive.

10.2.12. Other Types of Tests

-**Sensitization (immune) study**: guinea pig skin, administration of toxicant over 2-4 weeks, sensitization check 2-3 weeks after the last exposure.
-**Sub-acute study**: 14 days of exposure, 10 animals per sex in rodents. Clinical chemistry and histopathology, 14 days of observation after exposure.

10.3. Human Tests

Epidemiology uses existing human exposures to assess health impacts. Under many circumstances, the available data is insufficient. Also, epidemiology cannot provide data on chemicals not yet introduced, since there is no human exposure. To get data on new drug candidates, for example, some humans must be exposed at some point.
A company can make a new drug, and test it in test tubes and animals without telling anyone. Test-tube and animal tests are *preclinical*: they come before testing the new drug in humans. Legally, the company just needs to mark the packages, "Caution: Contains a new drug. ...Not for use in humans." Since the company's aim is to someday sell the drug for use in humans, it designs *preclinical tests* (*in vitro* and in animals) to get permission for the next testing stages.

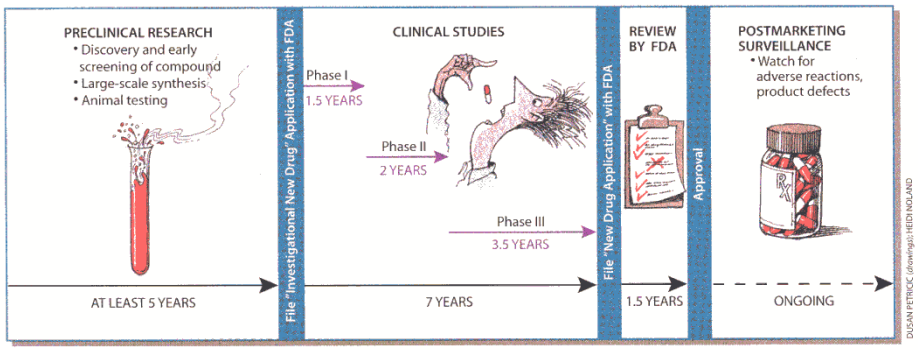
Successful preclinical testing (F10.21, left) shows that a drug is unlikely to poison humans. Over 90% of drug candidates effective in test tubes fail in animal studies. The drug does not act as intended, or is poisonous. Only 1% of the drugs that start the tests progress to the clinical stage. For those that do, the process took about three years.
For approval of an Investigative New Drug by the FDA, both acute (2 weeks) and sub-chronic (1-3 months) studies must be done in animals. If approved, clinical tests (I,II,III) can start as the chronic (3-18 months) animal tests proceed (F10.21, center).
To get permission for Phase I clinical studies, the company reports all about the preclinical stage, and describes the Phase I plan in detail. 8% of the drugs that start Clinical Tests are eventually approved, pushing the cost of developing a new drug to about 2 B$. About 40% of research costs relate to the *drug discovery* process, while 60% account for *drug development*.

10.3.1. Clinical Tests

10.3.1.1. Phase I: (10 M$, 1.5 years)

Phase I clinical tests are the first drug administration to humans, and are mainly about safety, not efficacy. Subjects in Phase I take a real risk, as animals do not “tell” you everything. Phase I tests are done on 10-100 healthy paid volunteers. Doses are carefully increased, and the effects measured, to establish the Maximum Safe Human Dose.

Clinical trials can often be confounded by placebo effects, an ill-understood phenomenon that is only beginning to be investigated. That is why clinical trials are often conducted double-blind.
Besides measuring safe doses, Phase I informs on mechanism of drug action. Keeping number of subjects small avoids unnecessary risks. If the drug is known to be toxic (AIDS and cancer drugs often are), patients with the disease are substituted for healthy volunteers.
Monitor: blood pressure, temperature, blood and urine samples, toxicokinetics.



F10.21. Time Course of Drug Development. Total cost for the development of a new drug is about 360 M$US. These costs can be lowered by improved screening.
Scientific American.

10.3.1.2. Phase II: (20 M$, 2 years)

Phase II clinical tests zero-in on the therapeutic range, below toxic effects.
200 patients with the targeted disease are tested, looking for proper therapeutic range, dosing regimen, appropriate end points of treatment and documenting drug efficacy.
Patient records: with control group, refine treatment dose and duration.

10.3.1.3. Phase III: (45 M$, 3.5 years)

Phase III clinical tests are mainly about the drug's practical use: write-up of the long, fine-print instructions that accompany medicines. Developing this information requires testing on 1000 patients, to assess and detect rare complications and find specific practical recommendations in drug administration. Requirements for this phase are especially demanding. Separate, independent tests must show the drug to be safe and effective. The company's report to government after clinical testing is often 100,000-plus pages. It then takes about two years for approval. 50% of drugs fail Phase III. 50% of failures are due to inability to outperform placebos, 30% due to safety risks, and 30% show no advantage over established therapies.

Some have suggested that Phase III trials should be replaced faster alternatives and post-market surveillance, and that current output of new drugs could be doubled by such a measure. After Phase II, patients and insurers would rate effectiveness, and drugs could then be handled based on that feedback. Though companies would still be liable for unforeseen side effects, patients and doctors would be warned that the drug was approved based on provisional research.

After approval, the drug is monitored by the pharmaceutical company itself for side effects that can only be discovered by use in many more thousands of humans.
The result should be an authoritative confirmation of drug effectiveness. But a number of drugs were taken off the market after their initial approval: Vioxx, Seldane, Rezulin and Baycol are examples. Some risks do not become evident before the drug has been used on millions of people, essentially because we do not know ahead of time what to look for.

The arthritis medication Vioxx was on the market for 5 years before withdrawal in 2004 due to increased risk of heart attacks and strokes. 55,000 Americans died while taking Vioxx, and other similar painkillers, such as Bextra, were also withdrawn. In 2007, a cardiologist showed that Avandia, a blockbuster antidiabetic drug, increased the risk of heart attacks. The claim was found to be true, but Avandia was kept on the market, because of its efficacy, but with a strict warning label.

It may be impossible to have all the information about the benefit-risk ratio of a drug at the end of the premarket phase, as clinical trials include only 500 to 3,000 patients. So the full

range of a medication's side effects is not likely to be revealed until it is used by the general population, which includes people with co-morbidities, pregnant women, and senior citizens. Drugs are approved based on incomplete information and inevitably, problems will emerge. Postmarket drug surveillance (F10.21) is there to detect problems early and efficiently.

10.3.2. Philosophical Questions on Clinical Testing

10.3.2.1. Use of human subjects

Is it feasible to fairly explain the potential risks to a patient in a clinical trial, when the purpose of the trial is to *learn* about these risks in the first place?

10.3.2.2. Breath of testing

How do you balance the need to test a drug *comprehensively* with the need to make it available as *quickly* as possible? Testing can be *two of the following* words, at the expense of the third.

Cheap	Trustworthy	Fast

10.3.2.3. Performance of Testing

Since 1992 in the US, pharmaceutical companies pay the FDA to defray the cost of reviewing drug applications, and this extra money cut the approval time from 27 to 15 months, perhaps bringing in more industry influence within the FDA.
But the proportion of drugs that later proved too dangerous rose from 2% (1989-1992) to 3.5% (1993-2000).
In 2004, the US FDA estimated that a drug entering clinical trials had an 8% chance of reaching the market, down from the historic 14%.
The poorer performance may be due to time compression, or it may indicate poor preparatory work.
Statistical methods of analysis may be incorrect, the test methodology may not be appropriate (choice of test system and response), and results may be selectively ignored to prop up drug development success. For a variety of practical reasons, an animal model may assess a chemical very differently than a clinical human model, leading to basic incoherences.

10.3.2.4. Conflict of interest

The drug company, which tests and monitors its own drugs, could aggressively disseminate data on the drug's effectiveness and closely guard information about its hazards. When hazards emerge progressively from data, it is tempting to imagine that early warning signs are statistical aberrations.
Panel members from government bodies such as the National Institutes of Health often have strong financial relationships with pharmaceutical companies, casting some doubt on their impartiality.
New drugs, most profitable to drug companies, end up being over-prescribed compared to older drugs that are as effective. Also, the risks of old drugs are usually better known than the risks of new drugs.

"Advertising may inadvertently minimize
the importance and power of medicines and their risks."
-William C. Weldon, Chairman of Johnson & Johnson

10.4. In Vitro Models

In vitro cytotoxicity and cell viability assays predict toxicity based on cell number, morphology, and metabolic activity.
In vitro testing is appropriate because of strategic reductionism, cutting the complexity of living systems approximately in two: there is an much complexity from the cell to the complete organism as there is from the cell to molecules.
In vitro methods are favored for ethical and economic reasons, and *can* provide better accuracy, sensitivity and resolution than *in vivo* methods.
It is however difficult to conduct *in vitro* tests under conditions that represent completely *in vivo* conditions. Primitive *in vitro*

tests do not provide the range of physiological conditions present in the body, nor the interactions among different cell types. Cells used in tests are often transformed or cancerous, exhibiting differences in gene expression and cell cycles.
In spite of this, high correlation coefficients are obtained when indicators for general acute *in vitro* toxicity are compared to LD_{50} values *in vivo*. So, it appears that toxicities depend mostly on actions at the intracellular level, as opposed to the extracellular level.
In vitro systems can use whole organs, tissue slices or cultured cells. Cultured cells are very popular because detailed microscopic observations are fairly easy when cells grow on the bottom of a dish.

10.4.1. Diversity of Cells

Eukaryotes had only about 10 cell types until 1.5 billion years ago, and 50 cell types until 1 billion years ago. The human body typically contains about 100 trillion cells that fall into about 300 specialized types.
Basic cellular functions are expressed at different degrees in different types of cells. For example, nervous system cells depend entirely on glucose for their energy requirements, while liver cells have high biotransformation as well as protein synthesis rates. These differences imply that complete *in vitro* tests for integral toxicity screening may require consideration of multiple cell types.
In vitro models of mutagenicity and genotoxicity (Chapter 13), are already well accepted. Although mutagenicity and genotoxicity testing would seem to be the ideal situation to minimize the need for numerous targets (all cells have similar DNA), even there, it has been found that results are enriched by the use of numerous models.

10.4.2. Obtaining Cell Suspensions

To easily manipulate cells, it is desirable to obtain them as a suspensions, as opposed to bulk tissue. This is usually achieved using proteolytic enzymes (trypsin, pronase, collagenase, papain) in diluted, calcium- and magnesium-free solutions. After digestion of the extracellular medium, mild mechanical manipulation of the cells will yield suspensions.
The extraction of the cells has undesirable consequences. In an animal tissue, cells have contact with the extracellular matrix, made up of protein (collagen, elastin), glycoprotein (fibronectin, laminin), mucopolysaccharides and lipids. Cellular messengers such as cytokines also influence cell behavior *in vivo*. As normal cells are extracted from an animal and placed in culture, they may lose some of their original characteristics in a matter of days, as they react to extraction from their original environment.
The experimenter must reassure himself that the characteristics critical to his toxicological investigation are retained in the model.

10.4.3. Selecting from the Cell Suspension

Virtually all cell types can be grown in the laboratory over the full range of durations required for toxicity testing. But some cells grow more easily than others. Cells obtained from animal tissue will contain a number of cell types. If the cells are grown for some time, the most hardy cell type may take over the culture. Because cells spread on a plate tend to automatically fill the available gaps, cells that divide rapidly tend to dominate *in vitro* systems as the cells grow over time.

If one requires a specific cell type, it is possible to select for them using selective growth mediums, growth surfaces or toxicants. A variety of techniques are used for further purification.

Another method, called *cloning*, is to mechanically capture a single or more cells and grow a culture from this material. This procedure produces a cell *strain*.
For example, if we try to grow bone marrow cells, their probability of forming colonies (*cloning efficiency*) are generally below 1%[15].

10.4.4. Normal Cells vs Cancer Cells

Normal cells have a limited lifespan *in vitro*. Consequently, unless aliquots of a cell suspension have been frozen, it is not possible to repeat exactly the same test twice.

Cancer or embryonic cells grow more readily than normal adult cells. This is probably because less specialized cells tolerate more easily the artificial environment of cell culture, specifically the plastic or glass vessel, variations in the concentrations of artificial nutrients and fluctuations in pH. Whereas normal cells can go through about 50 doublings (the so-called *Hayflick limit*), cancer cells will divide indefinitely. This makes repetition of tests even in the far future a possibility. The price for this convenience is that (1) they differ substantially from the normal tissue they originate from and (2) their karyotype is often abnormal.

10.4.5. Stem Cells

Stem cells can generate some or all of the cells in the body if properly controlled, and are therefore an important ingredient in regeneration of diseased tissue. However, stem cells are delicate to culture. They must be provided with fresh liquid nutrients at least once a day, passaged each week and have been successfully cultured only on nutrient layers of mouse cells until recently. These layers must be replaced every two weeks.

10.4.6. Primary Cultures

Primary Cultures are cells that were extracted from animals and have taken to growing *in vitro*. One can culture liver, kidney, gland and endothelial cells as well as fibroblasts from adult animals. Cells from the nervous system, heart and striated muscle are usually obtained from embryonic tissue, differentiating further *in vitro*.
When cells are obtained as primary cultures, it is necessary to characterize them, in order to define explicitly what we are working with. The definition will usually include morphological and growth rate observations as well as the functional-biochemical properties specific to that cell and that may contribute to end-point observations in the experiment.
A table (T10.22) lists the properties often probed to confirm such properties. The closer the cells falls in comparison to cells *in vivo*, the better. Although one would like such characterizations to be complete and encompassing, it is rarely possible to be completely reassured.

T10.22. Examples of variables that characterize *in vitro* models.

Hepatocyte	P_{450} activity
	Albumin production
	Gluconeogenesis
	Steroid hormone inducible tyrosine-amino-transferase and glutamine-synthetase activities
Proximal Tubule Kidney	Pronounced Microvilli
	Alkaline phosphatase activity
	Maltase activity
Neurons	Tetanus toxin binding
	D2 and D3 proteins
	Neurotransmitter synthesis
	Neurotransmitter receptors
	Acetylcholinesterase activity
	High-affinity transport for transmitters and precursors

10.4.7. Cell Lines

The difficulty of satisfactorily characterizing primary cultures has led many investigators to use a different tactic. If cells are successfully cultivated over time, the most rapidly growing type will become dominant, becoming a *cell line*. In some cases, the purity of cell lines can be increased by cloning. When obtained from **normal** cells, the line will have finite lifespan (60-80 doublings), an obvious disadvantage if tests need to be repeated in the future.
If the starting material was cancerous, a *continuous cell line* can arise, which can be cultured indefinitely, an obvious advantage for future experiments. However, cancer cells have diverse genotypes and phenotypes within the same culture, making them less than ideal for most tests, except tumor reduction experiments.
The ideal test object would be a normal cell that reproduces indefinitely, while expressing stable karyotype and phenotypes. In the past it has been possible to *transform* normal cell lines using viral gene sequences or chemical exposure. *Transformed* cell lines are immortal, and do not undergo senescence. However, transformed cells are genetically metastable, and can lose or gain whole or partial chromosomes. In many cases, transformed cell lines exhibit abnormal gene expression profiles that confound the study of biological mechanisms. Recently, cells modified with human telomerase reverse transcriptase (hTERT) have become available, which have unlimited division potential as well as stability in karyotype and phenotype. This continuity allows comparability of results from one experiment to the next, by freezing reference stocks to ensure an indefinite supply of cells.

A critical advantage of cell lines generally is that they have become standardized, and are being distributed to researchers at reasonable cost (American Type Culture Collection, Bethesda, Md, USA). Therefore, if one is willing to select from a long list, all the procedures of obtaining, selecting and characterizing cells can be avoided. Also, so much research has been done on the popular cell lines, that rich literature dossiers are available.

10.4.8. Cell Line Fidelity

Repeatedly culturing cells (*passaging* cells from a old container with depleted medium to a new one with fresh medium) selects genetic or epigenetic modifications for enhanced division that ultimately take over the culture.
Cell control mechanisms that would prevent this *in vivo* are not paralleled *in vitro*. As cells are exposed to toxicants, the composition by cell type as well as the properties of the cell culture may drift, particularly if a test lasts for many cell generations. Therefore, different experiments at different doses may finally contain different cell populations, even if the cells initiated from aliquots. This is also a problem *in vivo*, although the difficulty is less readily measurable, hidden under the term "toxicodynamics".

The problem of cell diversity within continuous cell lines is particularly evident in cancer cell lines. Under the microscope, many different cell phenotypes can be seen. When karyotypes are measured, substantial aneuploidy is documented. The histogram of chromosome numbers within a continuous cell line is in fact one of its characteristics (Chapter 14). Therefore, the continuous cell line can be viewed, under ideal conditions, as a stem cell producing a fixed proportion of various cell sub-types.
Human embryonic stem cells cultured for therapeutic purposes also accrue genomic changes, when cultured at length. After 22 to 175 passages, stem cells developed copy number aberrations (changed repeat number in genome). For example, 2 Mbase amplifications encompassing the *myc* oncogene, as well as

amplification of the whole 17q arm. Mitochondrial alterations occur as well: missense in NADH dehydrogenases, nonsense in ATPase 6. Higher promoter methylation is also frequent.[12]

10.4.9. Cell Culture Medium

The non-serum part of cell culture media is made of a number of pure chemicals mixed together to sustain cell cultures. This is often supplemented by *serum* (5-20%) or other poorly defined tissue extracts. The problem of purity existing for animal food (10.2.6) is paralleled in culture media, because the food of animals used to provide serum may not be tightly controlled, making the composition of their serum unreliable, even if known. When serum is purchased, the "batch" number is specified, in an effort to eliminate variability in terms of growth rates or immune components, for example.

Serum-free media eliminate serum supplements, improving the stability of results and reproducibility, but they also remove complex protective components such as albumin, a natural part of the buffering and detoxification action of the blood. To compensate, purified protein of human, animal or recombinant origin (many formulations are kept secret) are added in place of the serum (albumin, ferritin, transferrin, insulin, cholesterol). Albumin (often bovine serum albumin) being such a complex molecule, it is almost impossible to purify it completely, so its re-inclusion to supplement chemically defined media introduces variability. Rates of cell division generally also tend to diminish in serum-free media because of the absence of growth factors normally present in the blood. Some formulations will increase concentration of protein, notably insulin, to high non-physiological values, in an attempt to compensate. But increasing insulin levels decreases the activity of the SKN-1 protein, which normally promotes a set of Phase II detoxification enzymes (glutathione among them) that remove free radicals, leaving the cell more vulnerable to ROS[17].

Some components of the media will decay rapidly with time (glutamine), such that the shelf life of freshly made medium is limited. Both pH and osmolarity of the media must be controlled, a challenge in long duration tests. pH can be somewhat controlled by using CO_2 incubators or HEPES, an organic pH buffer. Osmolarity varies over time because culture flasks are not tightly sealed in the incubator to allow CO_2 and O_2 equilibration. As a result, some evaporation occurs unless the incubator is kept at 100% relative humidity, which in turn favors multiplication of bacteria and fungi, always a threat in the cell culture lab.

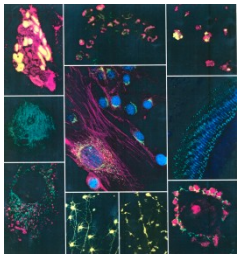
Certain types of cells are better maintained in culture at low oxygen (5%) rather than ambient oxygen (21%), which is thought to be due to decreased oxygen toxicity. On the other hand, some other cells types may not get enough oxygen in culture flasks, needing to build a tolerance for oxygen depletion. When testing for toxicants that affect oxygen metabolism, measured effects may rate as too high or too low, depending on circumstances.

10.4.10. Determining Test Doses for In Vitro Systems

If animals appear normal at the beginning of an *in vivo* test, this guarantees a physiologically valid baseline. In animal testing, the general appearance and behavior of animals also acts as an effective guide to proper toxicant dosing.

The corresponding guides in cell cultures are not so obvious. The normalcy of an *in vitro* situation is not as clear because many cell cultures represent quite artificial conditions. For example, cells in culture are adapted to strong changes in the levels of nutrients and metabolites as a result of cell culture medium renewal.

When doses of the toxicant are administered, doses could be used that would kill whole animals simply because the most susceptible tissues are not included in the test system.



To compensate, a larger range of doses may need to be tested *in vitro* to gain perspective, which has the advantage of being more informative.
One must be particularly cautious of toxicants with endocrine disruptor capabilities which are extremely potent, and may display flat or U-type dose responses. For example, in cell cultures, doses of TCDD as low as 10^{-11} M are toxic, with decreased viability observed even in nonproliferating cells.

10.4.11. Toxicokinetic Characteristics In Vitro

When toxicologists administer a drug to an animal at a certain dose, sight is often lost of the fact that the agent is metabolized and excreted, and it is implied that a single concentration is valid for the entire inter-bolus period. If specific toxic phenomena are indeed related to agent concentration, this assumption would tend to smear toxic transitions and retard detailed understanding.
The situation is similar *in vitro*, with the difference that a high ratio of medium to cell volume may stabilize the agent, as well as other concentrations. There is also the possibility for active control of agent concentration to clarify toxic thresholds. But there have been few serious attempts in the past to control *in vitro* environments actively and automatically, because of practical difficulties.

If the agent to be studied is sufficiently soluble in the culture medium, it can be added directly and perhaps even controlled by renewing the medium. If the substance is not, dissolution can be aided using an organic solvent such as ethanol or dimethylsulfoxide (DMSO), although these substance may introduce effects of their own, for example by allowing easier penetration through cellular membranes. For proper dosing, it should be verified that precipitates of the agent do not spontaneously form over time. Also, the amount of plasma protein available in the medium for binding of toxicants will affect effective concentration.
Penetration through cell membranes may take seconds to hours, depending on the properties of the agent.
As described in Chapter 8, biotransformation should be maintained *in vitro* to more closely represent *in vivo* situations. Ideally, an excretion route should be provided, although this is beyond the capability of most *in vitro* test systems.

10.4.12. Testing Techniques

Although there are many limits to *in vitro* testing, the attractions of high speed, sensitivity, reproducibility and economy are quite strong. As a result, there is considerable interest in developing *in vitro* techniques to increase the usefulness by astute selection of highly specific molecular probes or by using *in vitro* test batteries (multiple tests).
Whether cells are intoxicated can be measured in a variety of ways. We will outline 5 typical methods[11] below.

1. **Active exclusion of substances** such as Trypan Blue. Dead cells ultimately present holes in their membranes, because they are not maintained. Therefore, particles of Trypan Blue inside cells are a sign of cell death, since this pigment does not penetrate normal cells.
2. **Active uptake of substances** such as Neutral Red. Neutral Red is a supravital dye which accumulates inside the lysosomes of living cells. The pigment from dead cells is easily washed away.
3. **Leakage of cell constituents**, such as lactate dehydrogenase. Present in muscle, red blood cells and liver, the molecule will leak out if the cell is damaged, and can be found in the plasma.
4. **Loss of activity of intracellular enzymes**, such as in the tetrazolium stain test. This stain (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) is intracellularly reduced from a

pale yellow solution to a dark blue formazan crystals, which reveals metabolic activity.

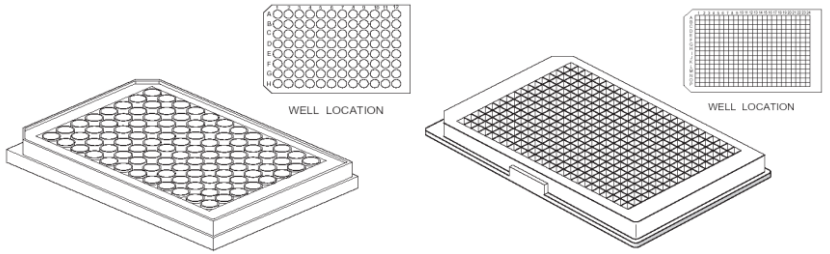
5. **Reduced synthetic activity** for molecules such as albumin. Made in liver cells and secreted extracellularly.

The EEC's policy of no animal testing for the development of novel cosmetics has given impetus to *in vitro* methods.
In the pharmaceutical industry, *in vitro* methods are frequently used in screening for new drugs. Therefore, *in vitro* success determines in large part the cost of drug development. More relevance can be obtained from *in vitro* testing by
- acquiring knowledge of basic physiology, so that relevant molecular targets are chosen and
- by multiplying the number of molecular targets and tests, taking advantage of automation (screening).

Many tests depend on simple colorimetric or cellular observations on material that is less complex than what they are intended to represent. For example, activation of a single heat stress gene may not have the same meaning as the activation of an array of genes as a result of thermal insult. Artificial skin tests, for the most part, barely make an attempt at reproducing the architecture of the skin. Can a dye penetration test represent well the most basic phenomenon present in skin, such as erythema?

10.4.12.1. Multi-Well Test Techniques

Automation of tests will provide more information per unit of human effort. Most of the traditional techniques of toxicology, for example necropsies and histology, are labor-intensive methods.
There has been a drive towards assay miniaturization, similar to Large Scale Integration in the electronics industry, aimed at the development of High Throughput biological assay

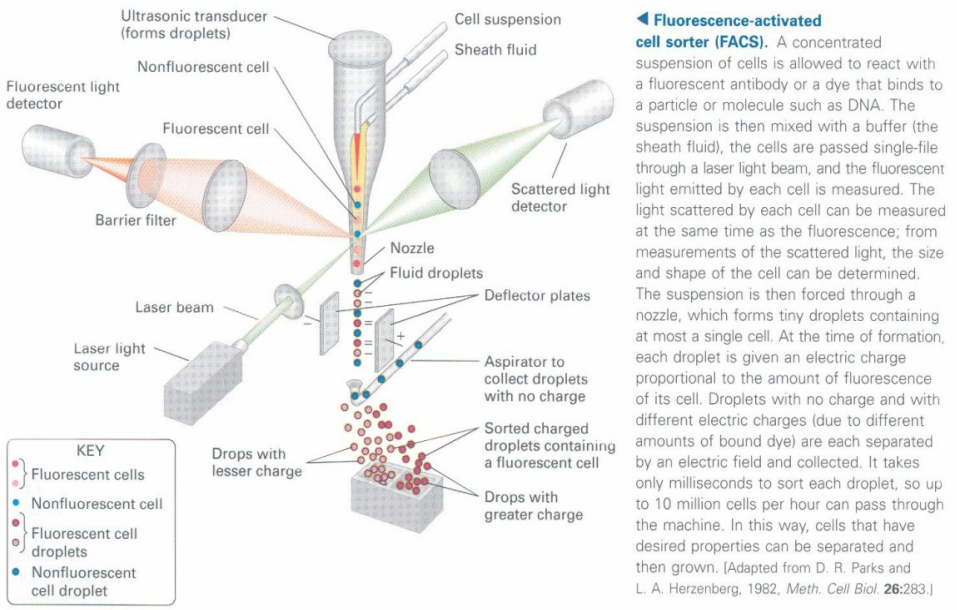


F10.23. Formats for high density bio-assays.

techniques. Multi-Well dishes (F10.23) for bio-assays come in two current sizes, 96 (~0.3 ml/well) and 384 wells (~100 µl/well). These dish formats are supported by an array of instruments for sample preparation, scanning readers and washers.
For example, multiwell-based fluorometric assays for the inhibition of the principal cytochrome P450s (CYPs) have been available since 1997.

10.4.12.2. Fluorescence-activated cell-sorting

In pharmacology, physiology and mechanistic toxicology, molecularly targeted information is obtained using chemical techniques dependant on the availability of specific fluorescent antibodies or dyes. The fluorescence enhances contrast with the background image.
Fluorescent techniques identify and localize specific molecules within a specimen. This leads to spectacular images with multiple stainings.
The "pretty pictures" are great for *localization*, but are somewhat weaker for quantitative information. Note that in publications, the pictures *always* seems to prove the point of the article, but that you much more rarely see a systematic analysis of *all* the available microscopy fields, together with quantitative analysis.



◀ **Fluorescence-activated cell sorter (FACS).** A concentrated suspension of cells is allowed to react with a fluorescent antibody or a dye that binds to a particle or molecule such as DNA. The suspension is then mixed with a buffer (the sheath fluid), the cells are passed single-file through a laser light beam, and the fluorescent light emitted by each cell is measured. The light scattered by each cell can be measured at the same time as the fluorescence; from measurements of the scattered light, the size and shape of the cell can be determined. The suspension is then forced through a nozzle, which forms tiny droplets containing at most a single cell. At the time of formation, each droplet is given an electric charge proportional to the amount of fluorescence of its cell. Droplets with no charge and with different electric charges (due to different amounts of bound dye) are each separated by an electric field and collected. It takes only milliseconds to sort each droplet, so up to 10 million cells per hour can pass through the machine. In this way, cells that have desired properties can be separated and then grown. [Adapted from D. R. Parks and L. A. Herzenberg, 1982, *Meth. Cell Biol.* **26**:283.]

F10.24. Principles of the Flow Cytometer.

If basic engineering problems of image segmentation, interpretation and analysis could be solved, the power of these techniques could be enhanced.

A somewhat more quantitative technique is flow cytometry (F10.24). In this technique, individual living cells are sucked through a fine capillary so that they line up. As they go through a laser beam, scattered light allows various determinations to be performed.

T10.25. Cell Lines in Common use in Cytotoxicity Testing.	
L929 Mouse fibroblast	Used for evaluation of biomaterials: well-characterized, easy to culture and reproducible.
WI-38 Human embryonic lung	When a human cell type is required.
HeLa Epitheloid carcinoma of the human cervix	
VERO Monkey kidney cells	Specialized tests for virucidal efficacy testing.

Flow cytometry is more easily quantified than classical microscopy, but is not as easily automated for time series analysis. Flow cytometry has been used for cellular work and to diagnose diseases of the blood and bone marrow, allowing discrimination based on fluorescent labels targeted to specific cellular markers.

10.4.13. Implementation of Cytotoxicity Testing

Mammalian cell cultures currently evaluate biocompatibility and toxicity of materials in medical devices and associated products. A variety of cell types (T10.25) which differ in relative sensitivity and in the time required to conduct the assay are used. These methods have shown good correlation with animal assays and are frequently more sensitive to toxic materials. Several of these cytotoxicity test procedures are widely accepted in biomaterial screening, quality control and audit programs.

T10.26. Correlation of Growth Inhibition with LD_{50} at 24h		
Chemical	**Growth Inhibiting Concentration$_{50}$ (μM)**	**LD_{50} (μM)**
Hypophosphate	9	8
Methylenehydroxydi-phosphate	125	300
Phosphoformate	1000	>4000
Phosphonoacetic acid	1000	>4000

10.4.13.1. Direct Contact and Agar Overlay

Mouse fibroblast cells (L929) cell cultures are grown to a standard monolayer. Duplicate samples of test material, as well as negative and positive controls, are placed in direct contact with the cell layer for 24 hours. The monolayers are fixed and stained and finally examined microscopically for the presence of morphological changes, decolorization, reduction in cell density or lysis induced by the test material.

10.4.13.2. Minimum Essential Medium Elution and End Point Titration

The test material is *extracted* for 24 hours in Minimum Essential Medium (MEM). Mouse fibroblast cells (L929) monolayers are exposed to test, negative and positive control extracts and examined for morphologic changes or cytolysis at 48 hours to determine a toxicity score within a range from zero (no effect) to 4 (nearly complete cellular destruction). The USP method requires testing in duplicate while the ISO method requires triplicate plates.

Extracts of test materials shown to be toxic in the Minimum Essential Medium Elution Test can be serially diluted (End Point Titration) to determine the highest dilution for which there is no toxic response.

10.4.13.3. Alterations in Cell Growth

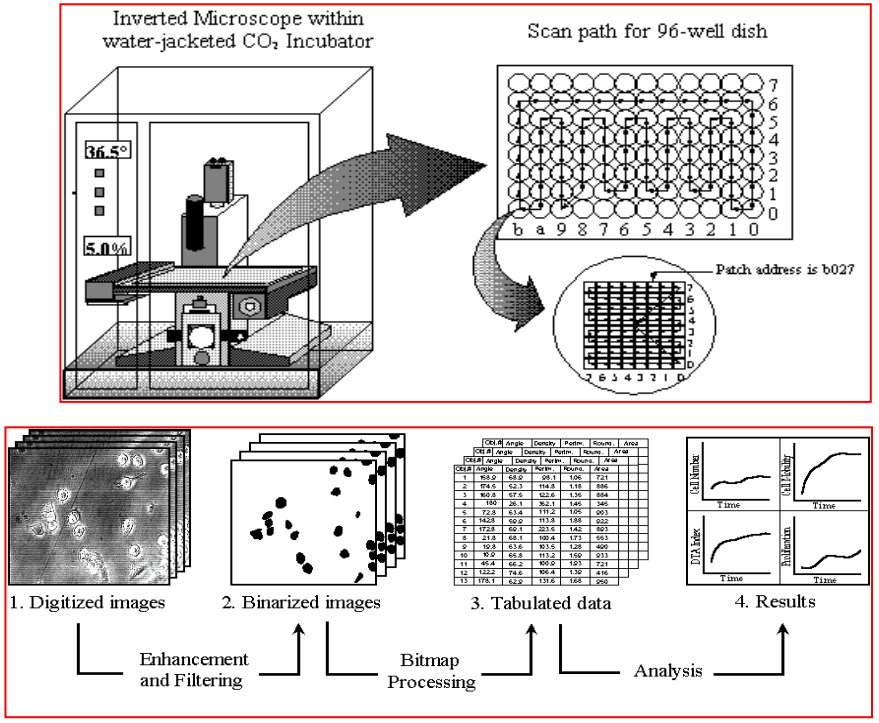
Water extracts of graded quantities of the test material are made and incorporated into tissue culture medium. The extracts or serial dilutions are evaluated for inhibition of cell growth by determining the protein content of monolayers at zero time and 72 hours after incubation under standard conditions. Data are expressed as the amount of test material which results in minimal inhibition of cell growth (less than or equal to 10%) or 50% inhibition of cell growth. This procedure can be used for evaluation of medical device plastics, ophthalmic solutions and similar products.

If toxicity is going to be measured in terms of cell growth, we have to interpret cell growth in terms of toxicity.

When immune cells divide quickly, it is usually because of a raging immune response.

On the other hand, all cells have complex growth mechanisms that are vulnerable to almost any type of metabolic toxicity, providing a wide-ranging net for toxicity detection. For example, oligodendrocyte precursors, when oxidized by agents such as methyl mercury, lead and paraquat, have a sub-population (25%) that stops growing[13].

Measuring the growth of cells over time (~a week) as they are exposed to toxicants is the gold standard of cytotoxicity measurements. It measures the ability of cells to divide and form colonies. A cell with damaged DNA may still live for a long time and give a false negative in a dye-exclusion assay. Cell counting, particularly if done automatically to insure statistical significance, has the potential to detect reliably both low-dose and high-dose effects of toxicants.



F10.27. Human cells in culture can be observed over time with automated computer vision systems. *Héroux, 1996.*

10.4.14. Advanced Applications of Cell Culture in Toxicology

Microscopy, computer imaging and automated cell tracking can be implemented for days on 96-well dishes contained in incubators (F10.27). This allows a continuous and detailed record to be kept in the form of thousands of images.
The advantage of such advanced systems is that they allow the simultaneous measurement of changes in cell division rate, reproductive sterilization, necrosis and apoptosis. Such detailed data on cell behavior is of great value for cancer research.
Even the gathering of a single measurement such as 50% cell proliferation inhibition is of value[8] in predicting LD_{50} in mice, as shown in T10.26. It is also correlates with reduced body weight[9] in animals.

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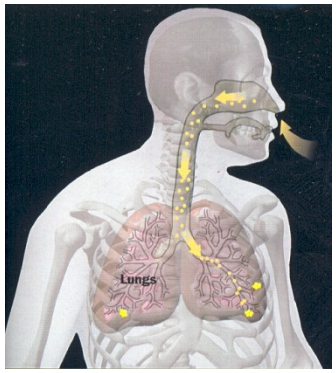
Pulmonary toxicity

11· Pulmonary toxicity

11.1. Anatomy



The general anatomy of the human respiratory system is depicted in F3.4.
Some characteristics of the human lung are shown in T11.1. Remember that the lungs can change several of these dynamic parameters quickly, given increased physical activity, a noxious odor, an irritant (thereby altering respiration rate, depth), etc.

T11.1. Characteristics of Human Respiration.
Levine, Pharmacology. Drug actions and reactions. Little, Brown, Boston 1978.

Human Pulmonary Characteristics	Value
Exchange Area (air)	200 m^2
Area of the Pulmonary Capillaries (blood)	90 m^2
Number of Alveoli	300-400 x 10^6
Air-Blood Barrier Thickness	0.5 -1 µm
Particle Size penetrating Alveolar Space	2 µm
Respiration Rates	6-200 liters/min 9-300 m^3/day

11.1.1. Cell Types

Pulmonary epithelium embryologically derives from *pharyngeal endoderm*, which differentiates into (1) an upper laryngobronchial epithelium and (2) a lower bronchiolo-alveolar lining. The bronchiolo-alveolar lining contains the airway epithelial cells, unique to the lung. Enzymatic activity is similar in both segments, both having a number of cell types in common.
More than 40 cell types can be found in pulmonary tissues, but most occur as well in other tissues of the body (vessels, blood cells, bone, nerve, etc). The functions of several cells are poorly understood.
Seventeen of these cells are epithelial (5 of these are not present in all species), 9 are in the connective tissue (7 of those are trafficking leukocytes), 7 are in blood vessels, 5 are in nervous tissue, 2 types of bone cells, 2 types of muscle cells, one pleural cell, one cartilage cell and one cell of the attenuated fibroblast sheath.
Of these cells, only 4 are unique to the lung: non ciliated bronchiolar cells or Clara Cells, squamous cells (Type 1 cells), great alveolar cells (Type 2 cells) and alveolar macrophages.

Every cell from the Type 1 to tracheal epithelial cells can now be cultured and studied, and tissue culture of lung cells is now common to study the lung.
A large amount of research is done with rodents, large animals are not often used.

11.1.2. Animal Anatomy

It is not easy to extrapolate data obtained from animal experiments, usually conducted by nose-only inhalation, to humans. Not only are the respiration rates and volume vastly different (T11.2.), but so is the structure of the respiratory tract. Significant differences exist in the deposition of particulate matter (T11.3.) among four routinely used species. Thus, animal experimentation must be carefully assessed when considering pulmonary uptake and toxicity in humans.

11.1.3. Bronchi

T11.2. Rates of respiration.
ARS/Sprague Dawley.

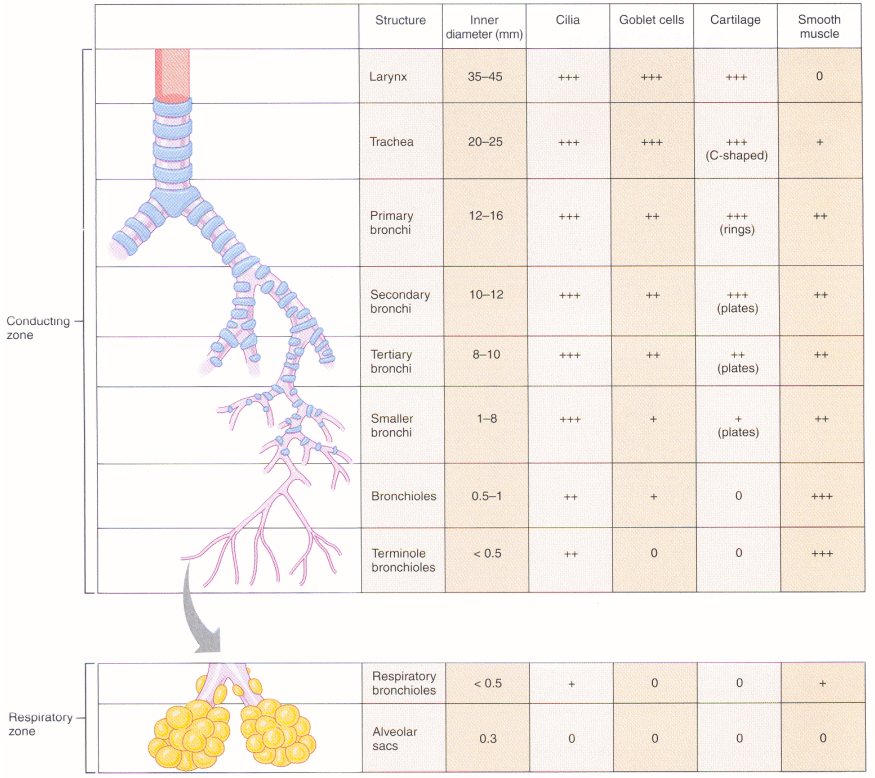
Species	Inspirations /minute
Human	12
Dog	10-30
Cat	20-30
Hamster	35-130
Monkey	40-60
Rabbit	40-65
Rat	65-110
Guinea pig	70-100
Mouse	80-240

A system of bronchial tubes leads air to 300 million alveoli in the lung. The bronchi (F11.5) are reinforced with cartilage and muscle cells, so that they collapse less quickly under pressure than the alveoli.
As air proceed into the lung, there is considerable lung surface expansion (F11.6) to 70 m^2. The airflow resistance as a result of reduction in airway channel diameter was discussed previously (Poiseuille's Law, page 3-6). Because of the considerable expansion of the air section of the airways, flow resistance is not concentrated at the lowest level of the lung. 85% of the flow resistance is in airways more than 2 mm in diameter.

Because of the considerable surface area, as well as because of rapid diffusion through gases, early systemic effects from pulmonary acquisition of a toxicant is usually as rapid as the sleep-inducing effects of a volatile organic anesthetic.
The agent is rapidly absorbed into the systemic circulation.

T11.3. Percent of total respiratory deposition following exposure to diluted cigarette smoke.
Binns et al. Toxicology 6, 97, 1976

Species	Larynx	Trachea	Lungs
Rat	5.8	4.2	72.1
Mouse	3.8	1.4	58.1
Hamster	6.1	3.8	71
Guinea pig	1.2	1.5	84.7

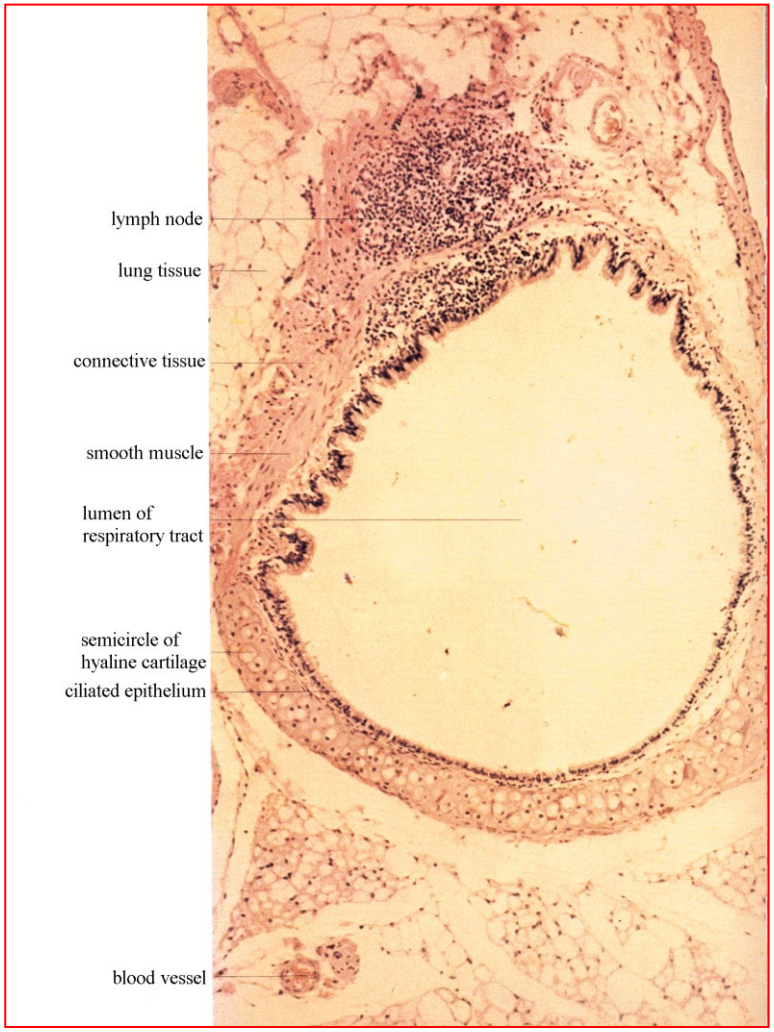


F11.4. Anatomy of the airways. *German & Stanfield, 2002.*

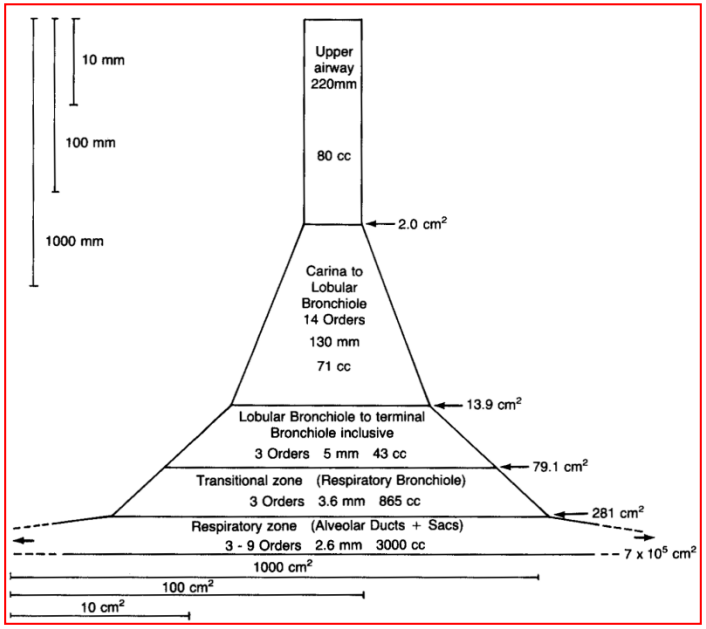
The blood returning from the lungs goes directly to the heart and is pumped out into the periphery, more than 20% directed to the brain. In the first "pass" through the peripheral circulation, the agent does not flow through the liver (only through the kidney). Systemic toxicity can therefore occur before biotransformation[1].

11.1.1. Alveoli

Lung tissue is essentially a foam (F11.7): it contains large alveoli of air lined with blood vessels for air to liquid-phase gaseous exchange. The walls of the alveoli are made of thin epithelium and elastic fibers.

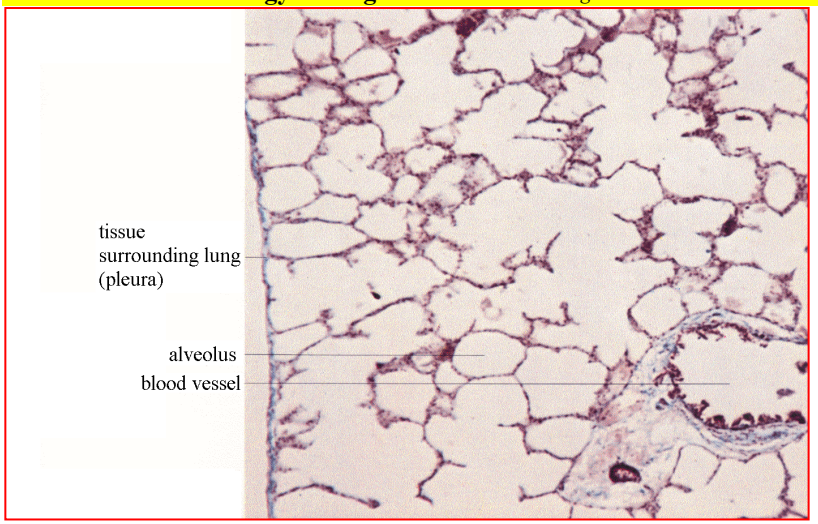


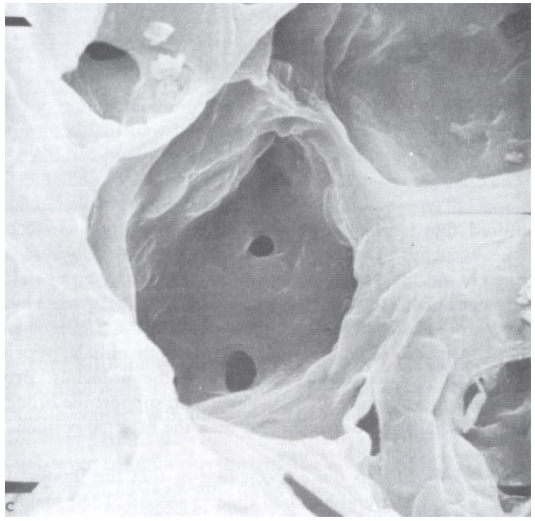
F11.5. Histology of bronchus. x 75. Biological Structures.



F11.6. A log-log graph showing the geometry of the total cross sectional interface of the lungs vs linear distance into it. *Williams and Burson.*

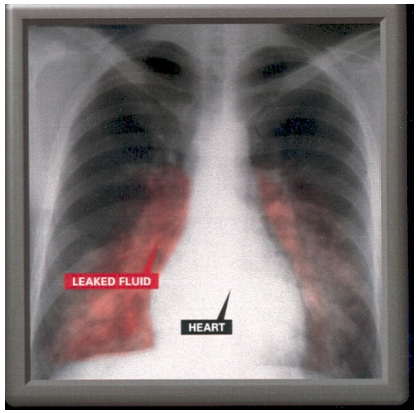
F11.7. Histology of lung tissue. x 200. *Biological Structures.*



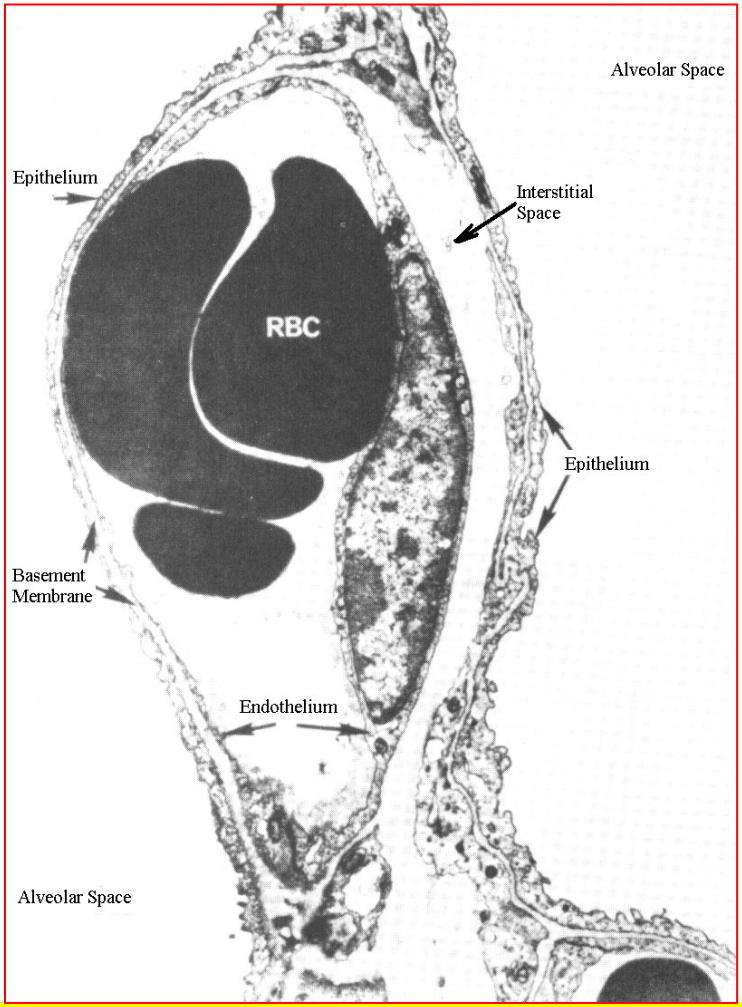


F11.8. Appearance of alveolus. The small (Kohn) pores are intended to equalize pressure between alveoli. *Williams and Burson.*

Air reaches the alveoli (F11.8) after more than 20 bronchial branchings.
100 ml of blood interfaces within the lungs with air through membranes as little as 0.14 µm in thickness. Red Blood Cells (RBC) are cruising in almost a single file in the capillaries, along the walls of the alveoli (F11.10).
The septum alone (between air and blood) is about 2 µm in thickness (lower part of figure 10.10). Acute lung exposures as well as oxygen deprivation often produce edema in the lung (F11.9), similar to drowning in your own body fluids, because of reduced transfer of O_2 and CO_2.



F11.9. Pulmonary edema: red areas show leaking of fluid from capillaries to the alveoli of the lungs. Nitric oxide can be administered to treat pulmonary edema.



F11.10. Blood capillary of the wall of an alveolus. The alveolar space is filled with air. The Endothelium contains the blood, the Epithelium lines the alveoli. *Williams and Burson.*

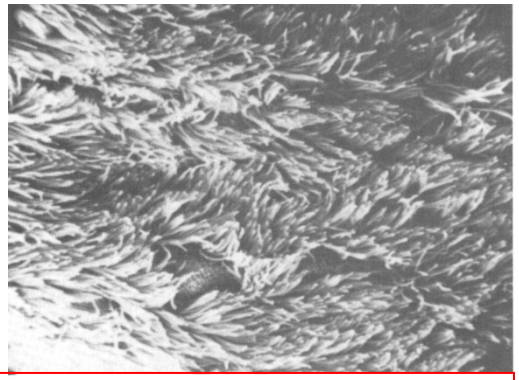
The airway mucosa brings inspired air to body temperature and humidity, and removes bacteria and particulates. If you breathe too fast in the winter, your deeper lungs can become irritated as a result of incomplete temperature and humidity equilibration.

11.1.2. Cilia

The mucus secreted within the airway is thin at the base of the cilia, so as not to impair movement, and thick at top to trap particles. Without mucus, the epithelium looks like shag carpet (F11.11).

F11.11. Cilia. The holes in the "carpet" are locations where mucus is generated.

Williams and Burson.



T11.12. Classes of Pulmonary Toxicants.

Particles

- fibrous material: silica, coal, asbestos
- non-fibrous material
  - inorganic (iron, Be, Sn)
  - organic -dust from raw cotton, gains
    -molds from bagasse, etc
    -proteins, detergents, etc

Irritants

- of upper, lower, or entire airway
- dependent upon solubility in mucus

Chemical Toxicity

- -cellular damage producing fibrotic tissue
  - (paraquat, BHT)
- -carcinogenesis
  - (PAHs, etc)

Cilium action is vulnerable to cigarette smoke, chlorine, ammonia, sulfur oxide, nitrogen oxide, cadmium, nickel and mercury fumes.

The lung's defense mechanisms to support bronchial clearance include **mucus, cilia** and **macrophages.** Immune cell activity is prevalent in the lung, as shown by the cytokines and other cellular mediators that are washed out of the lung in *vivo* experiments.

11.2. Aggressors

There are 2000 agents known to collectively produce various types of lung diseases. While gases may reach all the way to the alveolar sac, vapors will deposit along the secondary and tertiary airways (small bronchi and bronchioles), depending on the droplet diameter, while dusts and particles may penetrate beyond the bronchi or even the nose and throat, according to size (T11.13.).

11.2.1. Particulate Matter

11.2.1.1. Particle Size

Particles of non-soluble materials taken up by the lungs can be deposited in the lung by **impaction (>25 µm)**, **sedimentation (1-5 µm)** or **diffusion (<0.1 µm)**. The particles can be cleared by the lung in the sputum, can be dissolved by immune attack from macrophages or can remain in the lung as permanent irritants, potentially causing disease.

There is no dose-effect relationship on the basis of particle *weight* in respiratory diseases. Most of the mass of aerosolized dusts is made of large particles which precipitate quickly, trapped in the nose and throat. The important fraction is the number of particles per unit volume below 6 µm, because it can be deposited in the lung.

Ultrafine particles, for example from urban air, carry far more toxic combustion hydrocarbons per unit mass on their surface

than larger particles. Very small particles can lodge in the cell's mitochondria, inhibiting and killing them. Some substances that cause no damage as 10 µm particles are highly inflammogenic as sub-micron particles[5].

Cascade impactors have been developed to divide contaminated air samples into *respirable* and *non-respirable* fractions. In industry, general limits on dusts have been set at 10^6 particles/m^3. Levels above this standard indicate poor ventilation practices.

That size matters is obvious from the practices of the tattoo parlor. For tattoos to endure, the pigments have to be the right size. They must be small enough for macrophages to envelop, but not so small that they will be digested and cleared away by the cells. The optimal size for tattoo particles is 1 µm[9].

T11.13. Inhaled particles retention.
Goldstein Aronow & Kalman, Principles of Drug action, Harper and Row, 1968.

Region	Size (µm)
Mouth, pharynx, trachea	20
Bronchi, 2nd, 3rd bronchi	20-2
Terminal bronchioles	6-0.2
Alveolar ducts	6-0.2
Alveoli	2-0.2

11.2.1.2. Irritation Response to Particles

Particulate airborne pollutants, such as asbestos and silica act through a multiprotein complex known as the Nalp3 inflammasome which signals exposure of macrophages to internalized particles of asbestos and silica. This leads to the activation of a potent inflammatory response. In the absence of Nalp3, mice responded less vigorously to asbestos, supporting the idea that this inflammatory sensing complex plays a key role in the response to respiratory pollutants. The Nalp3 inflammasome is also involved in responses to bacteria.

11.2.1.3. Particle Pathology

The general particulate matter pathology term for the lung is *pneumoconiosis*, defined as the accumulation of dust in the lungs, and tissue reactions to its presence.

Dust deposition in the lungs often leads to *chronic restrictive disease,* because there is a reduction in *lung elasticity,* without significant *airway* disease. This occurs after many years of exposure to *fibrogenic* or *nonfibrogenic* materials.
The term *fibrogenic* describes the chronic deposition of scarred tissue (collagen) in place of normal lung tissue. The collagen is nonfunctional, and tends to reduce gas exchanges. The best examples are *silicosis* (miner's lung, coal worker's lung) and *asbestosis*, characterized microscopically by the presence of "ferruginous bodies". Those microscopic bodies are visible under appropriate stains and originate from unsuccessful macrophage attack of asbestos particles with iron-containing lysosomes.

11.2.2. Coal Mining Cases

11.2.2.1. Case 1.

A man began work as an underground coal miner in 1970, at age 22 years. He worked underground for 31 years, all but 2 years in coal-face jobs (the area of the mine where the coal is cut from the seam). In 2001, he began work in other areas underground, and his chest radiograph indicated 4 small category 2/1 opacities. In 2006, at age 58 years, his radiograph indicated progression to 2/3. His exposure history (limited exposure to silica dust) and slow disease progression were consistent with coal workers' pneumoconiosis.

11.2.2.2. Case 2.

A man began work as an underground coal miner in 1976, at age 18 years. After 23 years in coal-face jobs, in 1999, his chest radiograph indicated no evidence of pneumoconiosis. Seven years later, at age 48 years, his radiograph revealed category 2/2 small opacities and stage B progressive massive fibrosis. This rapid disease development is atypical of the usual clinical progression of coal workers' pneumoconiosis, which can take 20-40 years to develop, and is more consistent with silicosis. However, the man's disease developed without apparent exposure to silica dust.

11.2.2.3. Case 3.

A man began work as an underground coal miner in 1972, at age 18 years. By 2003, at age 49 years, he had spent 6 years at the coal face and 25 years as a roof bolter (drilling holes into the roof of mine passageways, often through siliceous rock, and inserting bolts to prevent rock falls), and a chest radiograph indicated category 1/2 small opacities, suggesting simple pneumoconiosis. During 2003-2006, the man continued to work at the coal face. In 2006, his chest radiograph indicated progression to category 2/2 small opacities. Although he had spent most of his mining years as a roof bolter, a job generally associated with silica-dust exposure, his disease development pattern was more consistent with coal workers' pneumoconiosis than silicosis.

11.2.2.4. Case 4.

A man began work as an underground coal miner in 1971, at age 20 years. In 2001, after 30 years working in jobs at the coal face and roof bolting, he had category 0/1 small opacities. After 5 more years of similar work, at age 55 years, his disease had progressed to category 1/2 simple small opacities and stage B progressive massive fibrosis. This exposure pattern and accelerated clinical course is more consistent with silicosis development than coal workers' pneumoconiosis.

11.2.3. Silicosis

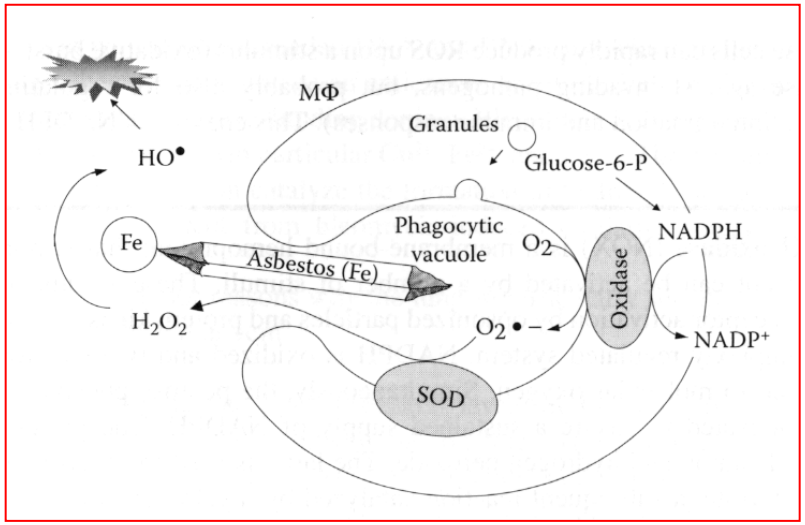
Silicosis results from repeated ingestion of particles of quartz rock dust (SiO_2) from such activities as sandblasting. Silicic acid (H_2SiO_3 or *silica gel*) is produced inside the macrophages that ingest the silica. They die, releasing toxic compounds that lead to fibrosis. Late stage disease may show right heart enlargement (congestive heart failure). It is a self-perpetuating problem once exposed. No treatment is available.
The acute form of the disease is rapidly fatal (1-2 yrs). The chronic form can take decades. The prognosis is quite poor in moderate to severe cases.

11.2.4. Asbestosis

There are various types of asbestos: crocidolite, amiosite, and chrysotile, that have been used in various types of insulation. Pathologically, 0.5 µm fibers appear to be the worst. Macrophages attempt ingestion of fibers in the lung (F11.14), which leads to diffuse lung damage and fibrosis.
While there is less elastin detected in asbestosis, more of the different types of collagen are found. The disease takes 20-40 years to develop, and the clinical outlook is poor. Asbestosis can also trigger mesothelioma and, in association with cigarette smoking, bronchogenic carcinoma (the rate is increased by a factor of 80). Controlling exposure is the only solution.
Clara cells with high xenobiotic metabolism will proliferate at the level of bronchioles and alveoli, even replacing mucus cells in an effort to proliferate and repair the lung's epithelium.
Asbestos is also linked with auto-immune diseases (rheumatoid arthritis, lupus, and scleroderma)[6].

11.2.5. Agents producing Reactive Airway Diseases

Nonfibrogenic material may be inorganic (talc, fibrous glass, metals) or organic (wood, raw cotton, flax bagasse, mouldy hay or grain, tea, tobacco).



F11.14. A macrophage attempts to dispense with an asbestos fiber. ROS are induced by NADPH oxidase and the high iron content in asbestos further catalyzes hydroxyl radicals. The cell then generates TNFα and other chemoattractants, stimulating fibrosis and tumor formation.
Boelsterli, 2007.

11.2.5.1. Byssinosis

While there is a strong component related to the mechanical effects of the dust particles in *byssinosis* (brown lung in cotton mill workers, silo worker's lung, farmer's lung), there is also an antigenic component, since the organic vegetable matter will draw an immunological response over time.
Cotton, flax or hemp fibers trigger mast cells to release histamine, constricting the bronchi. Histamine stores need to replenish, and symptoms are always worse when returning to work.
Intermittent reactions can occur over the course of 6 to 8 years. As time passes, the duration of symptoms (chest tightness, wheezing, shortness of breath) can increase.
There are significant changes in maximal expiratory flow rates from spirometry, after exposure. Symptoms occur intermittently at first. If protection is implemented, disease is reversible, but permanent airway constriction can occur otherwise.

11.2.5.2. Occupational Asthma

F11.15. Passing a battery of tests to measure the severity of asthma.

The longer you work with symptoms of occupational asthma, the less likely complete recovery becomes. These symptoms usually include coughing, wheezing, chest tightness and shortness of breath. The symptoms often start while at work, improve at home but sometimes awaken the person at night. Workers may also

experience itchy or watery eyes, stuffy, runny nose and skin rashes.
Woods (western red cedar, mahogany, teak, exotic woods from Africa), flour, polyvinyl chloride fumes, and isocyanates induce an immune response, with symptoms similar to asthma (T11.15).
Toluene *diisocyanate* is widely used as an additive in the production of polyurethane foam and paints. It reacts with water with evolution of CO_2. Once the individual is sensitized, very small concentrations are effective in producing obstructive reactions.

11.2.5.3. Hypersensitivity Pneumonitis

Pneumonia is often mistakenly diagnosed in Hypersensitivity Pneumonitis, also named Allergic Alveolitis (T11.16.). Over years, if the correct diagnosis is not made and if exposure persists, the lungs can be permanently damaged.
10% of the victims of this disease do not develop full-blown reactions, and they simply experience insidious shortness of breath. When the symptoms become more severe, and the situation is recognized, heavy lung damage has often already occurred.

Farming is a hazardous occupation, the lung hazards spanning mechanical effects, irritants, and chemical injury: ammonia, anhydrous ammonia, carbon dioxide, carbon monoxide, hydrogen sulfide♣, methane, nitrogen dioxide, grain feed, animal hair and skin, wastes, sandblasting residues, organophosphates as well as pesticide dusts, sprays, liquid pours, salves and dips all contribute.

T11.16. Causes of Occupational Asthma
from Scientific American

Hazard	Persons at Risk	Sensitizing Agent
Laboratory animals, birds, insects, other animal products	Laboratory workers, animal handlers, veterinarians Pigeon breeders, poultry workers, bird fanciers Grain workers Entomologists Crab and prawn processors	Rats, mice, rabbits, guinea pigs Pigeons, chickens, budgerigars Grain mites Moths and butterflies Crabs and prawns
Plants, wood dust	Bakers Food processors Tea workers Tobacco manufacturers Carpenters, sawmill operators, cabinetmakers	Wheat flour, rye flour Coffee beans, castor beans Tea leaves Tobacco leaves Wood dusts, including western red cedar dust
Biologic enzymes	Detergent industry workers Pharmaceutical industry workers, biomedical researchers	*Bacillus subtilis* Pepsin, trypsin, bromelain
Isocyanates	Workers with polyurethane, plastics, and varnish Automobile spray painters	Toluene diisocyanate Hexamethylene diisocyanate
Anhydrides	Workers with epoxy resins and plastics	Phthalic, trimellitic, and other anhydrides
Metals	Tanners Platinum refiners Metal platers	Chromium Platinum Nickel
Fluxes	Aluminum solderers Electronic workers	Aminoethylethanolamine Colophony
Drugs, other chemicals	Pharmaceutical workers Workers with plastics and rubbers Insulators Refrigeration workers Hairdressers	Penicillins, cephalosporins, methyldopa, spiramycin, tetracycline Azodicarbonamide Urea, formaldehyde Freon Persulfate salts, henna

♣ Hydrogen sulfide has a physiological role in the body as a vascular dilator, regulating blood pressure.

The involvement of the immune system in hypersensitivity Pneumonitis is highlighted in “detergent worker's lung”. In the manufacture of detergents, semi-purified proteolytic enzymes (papain) are added. After a certain time, an asthma-like condition results when the protein antigens are absorbed through nasal membranes or in the lungs. An immune response develops, with histamine release.
If substances do not cause either *pulmonary fibrosis* or *sensitization* of the pulmonary immune response, they are considered *inert*. However they can still load the respiratory clearance mechanisms (mucociliary, phagocytosis) to the point where physical blockage occurs, with a reduction in air flow, and labored respiration.

11.2.6. Asphyxiants

Simple asphyxiants such as CO_2, methane or nitrogen are inert. They are a threat because they replace oxygen. Chemical asphyxiants like CO, H_2S and cyanide on the other hand alter oxygen-carrying capacity or respiratory enzymes.

11.2.7. Irritant Gases

Water soluble gases do not go very far into the lung. Many gases such as ammonia, halogen acids (HF, HCl, HBr), oxides of sulfur, hydrogen sulfide, sulfuric and nitric acids, all are irritant to the upper respiratory tract because of their great solubility in the mucus of the nose and throat. Fortunately, these agents are sufficiently irritant to warn the exposed individual of their presence before dangerous exposures are reached. Exceptions to this rule are hydrogen sulfide and ammonium sulfide, where tolerance to the odor can occur and hypoxia strike without warning. T11.18 classifies respiratory airway irritants.

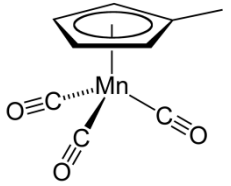
T11.17. Hypersensitivity Pneumonitis. *Scientific American*

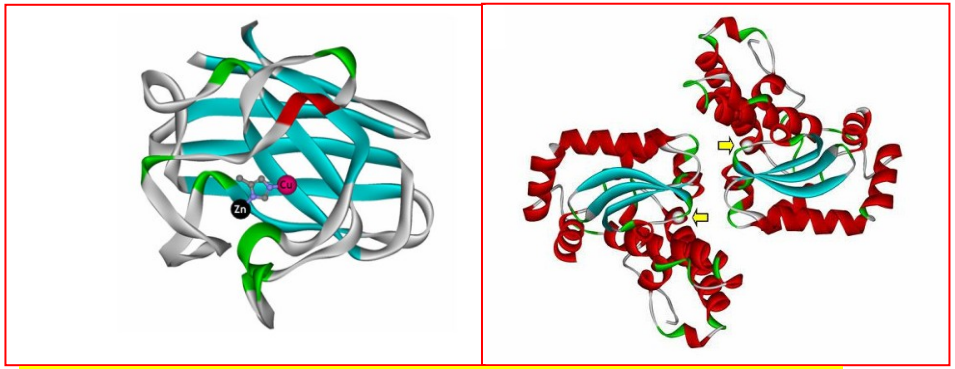
Disorder	Source of Antigen	Antigen Responsible
Aspergillosis	Aspergillus spores	Various aspergilli
Bible-printer's lung	Moldy typesetting water	Unknown
Bagassosis	Moldy sugarcane	Thermophilic actinomycetes (Thermoactinomyces vulgaris)
Bird (avian) contact lungs Budgerigar-fancier's lung Chicken-handler's lung Pigeon-breeder's lung Turkey-handler's lung	Parakeets Chickens Pigeons Turkeys	Unknown antigen (may be present in avian droppings or serum)
Cheese-washer's lung	Cheese mold	*Penicillium casei*
Coffee-worker's lung	Coffee dust	Unknown
Corn-farmer's lung	Corn dust	Unknown
Detergent lung	Detergents (process or use)	*Bacillus subtilis*
Farmer's lung	Moldy hay or grain	*Micropolyspora faeni, T. vulgaris, T. sacchari* IgG antibody reactions at peripheral bronchi.
Furrier's lung	Hair dust	Unknown
Humidifier/air cond. lung	Humidifiers/air conditioners	Thermophilic actinomycetes
(Wood) Joiner's lung	Sawdust	Unknown
Malt-worker's lung	Malt dust	Various aspergilli
Maple bark-stripper's lung	Moldy maple bark	*Cryptostroma corticale*
Mummy-handler's lung	Cloth wrapping of mummies	Unknown
Mushroom-worker's lung	Mushroom compost	Thermophilic actinomycetes
Paper mill-worker's lung	Moldy wood pulp	Various species of *Alternaria*
Paprika-slicer's lung	Moldy paprika	*Mucor stolonifer*
Pituitary snuff syndrome	Pituitary powder	Unknown
Sequoiosis	Moldy redwood dust	Various species of *Graphium*
Smallpox-handler's lung	Smallpox scabs	Unknown
Suberosis	Moldy cork dust	*Penicillium frequetans*
Tea grower's lung	Tea plants	Unknown
Wheat-weevil lung.	Wheat flour	*Sitophilus granarius*
Wood pulp-worker's lung	Moldy logs	Various species of *Alternaria*

T11.18. Respiratory irritants classified by vulnerable region.		
Upper Tract	**Tract and Lungs**	**Terminal passages and Air sacs**
Aldehydes Alkaline dusts Ammonia Chromic acid Sulfur di/trioxide Ethylene oxide HCl, HF	Br, Cl, F, I Ozone Chlorine oxides Sulfur chlorides P trichloro-penta- chloride Dimethyl sulfate Toluene diisiocyanate	Arsenic trichloride Nitrogen dioxide Nitrogen tetroxide Phosgene Organic solvents

SO_2 will penetrate the lung if adsorbed on particles, but NO_2, CO and H_2S can reach to the alveoli on their own. Such agents usually have a lower water solubility, or hydrolyze in the mucus to produce irritating secondary agents such as carbonyl chloride (phosgene) and oxides of nitrogen. Because they reduce pH progressively, the chemicals become highly irritant later (delayed effect). Some irritants alter respiratory function and these changes are distinctive in some cases.
Metals, particularly when freshly generated as fumes, are of small enough particle size to penetrate deeply into the lung, and will be efficiently absorbed. For example, approximately 40% of lead dust or "fume" reaches the lungs and 10% of that ingested is absorbed into the body.

"Metal fume fever" has been caused by inhaling fumes of antimony, arsenic, beryllium, cadmium, cobalt, copper, iron, lead, manganese, mercury, nickel, tin and zinc, creating a series of symptoms not unlike those of malaria or influenza. The worker develops chills, fever, nausea, cough, aches in bones a few hours after exposure, with the fever "breaking" after several hours, followed by profuse sweating and, by 24 hr after onset, little trace of the condition.
Organic forms of metals (tetramethyl and tetraethyl lead, alkyl aluminum, alkyl mercurials, metal carbonyls such as nickel carbonyl [$Ni(CO)_4$] or MMT (shown), manganese methylcyclopentadienyl tricarbonyl), and volatile hydrides (arsine, stibine, lithium, phosphorus-phosphine [PH_3]) all show greater toxicity than their respective metals or metalloids, due to their volatility, fine aerosols, and ready absorption through the lung because of their lipophilicity.





F11.19. Cu-Zn and Mn Superoxide Dismutase
(Mn at position of arrows)
Molecular Toxicology, Josephy, 2006

11.2.8. Pharmaceuticals

T11.20	Category I	Category II	Category III	Category IV
Occupational exposure ($\mu g/m^3$)	>500	500-10	10-0.1	<0.1
Toxicity and potency	Low	Moderate	Potent	Highly potent
Examples of drugs in this category	Naproxen, acetaminophen, erythromycin	Atorvastatin, nicardipine, oxycodone	Thalidomide, fentanyl	Leuprolide, nafarelin acetate, ethlnyl estradiol

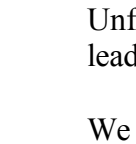
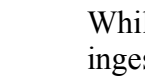
from Pharmaceutical Technology, 2006

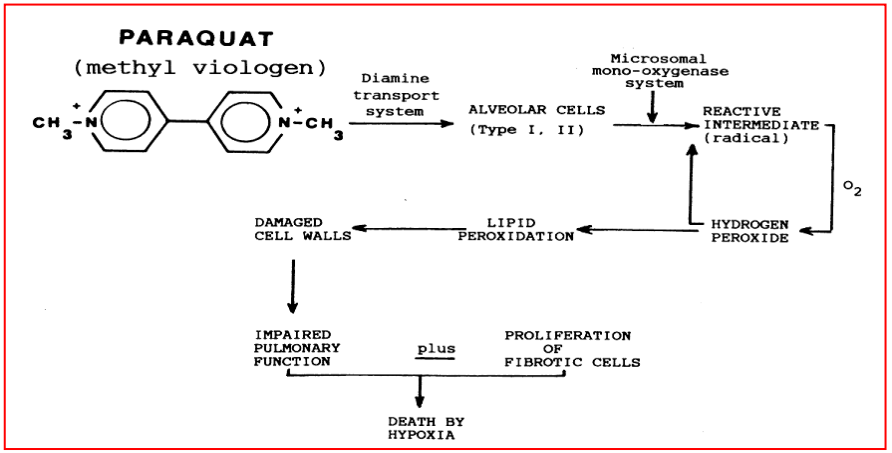
11.2.9. Agents inducing Chemical Toxicity

Vapors and gases can have devastating effects on the pulmonary tissue, damaging Type II Alveolar and Clara cells that contain the Phase I and II enzyme systems involved in xenobiotic biotransformation. In addition, as the Alveolar Type II and Clara cells die, their place is taken up by fibrotic cells. Unfortunately, fibrotic cells are not adapted to gas exchange, leading to hypoxia.

We have learned much about chemical toxicity in the lung through research on a herbicide, paraquat (F11.22). Paraquat, also known as methyl viologen, acts as a catalyst for cell damage, activating oxidation and lipid peroxidation. Oxidative stress is a major aggressor in the lung: breathing 100% oxygen destroys the lungs in about three days by superoxidation While highly polar when absorbed by inhalation or by ingestion of concentrated formulation, paraquat concentrates in the lung as a result of a diamine-polyamine transport system. There, it acts as a true catalyst, never broken down or destroyed. It produces oxygen radicals (superoxide anion O_2^-) that are converted to hydrogen peroxide by the tissue enzyme superoxide dismutase (F11.19.). These derivatives attack polyunsaturated lipids in cell membranes to produce lipid hydroperoxides which, in turn react with other unsaturated lipids to form more lipid-free radicals, thereby perpetuating the system. The resulting damage to cell membranes and to subcellular organelles (mitochondria) reduces the functional integrity of the cells, affects efficient gas transport and exchange and impairs respiration by destroying essential cell types (Alveolar Type II, Clara cells). This fosters the proliferation of fibrotic cells and a loss of elasticity of the lung. Paraquat intoxication is fatal within 3 to 4 weeks, with no cure.

T11.21. Chemical Warfare Agents
In the first world war, the use of chlorine, mustard gas and phosgene produced 1,250,000 casualties.

Agent	Appearance Odor	Absorption	Rate of Action	Lethal Dose (mg)	Effects
Mustard (blister agent) $C_4H_8Cl_2S$ 	Colorless to brownish liquid; slight odor of garlic or mustard	Skin contact & inhalation	Delayed for hours; long-term complication	1500 inhaled 4500 skin	Eye & airway irritation, tearing, chemical skin burns & blisters, pulmonary edema, respiratory failure
Phosgene (choking agent) CCl_2O 	Colorless gas; odor of fresh mown hay	Inhalation	Immediate to 72 hours	3200 inhaled	Eye & airway irritation, pulmonary edema, choking



F11.22. Mechanism of cellular action of paraquat.

The complexity of chemical-induced pulmonary toxicity is illustrated by trimellitic anhydride (TMA), a chemical used widely in the plastics industry and as an intermediate in the preparation of resin adhesives, polymers, dyes, and printing inks[3].
This agent induces four syndromes in humans, three of which are immunological, TMA acts as a hapten to induce

(1) TMA-flu;
(2) pulmonary distress anemia;
(3) immediate onset asthma;
(4) immediate irritant syndrome (non-immunologic).

11.2.10. Agents of Lung Cancer

Lung cancer is an important cause of mortality, and is caused by many factors. The cells of origin of many lung tumors are unknown. Cigarette smoke is an important cause. However, radiation (alpha, beta, gamma, etc), chemical carcinogens, particles, immune status of the cancer patient, exposures to other agents causing cell proliferation such as saline (intratracheal instillations), mineral dust, etc. can all be combined to result in cancer. Even stress and nutrition are implied as etiologic agents.
Some exposures, such as raw cotton dust, appear to reduce by 40% the risk of lung cancer, possibly by encouraging a chronic immune reaction[7].
In contrast to what was described above for chemically induced lung damage, carcinomas generally arise from long-term exposure to low concentrations of:

- volatile amines (convertible to N-nitroso compounds),
- polycyclic aromatic hydrocarbons (PAHs) from a variety of petrochemical products,
- combustion products of tobacco,
- asbestos, nickel, beryllium, cadmium, and
- low molecular weight, volatile chemicals capable of being converted *in vivo* to highly reactive and unstable intermediates that can mutate DNA.

Particulate material from combustion products (tobacco, wood, coal, oil, etc.) may have potentially toxic chemicals adhering to the surface of the particle. When inhaled and deposited in the lung, it is possible for the toxicant to desorb from the particle into the mucosal surface of the nasal passages, larynx, bronchi, etc. The transfer efficiency depends on the physicochemical properties of the agent (lipid solubility, molecular size, electrostatic charge, van de Waal's forces). This results in high concentrations of toxicant localized in small regions, for example the bifurcation point in a bronchus where particles build up. Mostly, lung cancer originates at the level of the bronchioles.
From the above, it is not surprising that the International Agency for Research on Cancer (IARC) has classified diesel engine exhaust as carcinogenic to humans (Group 1), based on sufficient evidence that exposure is associated with an increased risk for lung cancer and limited evidence for an increased risk of bladder cancer. The IARC Working Group also concluded that gasoline exhaust was possibly carcinogenic to humans (Group 2B). There are currently no limits on such emissions, but to control the cancer risk, they would probably have to be set below 20 $\mu g/m^3$.

11.2.11. Cigarettes

Effects of air-borne industrial agents on health are small in comparison with the effect of **cigarette** smoking.

The effects of smoking:

1. paralysis of cilia (one puff of cigarette smoke can paralyze the cilia for 20-40 minutes),
2. irritation of the mucosa,
3. increased secretion of mucus, leading to reduced effective bronchiole size,
4. hypertrophy of mucus glands encroaching on airway lumen and leading to increased air flow resistance,
5. disabling of macrophages' functions,
6. increase in macrophage and leukocyte population and decrease in level of proteolytic enzyme inhibitors, leading to progressive *digestion of the septa*.

Nicotine shifts cells of the body into high gear, mimicking acetylcholine. Nicotine prevents apoptosis and boosts cell survival. Dermatologists say that nicotine can speed dermal fibroblasts to go through a 10 week growth phase in only 10 days. In young animals, nicotine consumption increases probability of addiction to other drugs[8].

About 600 additives have been used in cigarettes, one of which is ammonia. Many were used for flavoring. Nicotine in cigarette smoke is initially in the form of an electrically charged non-volatile acid, preventing passage into the blood. into a gas. Ammonia converts the acid to a free base or uncharged alkaline form, enhancing transfer into the lungs by as much as 100 times.

A similar procedure is used to treat cocaine with alkaline materials to produce *crack*, which is much more lipid-soluble[4]. Effect 6 above is more familiarly known as **emphysema**, which is digestion of the walls of the alveoli through proteolysis. As a result of disturbed alveolus anatomy, there is accumulation of uncirculated air pouches in the lung.

90% of lung cancers are due to smoking. The death rate is 90-95%.

11.3. Evaluation of Lung Function

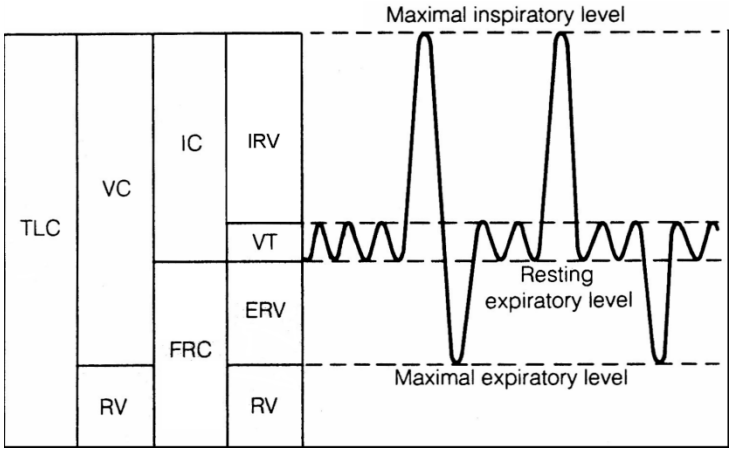
11.3.1. Bronchial Provocation

In asthma, the bronchi respond to a number of stimuli. *Bronchial responsiveness* can be important in establishing a diagnosis of asthma. Acetylcholine, histamine or other chemicals can be used to provoke an *obstructive episode*. But dry, cold air is the safest agent to use.
Asthma and bronchitis primarily affect the bronchial tubes. Asthma is an **obstructive** (obstruction to air flow) lung disease, as opposed to fibrosis which is **restrictive** lung disease (mechanical compliance of the lung is reduced – *lung stiffens*).

11.3.2. Spirometry

Spirometry is the measurement of expired and inspired lung volumes. There are four *subdivisions of total lung volume* (see F11.23:

- Tidal volume (VT), the volume of air inspired and expired with each breath,
- Inspiratory Reserve Volume (IRV), the maximum volume of air that may be inhaled beyond the normal tidal breath,
- Residual Volume (RV), the volume of air that remains in the lungs after maximal expiration, an
- Expiratory Reserve Volume (ERV), the maximum volume that may be exhaled between resting end-tidal position and residual volume.

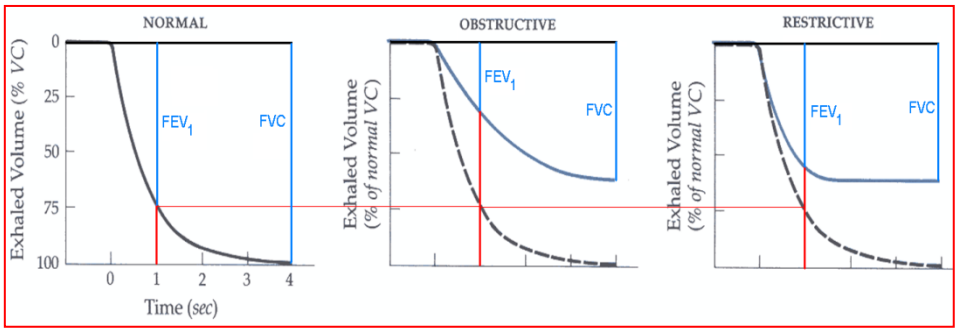


F11.23. Lung Volumes.

On the basis of these four volumes, four *lung capacities* can be described:

- Total Lung Capacity (TLC), the amount of air in the chest after maximum inspiration, equal to the sum of all four lung volumes (TLC = RV + ERV + VT + IRV),
- Vital Capacity (VC), the maximum amount of air expired after a maximum inspiration (i.e. the total amount of air that can be moved in and out of the lungs (VC = ERV + VT + IRV = TLC – RV),
- Functional Residual capacity (FRC), the amount of air remaining in the lungs at the end-tidal position (FRC = RV + ERV),
- Inspiratory Capacity (IC), the maximum volume of air inspired from end-tidal position (IC = TV + IRV).

The *forced vital capacity* (FVC) curve is determined by asking the subject to give a maximum expiration *as rapidly as possible* after a maximum inspiration.
The forced expiratory volume in 1 second (FEV_1) and the FEV_1/FVC ratio are derived from the FVC curve.



F11.24. A normal spirogram compared to those in obstructive and restrictive lung disease. *modifed from Scientific American.*

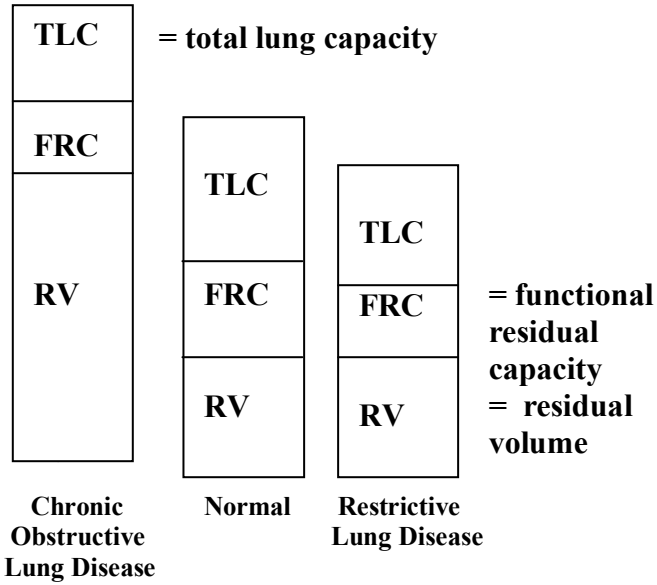
The FVC, FEV_1, and FEV_1/FVC ratio can be predicted from Knudson's equations (Am Rev Resp Dis 1983;127:725-734).

White Male, Age < 25
FVC = -6.8865 + (.059 * HT) + (.0739 * AGE)
FEV1 = -6.1181 + (.0519 * HT) + (.0636 * AGE)
FEV1/FVC% = 100.4389 + (-.0813 * HT)
White Male, Age ≥ 25
FVC= -8.7818 + (.0844 * HT) + (-.0298 * AGE)
FEV1-6.5147 + (.0665 * HT) + (-.0292 * AGE)
FEV1/FVC% =86.6862 + (-.105 * AGE)
White Female, Age < 20
FVC = -4.447 + (.0416 * HT) + (.0699 * AGE)
FEV1 = -3.7622 + (.0351 * HT) + (.0694 * AGE)
FEV1/FVC% = 109.9739 + (-.1909 * HT) + (.6655 * AGE)
White Female, Age ≥ 20
FVC=-2.9001 + (.0427 * HT) + (-.0174 * AGE)
FEV1=-1.405 + (.0309 * HT) + (-.0201 * AGE)
FEV1/FVC% = 121.6777 + (-.1852 * HT) + (-.1896 * AGE)
Height (HT) is in cm; AGE is in years.

The FVC, FEV_1, and FEV_1/FVC ratio are usually reduced in subjects with *obstructive lung disease*.
Subjects with *restrictive lung disease* will have reduced FVC and FEV_1 but a normal or increased FEV_1/FVC ratio, because both the FVC and FEV_1 are reduced by similar amounts. Spirometry test results are compared to tables of predicted values derived from a normal population of non-

smoking adults. The predicted values are based on height, age, gender, and race/ethnicity. Results are in liter and expressed as a *percentage of the expected Vital Capacity*.

	FEV_1	FVC	$FEV_1/FVC\%$
Normal	**$\geq$ 80%**	**$\geq$ 80%**	**$\geq$ 75%**
Obstructive	**< 80%**	**< 80%**	**< 75%**
Restrictive	**< 80%**	**< 80%**	**$\geq$ 75%**
Mixed Obstructive/ Restrictive	**< 80%**	**< 80%**	**< 75%**

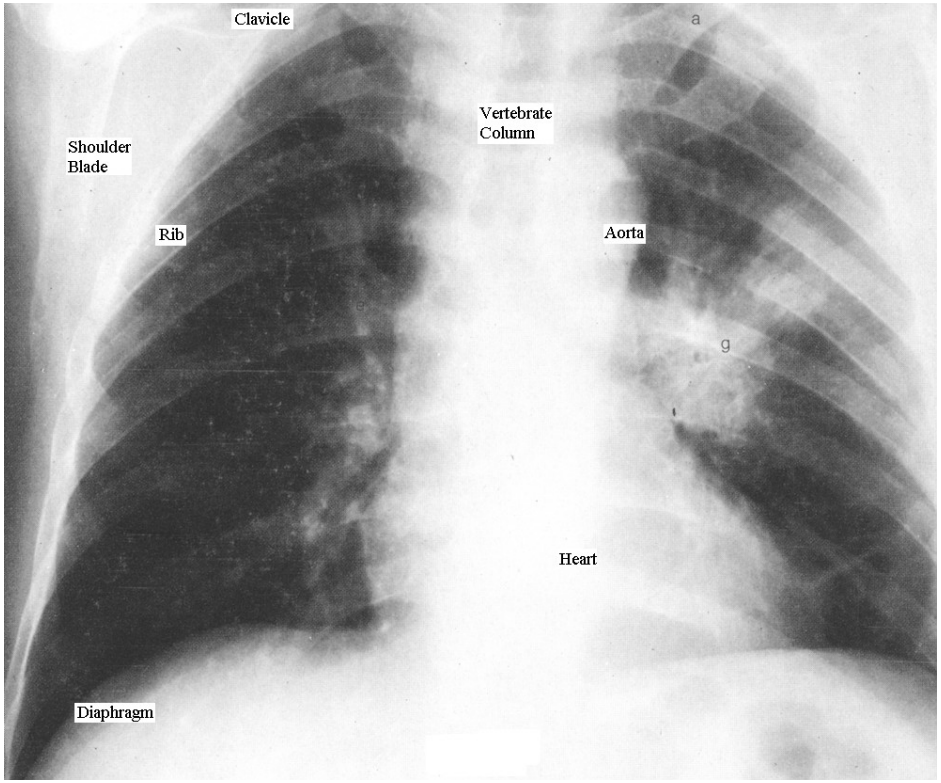


Lung function abnormalities in classic, simple silicosis are uncommon in the early stages, even when the chest x-ray shows small rounded opacities. Multi-factorial effects of cigarette smoking, the type of dust involved (such as a mixture of dusts), the dose of the dust and the duration of exposure, and the presence of other pulmonary diseases such as tuberculosis may contribute in the alteration of an individuals patient's pulmonary function. Pulmonary function testing in workers with advanced (PMF) disease will show severe restriction or mixed pattern of obstructive/restrictive defect, loss of pulmonary compliance, and hypoxemia.

In acute silicosis there is usually a rapid progressive decline in pulmonary function with eventual respiratory failure.

F11.25. Volume (accumulates air) and Flow (measures flow) Spirometers.

11.3.1. Chest X-ray



F11.26. Chest X-ray showing an inflammatory process in the left lung ("g"). *Biological Structures.*

Another important diagnostic procedure is the chest X-ray (F11.26).

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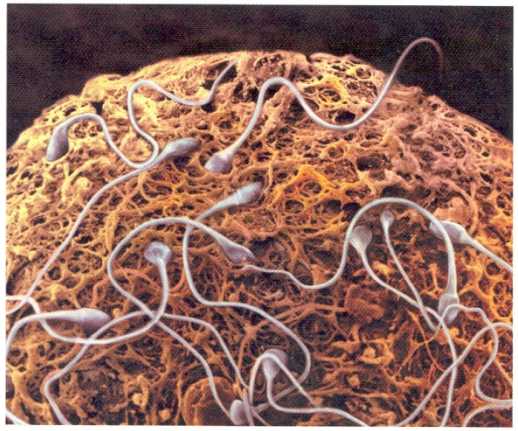
Reproductive toxicity

12· Reproductive toxicity



Reproductive Toxicology studies the occurrence, causes, manifestations, and sequelae of adverse effects of exogenous agents on reproduction.
The potential for an interaction between a chemical and one of the many stages of reproduction is almost inexhaustible.

12.1. Vulnerability of Reproduction

In humans, 1out of 8 pregnancies spontaneously miscarry because of structural chromosome abnormalities in the fetus. Other known causes are hormonal imbalance, infection, alcohol, smoking and drug abuse. Mother's immunity may account for as many as 80% of unexplained miscarriages.

1/200 people have chromosomal abnormalities at birth.
Drugs and environmental chemicals have traditionally accounted for *only a very small percentage* of malformations in man (T12.1), and the situation was believed the same for fetal wastage (spontaneous abortions).
This may seem surprising, until one appreciates the *normal extent* of early adverse reproductive outcomes (T12.2).

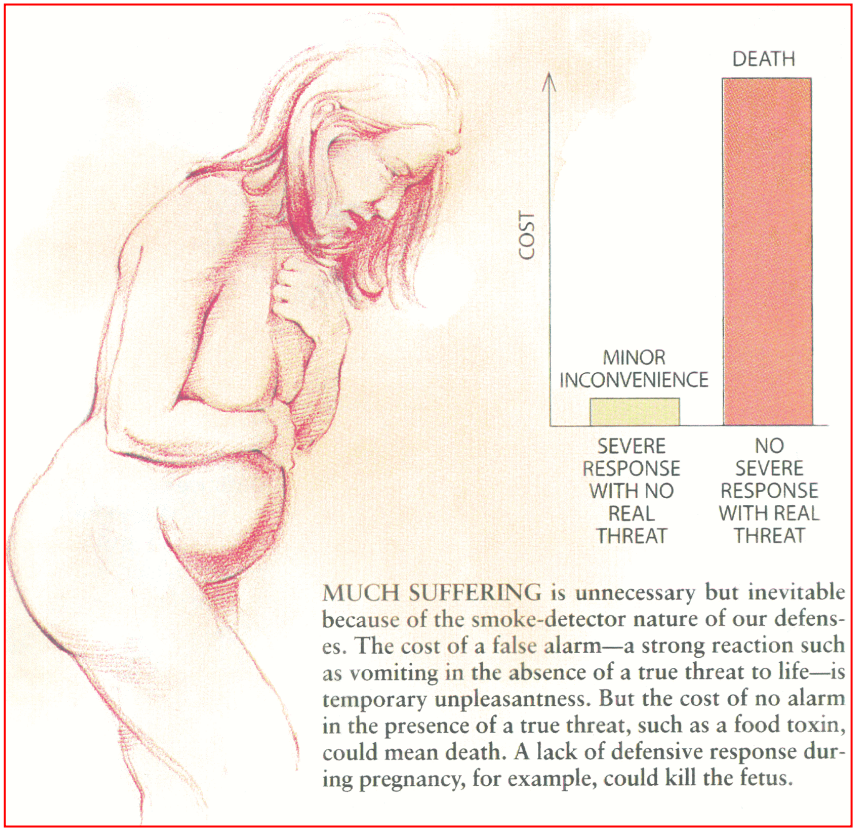
Since adverse reproductive outcomes are common, the detection of those that are specifically related to chemicals is difficult. But recent evidence points to more troubling results. An English study found that living within 3 km of a hazardous waste landfill increases birth defects by 41%[9].
Reproductive success is influenced by a wide range of factors, from neurological components (loss of libido) to the complex hormonal controls of both spermatogenesis and oogenesis, specifically luteinizing hormone (LH) and follicle-stimulating

T12.1. Causes of malformation in man.
Wilson, Fed. Proc. 36, 1698-1703, 1977

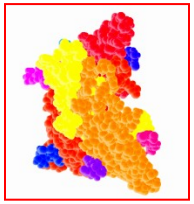
Mechanism	% Incidence
Genetic transmission	20
Chromosome aberration	3-5
Environment	
radiation	1
infection	2-3
maternal metabolic imbalance	1-2
drugs and environmental chemicals	4-6
potentiation interactions	?
Unknown	**65-70**

T12.2. Reproductive Outcomes in Human Development

While women are born with 1 million oocytes, only about 500 turn into full-fledged eggs over their lifetime.
40% of all human embryos die before implantation
60% of all fertilized human embryos die during a normal pregnancy
7.5% of all delivered infants have developmental abnormalities that interfere with survival or quality of life
Among wasted feti:
- -chromosomal aberrations (65% of all first trimester abortions)
  - -35% are karyotypically abnormal
  - -30% have abnormal morphological development



F12.3. Enhanced Toxicity Defense in Pregnancy.
Scientific American.



hormone (FSH, shown).
In spite of all precautions, test-tube babies are twice as likely as naturally conceived babies to have multiple major birth defects and are more likely to be under weight at birth.

It is believed that in pregnancy, to preserve the reproductive process, toxicity defenses of women are hyperactive, leading to such symptoms as chronic nausea (F12.3). In this case, the sensitivity of toxicant detection is magnified to the point where some specificity is lost.

12.2. Vulnerability of Cell Division

The process of giving birth is composed of gametogenesis, fertilization and embryonic and fetal development, all events that depend heavily on **cell division**.
Gametogenesis, for example, is vulnerable because the stem cells involved in spermatogenesis and oogenesis undergo several cycles of mitosis (replication as diploid cells) followed by stages of meiosis (reduction in the number of chromosomes to a haploid state).
To carry out these activities, the nucleic acids (DNA) must be dissociated from the protective protein (histones) that normally surround the nucleic acid, leaving them exposed to chemical attack.
Cell division is resource, hormone and cytokine dependant, and division *speed* influences the outcome of embryo structures. Any toxicity, even only changing the speed of processes, can induce problems in embryo structure.
Considering the level of cell division that occurs in the process of reproduction, it is surprising that so few cancers develop in association with the process.

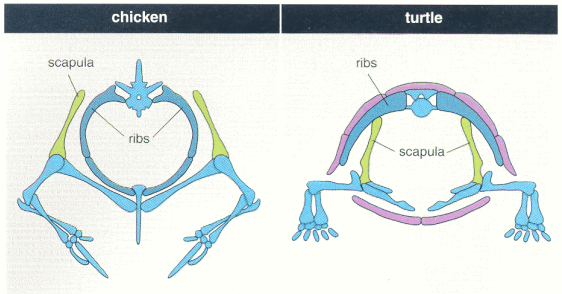
12.3. Reproductive Toxicity Mechanisms

12.3.1. Timing of Events in Reproduction

Any agent capable of altering the speed of cell processes, such as so-called cell division *checkpoints*, is a potential teratogen. There are specific reasons for this: the Hox genes (homeobox) make up a set of conserved genes that setup an animal's body plan. The physical order of the Hox genes along the chromosomes corresponds to body sequence: head, thorax then abdomen. This is also the order in which genes are activated during development, so timing is important. Small differences

may be tolerated: much of the observed variations in dogs result from genes that alter development speed. But when pregnant sheep eat the "corn lily" mountain flower, they give birth to lambs with grotesque birth defects, such as a missing eye. The chemical responsible was named *cyclopamine*. It is known that *cyclopamine* specifically inhibits the growth of certain brain cancer cells (medulloblastoma)[11].

In *heterochrony*, the timing of developmental events is changed, and as a result radically different structures can emerge. *Heterochrony* can result in *heterotopy*, which means that a developmental event occurs in a new location. Some structures could, as a result, come in contact with other structures from which they are normally separated. *Heterotopy* was shown in animals to result in a variety of phenomena, such as extra set of legs or wings, or entirely unique structures. The bone structure of chickens and turtles uses the same building blocks, but *heterochrony* and *heterotopy* produce entirely different body plans. For example, the relative location of ribs and scapula (shoulder blade) is completely different in each animal (Fig).

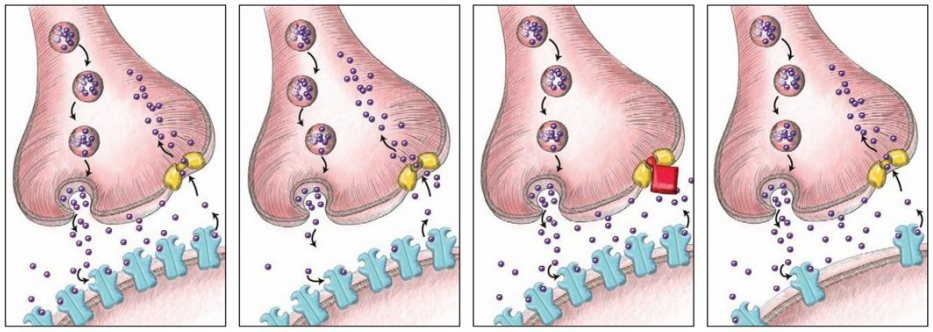


F12.4. The scapula (shoulder blade) ends up in different locations in different animals as a result of different developmental timing. *American Scientist, May-June 2010.*

This explains why reproduction is so vulnerable: small changes in the speed of processes individualize a person, but larger changes can lead to malformations. When malformations can be stabilized into a coherent whole, a different body plan may emerge, but understandably, most alterations lead to functional reductions.

12.3.2. Up- or Down- regulation of Nerve Transmission

It is thought that exposure of developing fetuses to certain drugs, for example, serotonin reuptake inhibitors, can leave a permanent imprint in their brains (see Figure below).



No mood disorder Serotonin (purple) is made in the nerve cells and released into an area called the synapse, where it hits the next nerve and is then reabsorbed.

Depression People with depression have a lack of serotonin in the synapse, either because not enough is produced or because too much is reabsorbed (shown).

SSRI treatment Selective serotonin reuptake inhibitors block the absorption of serotonin, leaving more in the synapse. This means more can hit the next nerve cell.

Developing baby Some animal studies suggest that a developing baby exposed to SSRIs will set its serotonin system differently, resulting in too few nerve cell receptors.

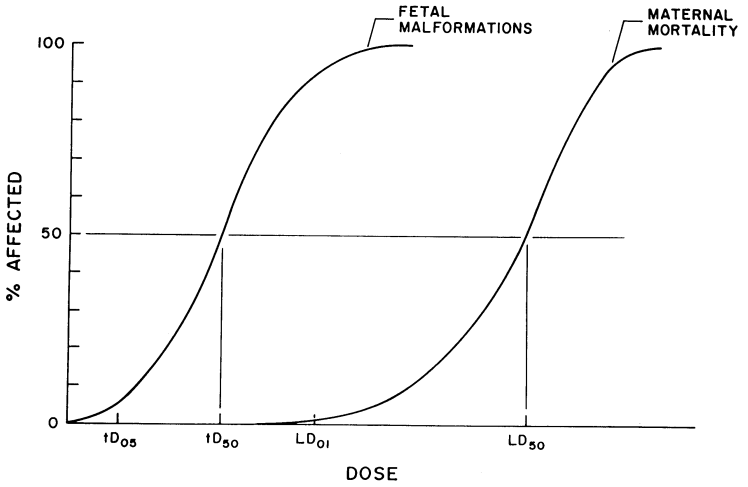
F12.5. A developing baby exposed to Selective Serotonin Reuptake Inihibitors may set its serotonin system diffrently. *Science News, June 2010.*

12.4. Teratogenicity Index

What levels of exposures can produce malformations, as compared to generally detectable toxicity?
The definition below relates an incidence of some form of teratogenicity to some form of toxicity (maternal mortality, in F12.6).

$$\textit{Relative Teratogenicity Index} = LD_{01}/tD_{05}$$

An RTI of 1 means that toxicity is not specific to the embryo, because a number of mothers also suffer toxicity. An RTI of 4 is the formal threshold indicating that the embryo is more sensitive than the adult.



Dose-response curves for fetal malformations and maternal mortality in a hypothetical teratogenicity experiment. The tD_{50} and LD_{50} are, respectively, the doses at which half the fetuses are malformed and half the mothers killed. The tD_{05} and LD_{01} are derived in the same manner, but at the 5 and 1% levels, respectively. This compound would be considered a teratogenic hazard because the ratio of the LD_{01} and tD_{05} is about 4.

F12.6. Teratogenicity Index determination. *Scialli.*

The outcome of the teratogenicity can be interruption of the reproductive process, or alteration of the newborn (classical teratogenesis).

12.5. Sperm

Eggs normally repair any damage in their fertilization partners, so scientists have been more concerned with eggs than sperm. Eggs remain viable for 12 to 24 hours (ovulation), while sperm can fertilize eggs for up to 5 days in the female reproductive tract.

Glutathione peroxidase 5 (GPX5) prevents oxidative damage to sperm DNA by fighting hydrogen peroxide. Without GPX5, older mice (and maybe men) have a higher risk developmental defects. A related protein, GPX4, is associated with infertility in people[34].

Spermatozoids are produced on-goingly from stem cells in a 74 day process. Each year after puberty, sperm producing cells replicate about 23 times.

Sertoli cells are the elongated cells in the tubules of the testes to which the spermatids are initially attached (F12.8). They provide support, protection and nutrition. Using tight junctions, Sertoli cells also provide the *blood-testis barrier* which maintains different fluid compositions in the blood and in the lumen of the seminiferous tubule, which is important for sperm development. The barrier also prevents immune attacks on sperm by the male's immune system.

T12.7. Pathology at Conception.

Male function: sperm production and transport
Female function: maturation-release of ova and function of the corpus luteum

- stress, infections, trauma (physical, mental)
- neurological and hormonal components that govern libido, performance, activity
- stress, endocrine disorders, strenuous exercise and malnutrition in females
- drugs, therapeutic agents, industrial chemicals

In spite of this, a large proportion of abnormal sperm are produced. Men younger than 20 and older than 30 produce more abnormal sperm. If as much as half of the sperm is abnormal in morphology or motility, the male is usually infertile.

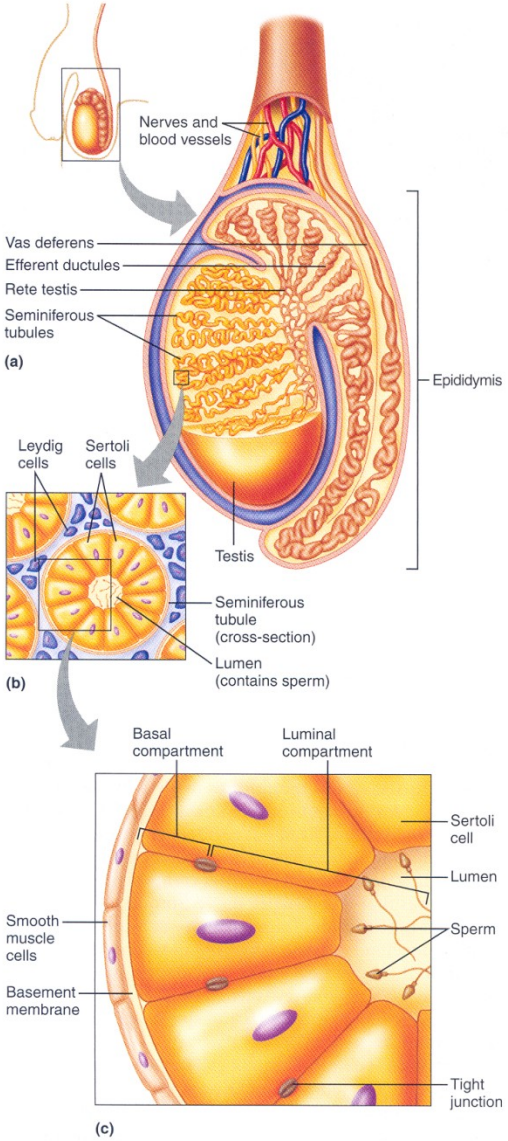
F12.8. Testis.

a: Seminiferous tubule inside the human testis.

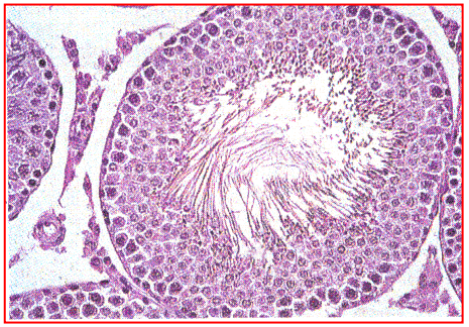
b: section of seminiferous tubule with location of cells at low magnification.

c: portion of seminiferous tubule, showing association of Sertoli and germ cells.

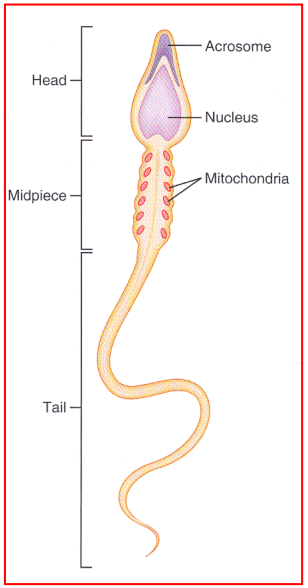
Germann & Stanfield, 2002.

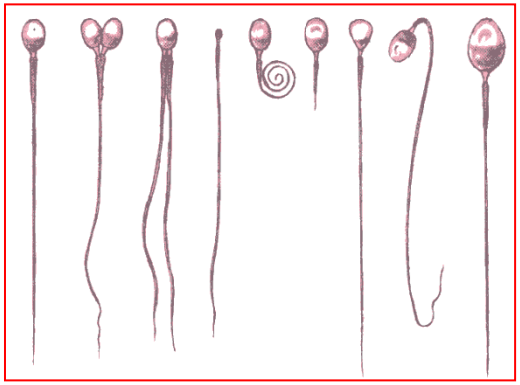


F12.9. In the rat, seminiferous tubules are about 150 µm in diameter. Hair-like structures are flagella of nascent sperm.

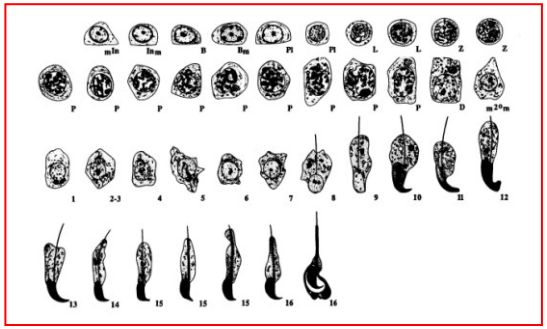


F12.10. Human sperm anatomy. >
Germann & Stanfield, 2002.





F12.11. Abnormal sperm compared to Mr. Right (on the right). *Guyton.*



F12.12. Stages of development of mouse spermatozoa.

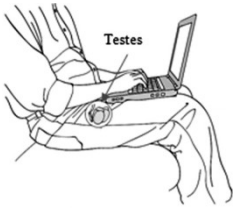
The importance of sperm mobility in determining conception may seem at first an odd Olympic event at the microscopic scale. However, sperm mobility is largely determined by ATP, and this competition is probably nature's way of selecting for effective ATP variants.
One approach to male contraception is to give men doses of testosterone. This turns off production of FSH and LH that are necessary for sperm development.

12.5.1. Chemicals

Animals studies show that drugs, alcohol, radiation, pesticides, solvents and other chemicals produce effects handed from father to son.
Male mice exposed to cocaine pass on memory problems to their pups[40].
Male rats exposed to a fungicide (vinclozolin) in the womb can pass tumors and diseases of the prostate and kidney down for at least 3 generations[39].
Fathers who smoke or are exposed to PCBs increase offspring risk of brain tumors.
Babies of firefighters, painters, woodworkers, janitors and men exposed to solvents in the workplace are more likely to be miscarried, still born or to develop later cancers.

In the male, sporadic exposure to a reproductive toxicant may only affect the developing spermatozoa presently at certain stages of maturation, having little or no effect on other stages. Most spermatozoa would still appear normal. This is why developmental maps of spermatozoa (F12.12) are useful in identifying the vulnerable stage. The stem cells from which sperm arise are tough and quite resistant to damage since, at the level of the spermatogonium (Type A), some of them appear to be recycled back to primordial cells, or at least to some intermediate to await the initiation of the next wave of spermatogonia. However, repeated, frequent exposures can markedly suppress spermatozoal production, with a dramatic loss in fertility, observed as morphologically abnormal spermatocytes and low motility[1] (PCBs[5]).

12.5.2. Wi-Fi



Electromagnetic radiation from Wi-Fi connections used in laptop computers decreases human sperm motility and increases sperm DNA fragmentation[44]. Such effects can be explained by alterations in ATP production by high frequency radiation[45, 46].

12.5.3. Temperature

During their 10 weeks of development, spermatozoa are acutely sensitive to high temperatures. Fathers exposed to excessive heat from saunas or electric blankets before they conceive children seem to have higher rates of brain cancer in their offspring[30]. The scrotum provides a temperature slightly below body temperature, which is important to maintain sperm production. Once produced, sperm can survive at normal body temperature.

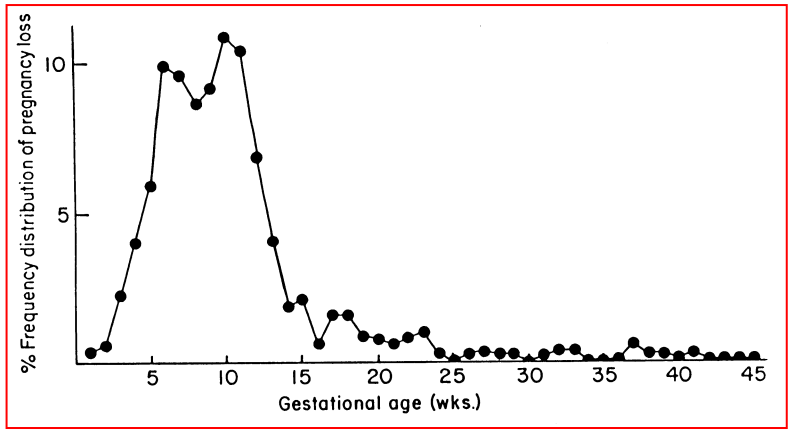
12.5.4. Age

The offspring of teenage fathers have increased risk of premature birth and death and low birth weight[38].

Parents over 40 have increased offspring incidence of autism and schizophrenia, and 6 times more likely to have children with Down syndrome[37].
Fathers over 50 are about 30% more likely to have offspring suffering from autism, schizophrenia and Down syndrome, and to have daughters who develop breast cancer.

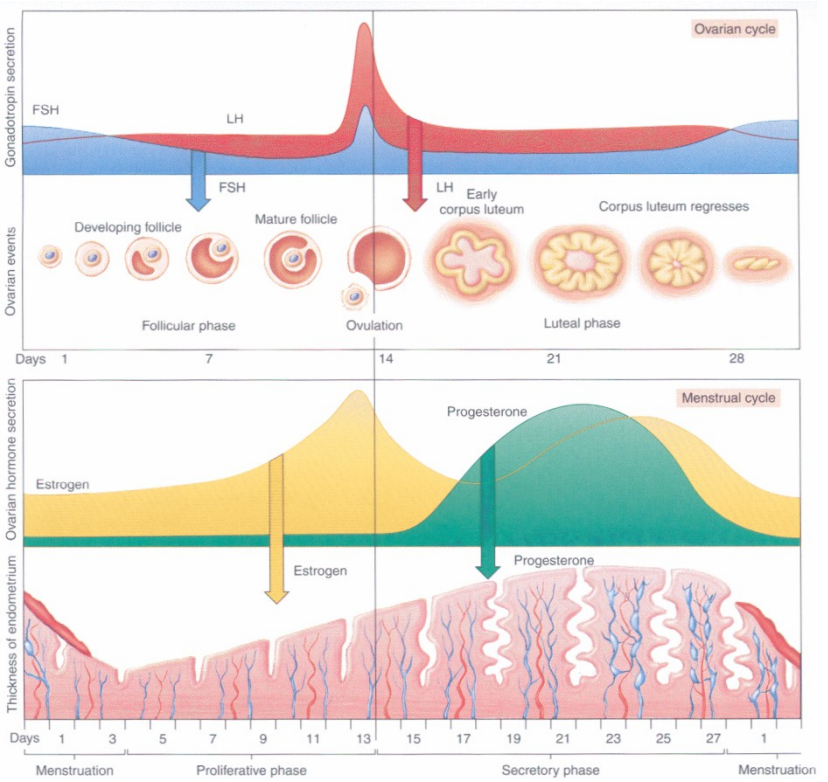
12.6. Time-Course of Gestation

In early gestation, effects of chemicals are likely to be drastic, resulting in sterility or in loss of the embryo.
Sterility can result from interference in the synthesis of gametes (sperm or ova), or from a lethal defect in fertilization.
Death and loss of blastocyst or embryo can also occur pre- or post-implantation (F12.13).



F12.13. Frequency distribution of 723 losses among 4351 pregnancies, by gestational age. *Mattison.*

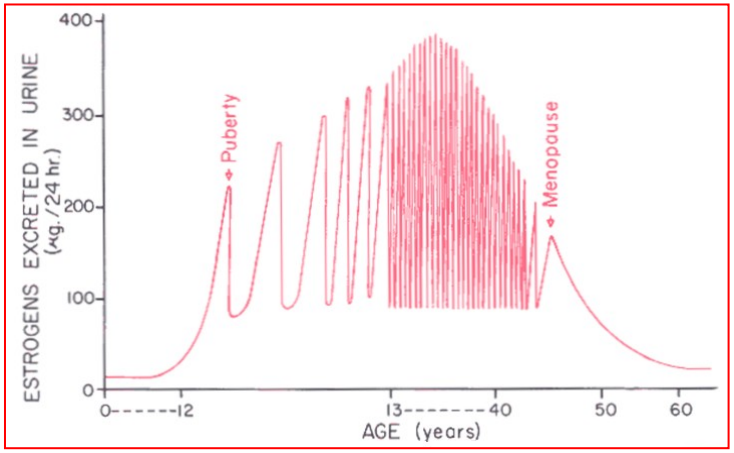
In the latter stages of gestation, structural or functional abnormalities are more frequent.
There are many examples of functional effects. We will mention later the effects on alcohol on the developing brain.
Another one is the influence of prostaglandin E2 blockers



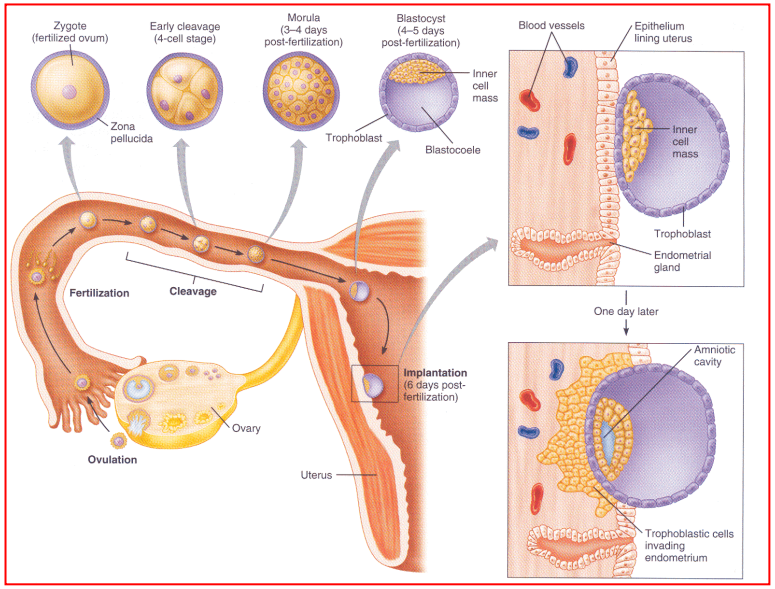
F12.14. Gonadotropins and ovarian hormones during the female cycle. *Guyton.*

(such as aspirin, acetaminophen and indomethacin) on synapses in male's preoptic area. These alterations affect sexual behavior in later life[23].
Until recently, it was thought that female mammals carried the entire complement of ova that they would use during their breeding lifespan. These ova come from primordial germ cells formed during fetal development *in utero* which results in several million oogonia, the bulk of which become atresic (degenerate). A few survivors undergo meiotic reduction to the haploid state and become arrested in a meiotic prophase state

until puberty. Ovarian tissue may be affected toxicologically while *in utero* (the developing female fetus is an exposed future mother) or following exposure *ex utero* at any postpartum stage of development.
It now appears that germline cells persist in the ovaries of adult mice, and the same might be true in human females[24].
Hormonal events in the female are depicted in F12.14 and 12.15. In the middle of the cycle, a surge of LH floods the ovarian follicle, triggering maturation and release of the egg. The first week of the cycle leads to light implantation (F12.16), while 1 month leads to strong implantation.



F12.15. Estrogen secretion through life. *Guyton.*



F12.16. The first week of embryonic development in the oviduct and uterus: ovulation, fertilization, cleavage, formation of morula and blastocyst and beginning of implantation. *Germann & Stanfield, 2002.*

Why doesn't the mother reject the fetus?

The fetus may use antigens from the Major Histocompatibility Complex derived from his father's genes. What if these genes are incompatible with the mother's immunity?
There has to be a mechanism inhibiting rejection. Is it

- a placental barrier?
- an antigen presentation modification?
- that the mother's immune system is forced to tolerate the fetus?

Known facts[8]**:**

- IDO, indoleamine 2,3-dioxygenase, is an enzyme that immune cells use to suppress other immune cells by breaking down *tryptophan*, which immune cells may need to proliferate.
- Cells at the *placental interface* produce IDO.

Hypothesis: fetus actively suppresses his mother's immune cells.
Observation: IDO inihibitors given to pregnant mice cause them to abort genetically distinct fetuses ONLY.
Question: What would happen if a toxicant could inhibit this enzyme?

12.7. Vulnerable Periods

Following implantation, the embryo (so named in the first 3 months) develops in steps of organogenesis into a fetus (so named in the last 6 months). To each of these organogenic steps corresponds a "**toxic windows**" where particular replicating cells are hypersusceptible to chemical attack, frequently at a concentration of agent far below that required for normal toxicity.

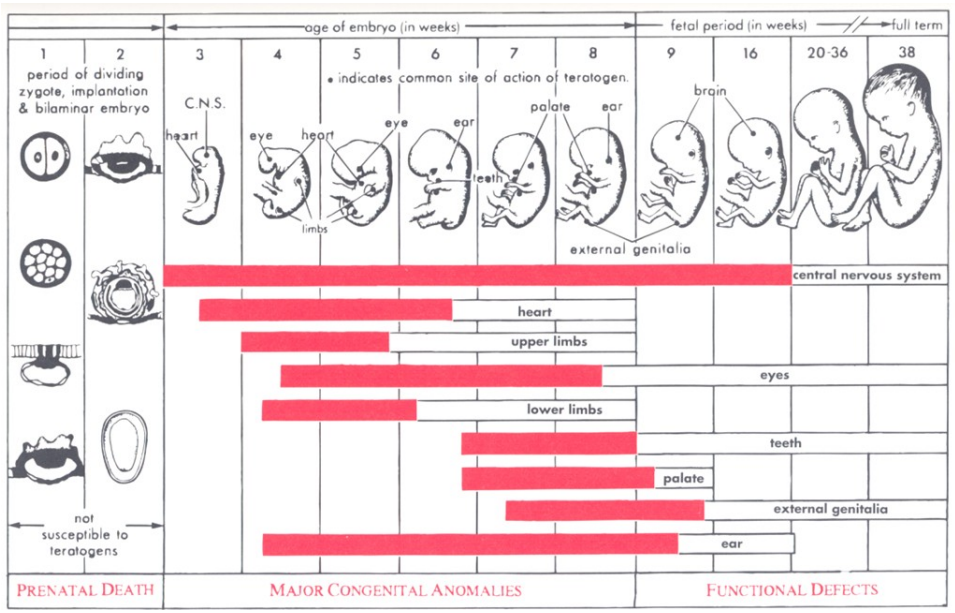
3% of newborns show some teratogenic anomaly at birth and an additional 4% are labeled as defective at the end of the first year of life. The later defects are *functional* ones that appear as the baby grows.

For an effect from the agent to occur, it has to be present at the right time and at the right concentration. The time interval of greatest susceptibility is the period of organogenesis.

In the human, many organ systems develop over a span of 12 to 30 days, during which they may be susceptible to chemicals.

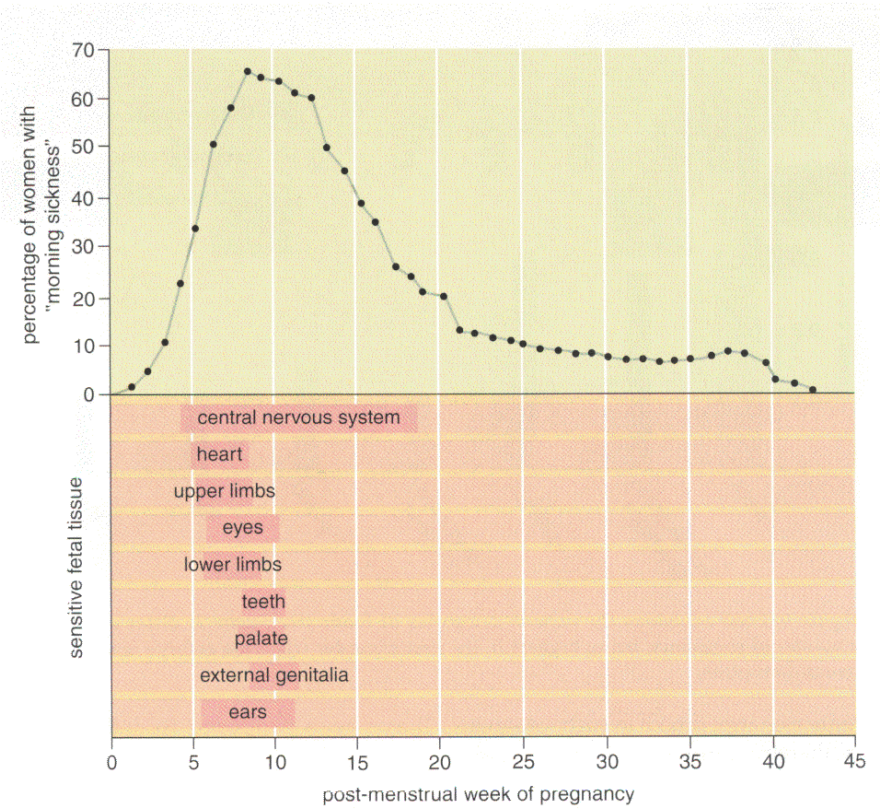
Organogenesis in humans is in the first 60 days after fertilization, unfortunately at a time when a woman may be unaware of her condition. After these 60-days, the effects of chemicals on the fetus are more likely to be cancer, organ dysfunction, behavioral and developmental abnormalities.

Coming to the health specialist for relocation after finding out about a pregnancy can provide only partial protection, as exposure has likely already occurred. This is a problem in occupational health, with a workforce that is 45% female, a large percentage of which in child-bearing years. Parents (male or female) should be protected from exposure if they are going to bear children. As one might expect, the periods of organogenesis vary between species (F12.19, 20, 21). In laboratory animals, as is shown in T12.19 and F12.20, organ development is condensed into much shorter periods, often only of 24 hour duration.



F12.17. Action of teratogens during embryogenesis. The red bars indicate major malformations, the white bars indicate functional alterations. *Environmental Toxicology, 1996.*

Standard teratology testing protocols now demand that the pregnant test animal be exposed to the agent between days 6 and 15 (for mouse and rat) and between days 6 and 18 (for the rabbit), thus covering the major span of organogenesis.

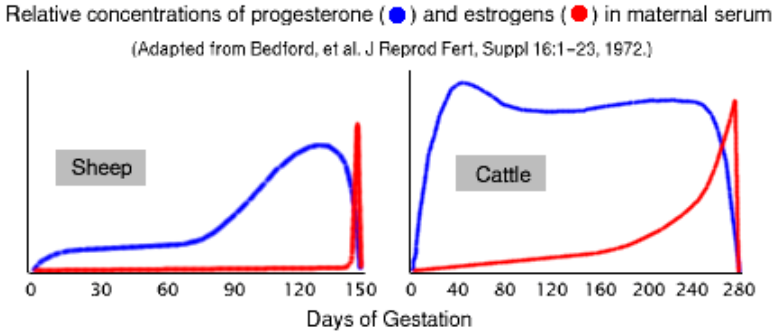


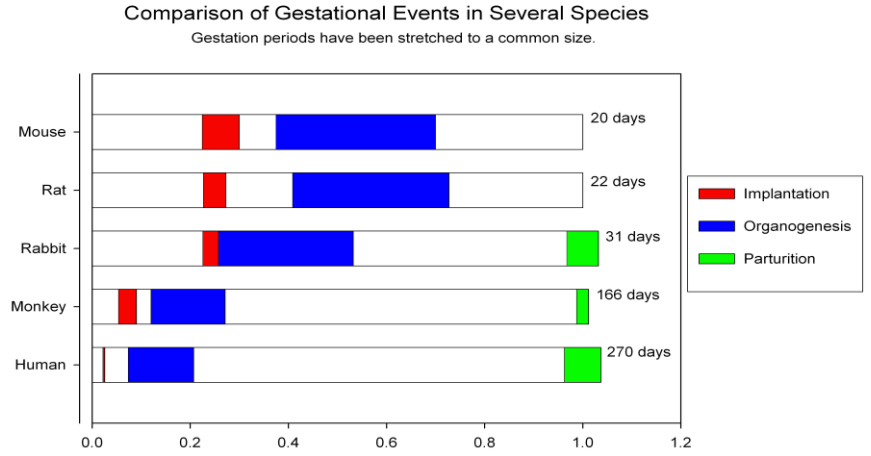
F12.18. Nausea and vomiting tend to peak between the 8th and 12th week of pregnancy, the crest sensitivity of fetal tissues. *Scientific American.*

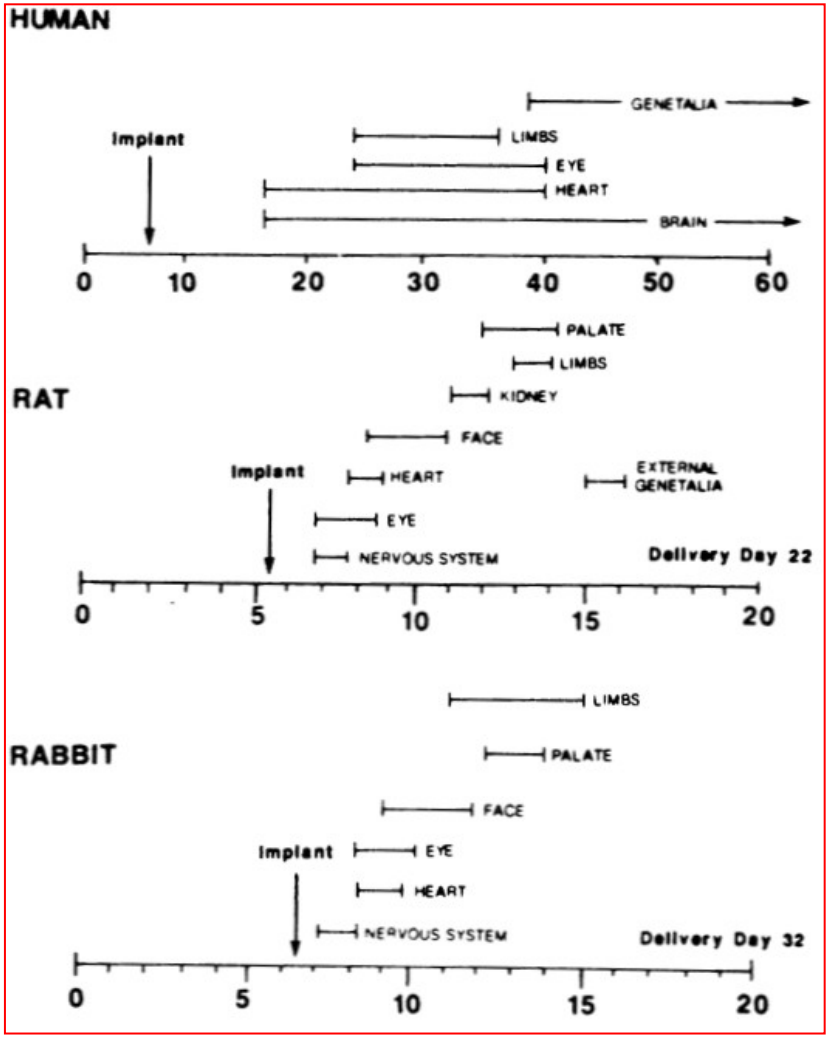
INTER-SPECIES DIFFERENCES IN GESTATION

T12.19. Gestational Events in Several Species. *Williams & Burson.*

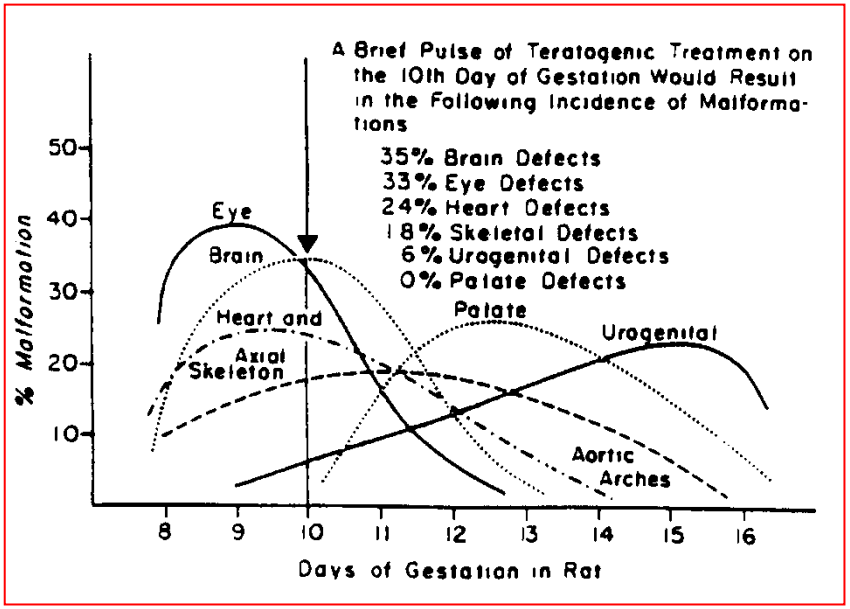
Number of Days after Conception			
Species	Implantation	Organogenesis	Parturition
Human	6-7	20-56	260-280
Monkey	9-15	20-45	164-168
Rabbit	7-8	8-16.5	30-32
Rat	5-6	9-16	22
Mouse	4.5-6	7.5-14	20



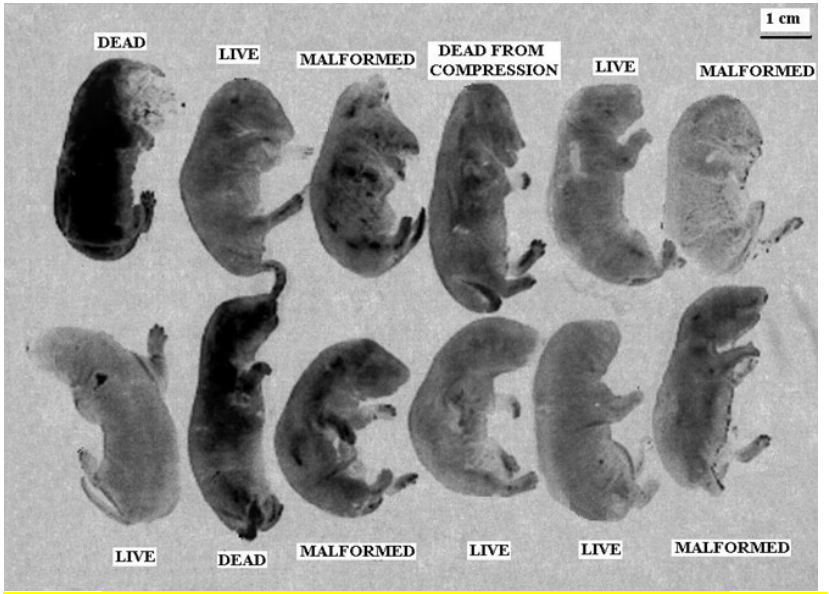




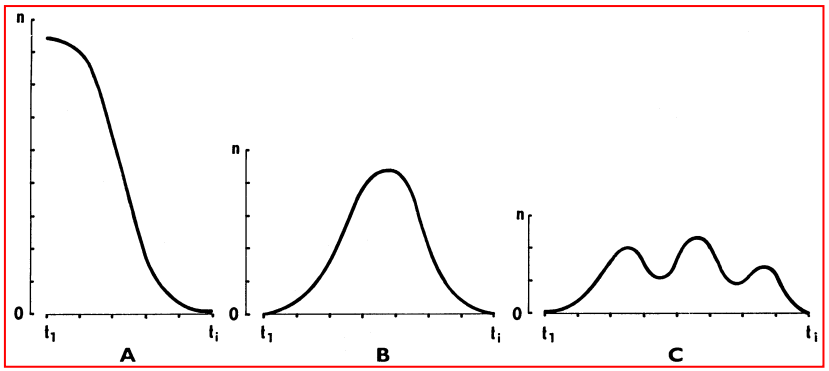
F12.20. Critical periods of embryogenesis. Periods of vulnerability are short in many animal models. In the standard teratological study protocol, pregnant females are exposed to three levels plus control from day 6 to day 15 (mice and rats) or from day 6 to 18 (rabbits).



12.21. Rat responses to irradiation according to time of administration. *Chemical Exposure and Toxic Responses.*

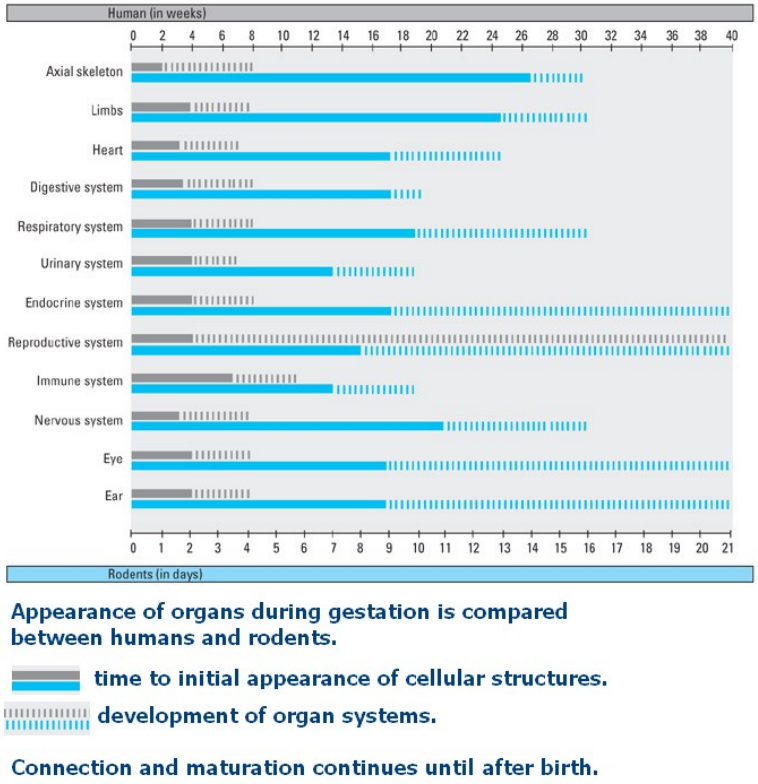


F12.22. Animals from the same litter do not necessarily share the same fate. *Hayes, 2001.*



F12.23. Time-dependence of malformation rates. *Persaud.*

Specific malformations associated with a chemical exposure will occur in a particular species at the critical periods corresponding to appearance and development of cellular structures (F12.24).
The time course of malformation probability in a specific organ system can follow different unfoldings, as shown in F12.23.
In "A", the foundation of an *organ system* is put into place, leading to a sudden rise in malformation susceptibility.
Curve "B" reflects malformation rates corresponding to the exponential phase of *organ* development.
When more than one cell population is involved in an organ, the curve can be multi-phasic ("C").



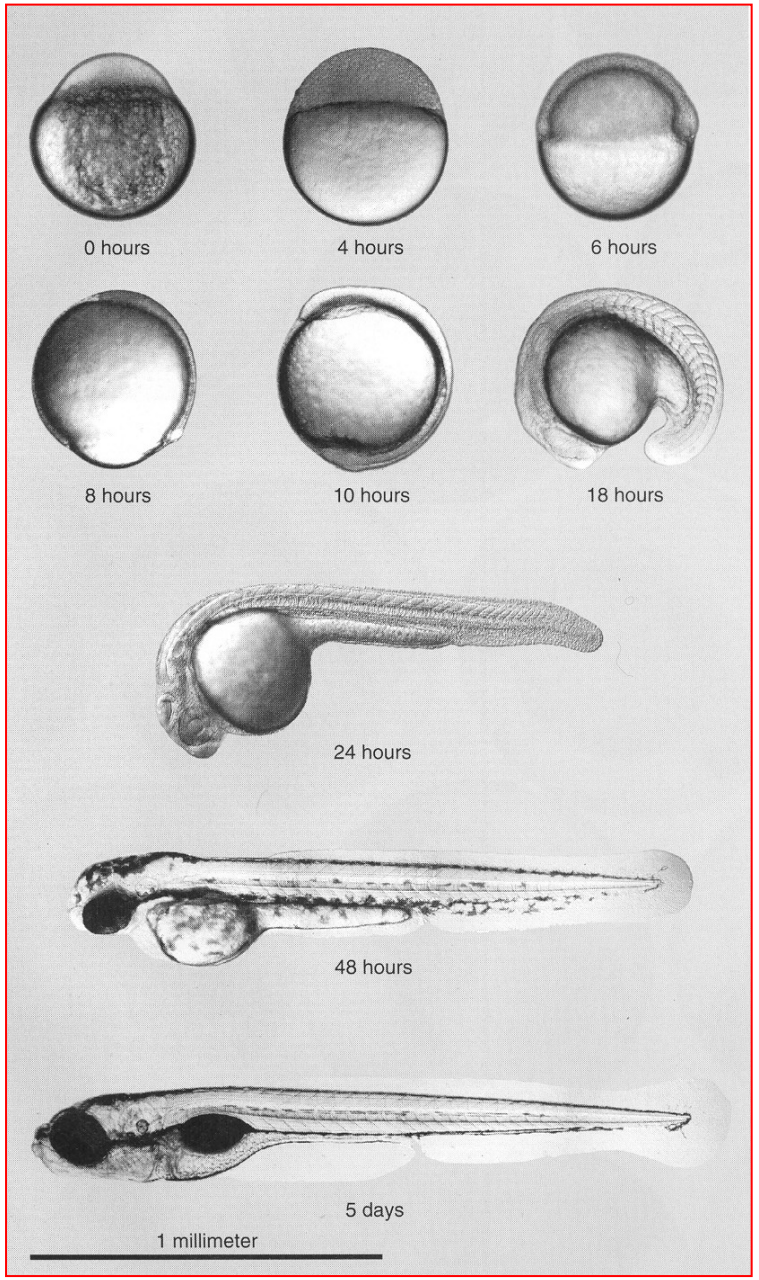
F12.24. Critical periods of gestation compared between human and rodent.

F12.25. Rapid changes in the zebrafish embryo. >
From American Scientist, Sept-Oct 2006.

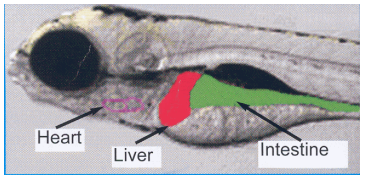
While animal experiments can be controlled closely, epidemiological investigations of teratogens in humans require an association between exposure and a "congenital" defect. In the case of thalidomide, the number of phocomelia cases was orders of magnitude increased from the previously known incidence rate of this rare birth defect. It took only a few months to identify thalidomide as the inducing agent. But most chemicals induce birth defects at a low rate, the risk ratio or odds ratio usually being below 2^2. With so many confounding factors in epidemiological studies, it is difficult to obtain the tight associations needed to identify culprit chemicals in full confidence.

When testing for teratogenicity, it is often convenient to use models that develop rapidly and that are convenient to observe. In the zebra fish, the first structures, such as the trunk muscles and the head, begin to form 10 hours after fertilization. The heart pumps blood and the fish begins to move its tail within the first day. Other organs-eyes, ears and brain-start to form during the first 24 hours. The larvae hatch and start to swim when they are just a little more than 48 hours old. 3 days after fertilization, the zebrafish embryo is fully formed (F12.25). These 3 days are equivalent to 3 months in human embryos.

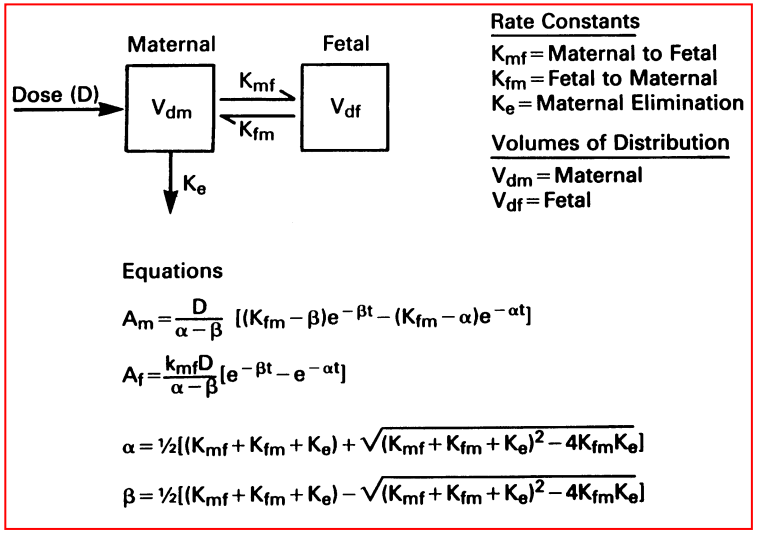
By day 5, the yolk is largely consumed and the embryo has been transformed into an independent organism. By comparison, a 5-day-old human embryo is merely a blastocyst-a hollow ball of cells that has yet to attach itself to the lining of the mother's uterus.



F12.26. Transparent zebrafish embryo.



The vulnerable periods delimit times at which embryonic tissues have special properties relevant not only to toxicity but also to regeneration. In a pig model, it was found that specific times were optimal to maintain the activity of xenotransplanted tissues: day 28 for liver, 56 for lung and 42-56 for pancreas.[21]



F12.27. A two compartment toxicokinetic model adapted to explore the disposition of a xenobiotic during pregnancy. *Needleman.*

Prenatal exposure to dioxin-like polychlorinated biphenyls (PCB) congeners 118 and 156 is associated with strong detrimental effects on fetal neurodevelopment in children. Non-dioxin-like PCBs affect motor development, but not mental functioning. This data is interesting in that some prenatal periods for humans correspond to postnatal exposure time windows for animals[43].

12.8. Fetal Compartment

Since the fetus and placenta temporarily become supplementary organs in the female, compartmentalization is altered. This can be studied by the classical methods of toxicokinetics (F12.27). However, compartmentalization is not complete. A small concentration of fetal blood cells, as well as fetal DNA fragments, circulate in the mother's blood.

12.9. Toxicity on Fertilization-Migration

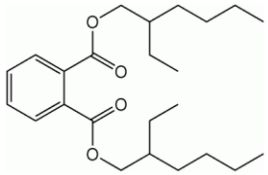
There are many factors that can affect conception (T12.5). For example, female rats exposed to PCBs in the womb are more reluctant to mate.

Fertilization occurs when the ovum meets the upward-migrating sperm in the region of the ampulla of the Fallopian tube. To penetrate the layers of the oocyte (the thick acellular zona pellucida and several layers of granulosa cells), the spermatozoon must have matured, becoming highly motile and fertile, and achieved *capacitation* (changes in the sperm surface and chemical changes within). This involves two distinct reactions. The first, the acrosome reaction, results in a series of fusions between specific membranes, with the release of hydrolytic enzymes capable of penetrating the oocyte granulosa cells, digesting them. The second, an enhanced or activated motility, provides the extra flagellar activity to thrust the sperm through the digested "tunnel". One can visualize that a highly reactive chemical in seminal fluid could interfere with these procedures, thereby reducing the fertility of the spermatozoa.

The migration of the fertilized ovum down the Fallopian tube occurs during a period of 4 to 5 days during which time the cells double, double again and again from 1 cell to 2 cells to 4 cells, to 8 cells, to 16 cells, and to 32 cells, at which time the cellular organization changes to produce the blastocyst (blastocele). This rapidly dividing cell mass is susceptible to chemical attack and may die at later stages following implantation. This will result in spontaneous abortion during

the 1st to 2nd months of gestation. Less drastic DNA damage in these rapidly dividing cells may result in implantation, but in loss of the fetus (early or late fetal death) or carriage of the affected fetus to term, resulting in some structural or functional anomaly.
Detailed genetic assessment of blastocysts reveals that many of the cells within them have major chromosomal abnormalities, confirming a high spontaneous incidence of mozaicism[5].

- Exposure to pesticides makes men less fertile.
- Males exposed to TCDD (dioxin) in Seveso Italy are permanently less likely to have male children. If exposed before the age of 10, their sperm is feeble and depleted. If exposed between 10 and 17, there is more sperm with higher motility. When exposed as adults, there is no obvious effect on sperm[33].
- There are plans under way (EPA) to assess the effect on reproduction of endocrine disrupters of estrogen, androgen and thyroid hormones. Because male embryos are more delicate, environmental pollution may contribute to the observed decrease in male births.



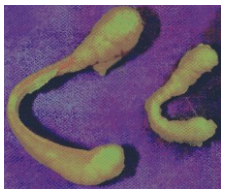
Phthalates are used as a softening agent in PVC products (infusion bags). The chemical (figure) most used and of greatest concern is DEHP: di-2-ethylhexyl phthalate♦. In infusion bags, the chemical apparently protects blood components from decay, possibly by inhibiting certain reactions[7].

Exposure in rats reduces the size of the sperm-storing organs. The testes are shriveled, smaller, or can become a sac filled with blood. Boys exposed in the womb to higher concentrations of phthalates (diethyl, dibutyl benzyl, butyl benzyl and diisobutyl) had shorter anogenital distances, smaller penises and had testes that did not properly descend into the scrotum (this suggests impaired production of testosterone)[28]. One quarter of US women have body concentrations sufficient to trigger theses effects.

Monobutyl phthalate and monbenzyl phathalate urine concentrations in human males have already been inversely associated with sperm count and motility. Malformations increased with concentration[14].

Phthalates are also associated with low birth weight[35].

DEHP exposure in children (from dust) is correlated with a 2.9 times rise in incidence of asthma. Other phthalates are correlated with rhinitis and eczema. It appears that phthalates can amplify the body's response to allergy-causing compounds[20].



A study of 1450 adult men has shown that higher phthalates in urine correlates with obesity[31].

F12.28. Epididymis♣ in the rat. Unexposed (left) is more than 3 times the size of that exposed to DBP (dibutyl phthalate) in the womb.

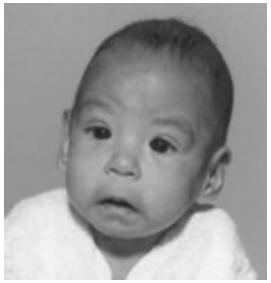
♦ The chemical is regulated in California since late 2003. It is expected to be replaced by epsilon-caprolactone.

♣ Epididymis is a cordlike structure on the posterior edge of the testes where storage, transit and maturation of spermatozoa occurs.

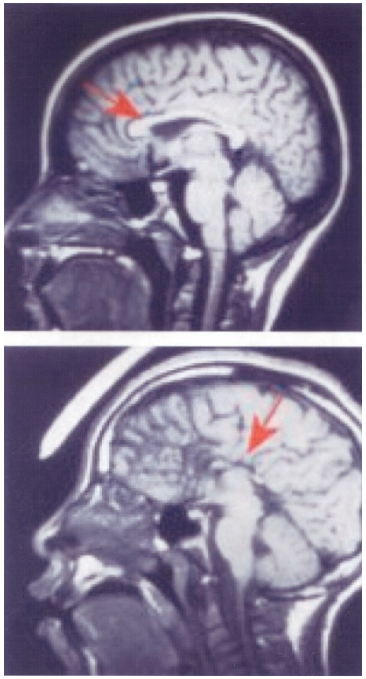
12.10. Teratogenicity Examples

12.10.1. Fetal Alcohol Syndrome.

F12.29. Facial Features of FAS.



- Epicanthal folds
- Small, widely spaced eyes
- Flat midface
- Short, upturned nose
- Smooth, wide philtrum
- Thin upper lip
- Under-developed jaw



F12.30. Corpus Callosum difference between normal (top) and alcohol-exposed infants (bottom).

A cell-adhesion molecule called L1 guides cell migration in the developing brain. Alcohol prevents cells guided by L1 from adhering to each other. A single episode of alcohol exposure lasting 4 hours is enough to kill groups of cells by interfering with GABA, glutamate and serotonin. Exposure to alcohol also diminishes the size of the corpus callosum in human infants (F12.30).

There have also been indications in rat studies that alcohol consumption in mothers can increase the risk of breast cancer in offspring[22].

FETAL ALCOHOL SYNDROME PHYSICAL ABNORMALITIES
(Hermann Loser, University Children's Clinic, Munster, Germany)

98% are under normal height and weight
84% Microcephalic
89% Mental and Motor Retardation
80% Speech impediments
20% Hearing problems
20% Swallowing/Feeding problems
72% Hyperactive
58% Slack muscles
20% Autism/Aggressive/Social Problems
95% Facial anomalies
29% Heart defects
10% Kidney defects
46% Genital deformities
25% Eye problems
16% Bent crooked little finger
51% Shortened and bent little finger
13% Under-developed fingers
9% Hip deformities
16% Small teeth
30% Pigeon Chest
7% Concave chest
7% Cleft palate
44% Spinal dimple
12% Hernia
35% Hair growth on back of neck

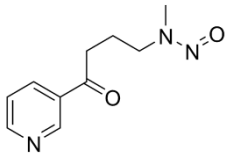
12.10.2. Cigarettes

Women who smoke reach menopause sooner than non-smokers, possibly because their supply of eggs is smaller.
It is likely that smoking before pregnancy or during nursing also impairs the offspring's supply of eggs.
In mice, weekly administration of benzo(a)pyrene and 7,12-dimethyl-benz(a)anthracene over 3 weeks in doses representing one pack of cigarettes per day caused offspring to have one-third fewer eggs. This happened whether the mother was

exposed before pregnancy or during lactation. If exposed during both periods, two-thirds of the eggs were missing[32].

F12.31. Cigarette publicity.

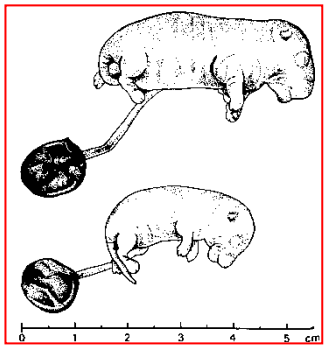
The metabolite of nicotine nitrosamino ketone (NNK, shown) may increase the incidence of cancer in the offspring.



Women who complain of headaches or breathing problems associated with exposure to organic chemicals such as phenol, xylene, acetone and trichloroethylene are much more likely to have premature births or show birth defects in their offspring.

12.10.3. Sound

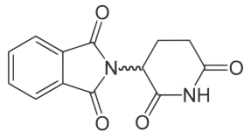
Postnatal growth in fetuses exposed to high levels (100 dB) of continuous sound is slower in exposed than in controls. In rats, exposure to pulsed noise for 7 hours induced serious retardation of embryo growth (F12.32). A large variety of resorptions were found.



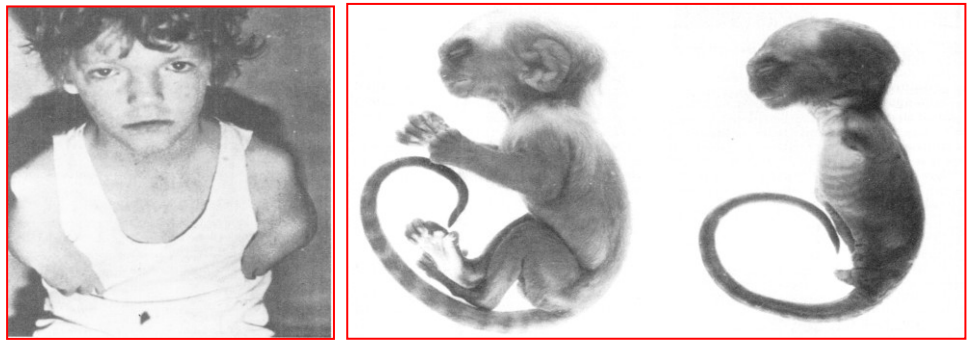
F12.32. Sound-exposed mother's fetus, 14 days post-conception, is at the bottom.
Persaud.

12.10.4. Thalidomide

Thalidomide, originally developed as a sedative in 1957, was widely used, particularly in England and Germany, to stem morning sickness and nausea in pregnant women, with dramatic consequences.
Thalidomide was not marketed in the US on the basis of a handwritten memo by Frances Kelsey mentioning peripheral neuritis symptoms in adults (a tingly numbness in the fingers). 7,000 children were born with phocomelia (seal limb) or amelia (no limb) across Europe in the early 1960s.



Thalidomide focused attention on chemical-induced birth defects and led regulatory agencies to introduce specific protocols to test for organ-specific teratogenicity.
Given before or after a crucial time period (days 24-36 of gestation), thalidomide has little effect, but during that 12 day time span, when limb buds develop, the drug halts further growth of long bones.



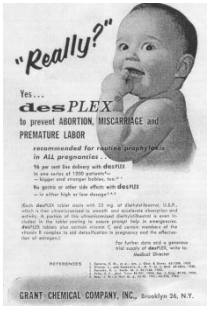
12.33. Phocomelia in a child. Marmoset (normal at left) and Thalidomide-exposed between days 38 and 52 (at right). Rats and mice would show no effects under the same exposure.
Essentials of Environmental Toxicology.

Thalidomide is a strong teratogen in humans, but a weak one in animals. Aspirin is a strong teratogen in rats. It has also been linked to miscarriage in women, although acetaminophen was not[19].Thalidomide is approved since 1998 to treat complications of leprosy, and may be useful in the treatment of multiple myeloma or myelodysplasias, blood cancers notoriously resistant to chemotherapy as well as rheumatoid arthritis, tuberculosis and AIDS[4]. Thalidomide-affected babies continue to be born in developing countries where it is used for the treatment of leprosy.

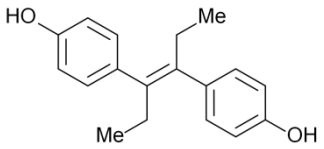
Thalidomide's actions are multiple and mysterious, but probably require bio-activation, since the drug is largely ineffective *in vitro*. A prominent anti-angiogenesis effect may explain its teratogenicity (developing bones need extra capillaries) as well as its effect in multiple myeloma (squelching new blood vessel formation should starve cancer in the bone marrow). Thalidomide is of interest because it regulates TNF-α. TNF-α encourages the demise of tumor cells.

12.11. Delayed Effects on the Fetus

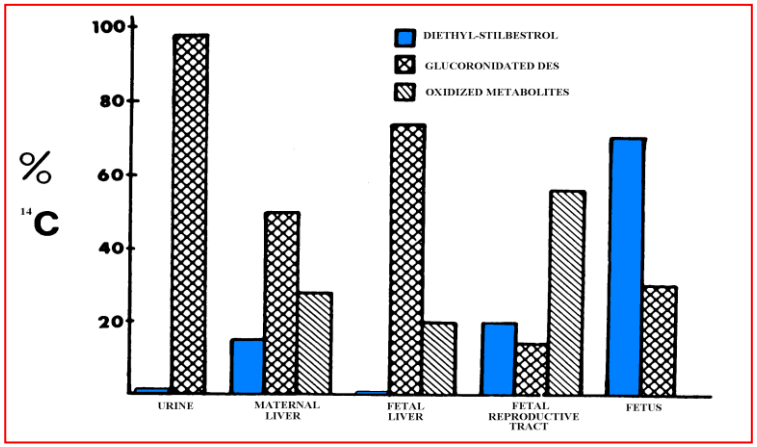
12.11.1. Delayed Teratogen



F12.34. 1957 advertisement promoting DES.



Between 1940 and 1971, over 2 million women were given diethyl-stilbestrol, DES, the first synthetic version of estrogen, as a treatment for high risk pregnancy. It was believed at the time that insufficient amounts of estrogen led to miscarriage. DES turned out to be a "delayed teratogen" in daughters between 17 and 22 years of age, inducing clear cell adenocarcinoma in the vagina and cervix. The next generation of daughters may also be affected. It was found in 1999 that DES suppresses the activity of the **Wnt-7a** gene, influencing the development of the male and female reproductive tracts. The repartition of DES is not similar in the tissues of embryo and mother, a good example of fetal compartmentalization (F12.35).

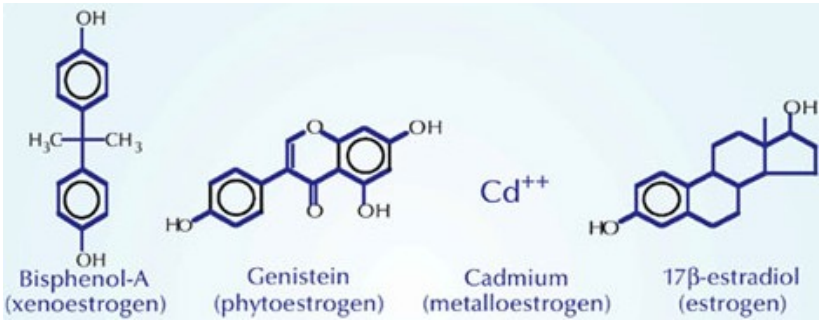


F12.35. DES and metabolites in fetal and maternal tissues. *Mattison.*

12.11.2. Endocrine Disruptors

Endocrine disruptors are a diverse class of chemicals (DDT, DES, soy estrogens, atrazine, BPA and PCBs) who disturb normal endocrine functions at *very low concentrations*, and display unusual dose-responses, making their detection difficult in experiments where large doses are typically used. In adults, it is believed that interference with hormone action is mostly reversible, but that it may not be in the developing fetus or young child, permanently altering the organization of many critical body tissues. Endocrine disruptors have effects on male and female reproduction, breast development and cancer, prostate cancer, neuroendocrinology, thyroid, metabolism and obesity, and cardiovascular endocrinology. To study the neuro-developmental impacts of endocrine disruptors, the volumes of

sexually dimorphic nuclei ("SDN") in the hypothalamus are commonly used as a bio-marker.



- Women who have had substantial exposure to endocrine disruptors such as estrogen mimics are more likely to bear sons who develop testicular cancer. The chemicals involved are hexachlorobenzene, PCBs and chlordanes[12].
- A important estrogen disruptor is **bisphenol A** (BPA, above), a precursor in the manufacture of polycarbonate plastics, making them harder and more resilient, and epoxy resins that line the interior of metallic cans. BPA easily leaches into food containers when heated.

  BPA mimics estrogen, and has been found in animal studies to alter prostate gland size, and shift the onset of sexual maturity. It could cause miscarriages and mental retardation (Down's syndrome)[15] in humans, as well as insulin resistance in mice[27]. BPA can also alter brain formation in female mouse fetuses, and later produce male behavior in adult females at very small doses, 25 ng/kg.day. The average person's daily exposure to bisphenol A is similar to that dose[29,41]. BPA may produce heart arrhythmias in females because at estrogen levels typically found in premenopausal women, the addition of BPA would spike vulnerability to arrhythmias, causing a higher mortality rate after heart attacks in premenopausal women, compared with men[36]. BPA may also produce permanent damage to a gene important for reproduction, HOXA10, by loss of a methyl group, permanently altering its activity and rendering uterine tissue hypersensitive to the effects of estrogen[37]. Exposures vary from 1 to 15 µg/kg.day, and urine and blood levels fluctuate following consumption of fluids from BPA-containing polycarbonate bottles. The US EPA reference dose is 50 µg/kg.

- When released in the environment, ethynylestradiol from birth control pills can alter the fertility of trout eggs at a level of 10 ppt[16].
- Trace amounts of cadmium mimic estrogen and alter the reproduction of female rats by binding to estrogen receptors[17].
- Exposure to cortisol, a hormone that crosses the placenta into the fetus, between the 5th and 7th week of gestation elevated blood pressure in offspring by 15 mm of Hg. They also had fewer nephrons in their kidneys[25]. Stress to mothers reprograms a baby's physiology, making it susceptible to high blood pressure later in life.
- The fungicide vinclozolin promotes an epigenetic transgenerational phenotype involving a number of disease states. Vinclozolin produces male infertility by acting transiently at the anti-androgenic time of embryonic sex determination to promote in the F1generation a spermatogenic cell defect and subfertility in the male. This phenotype was transferred through the male germ line to all subsequent generations analyzed (up to F4)[42].

Many troublesome substances originate in food. When individuals were switched for 3 days from their normal food sources to the same foods freshly sourced and unpackaged, the levels of bisphenol A and a key phthalate in their urine decreased by 65% and 53% respectively[47].

12.12. Types of Reproductive Studies

Because of the complexity of reproduction, diverse types and lengths of experimental studies of toxicity can be conducted (T12.36).

T12.36. Types of Reproductive Toxicity Studies in Males and Females.

MALE	FEMALE
Sperm production study	Pre-Implantation Studies
Serial mating study	Single generation study
Extended mating study	Multiple generation study
Dominant lethal assay	Fertility in continuous breeding
In vitro tests	Teratogenicity

In a *Serial Mating Study*, 50 females per male may be used.
Extended means that the agent is administered over 6 spermatogenic cycles (one cycle in the rat is 58 days).
In a *Dominant Lethal Assay*, the agent is dosed to result in fetal lethality from genetic mutations.
In *Fertility in Continuous Breeding,* breeding is forced throughout the lifetime of the animal.
There is a fundamental distinction between *fertility* studies and *teratogenicity* studies.
Note that it is not easy to uncover reproductive toxicity in males, in spite of a large number of available tests on semen (T12.37).
The gold standard test is still whether healthy offspring can be obtained.

T12.37. Semen Profile for Reproductive Toxicity.
from Chemical Exposures and Toxic Responses

Sperm concentration
Sperm viability
- Vital stain
- Hypo-osmotic swelling

Sperm Motility
- Percent motile
- Curvilinear velocity
- Straight-line velocity
- Linearity
- Lateral head amplitude
- Beat cross frequency

Sperm size and shape
- Morphology
- Morphometry

Semen parameters
- pH
- Volume
- Marker chemicals from glands
- Toxicant or metabolite concentrations

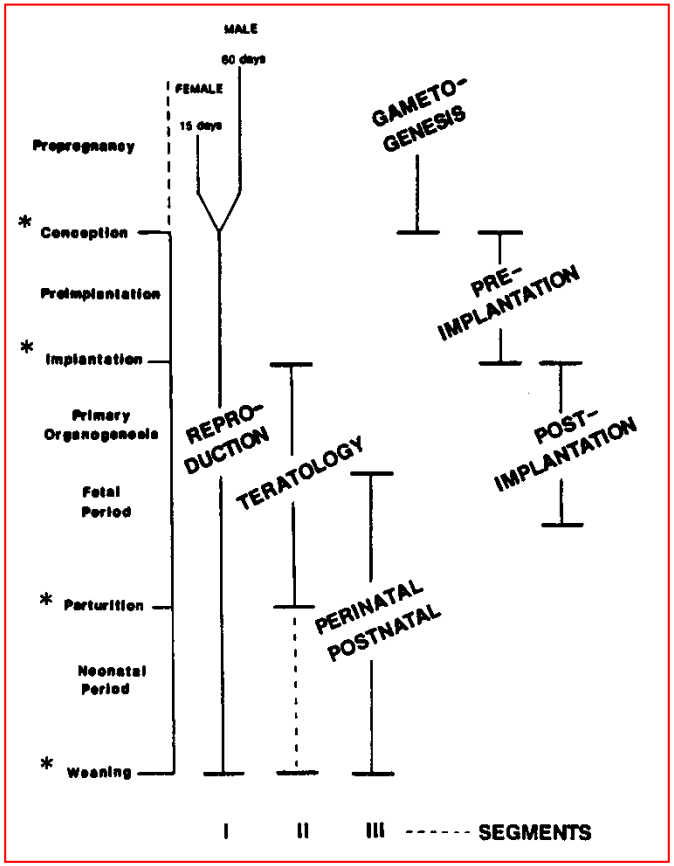
12.12.1. Single Generation Studies

Study formats can cover various Segments within a single generation (F12.38).

12.12.2. Multiple Generation Studies

Multi-generation testing is justified on the basis of

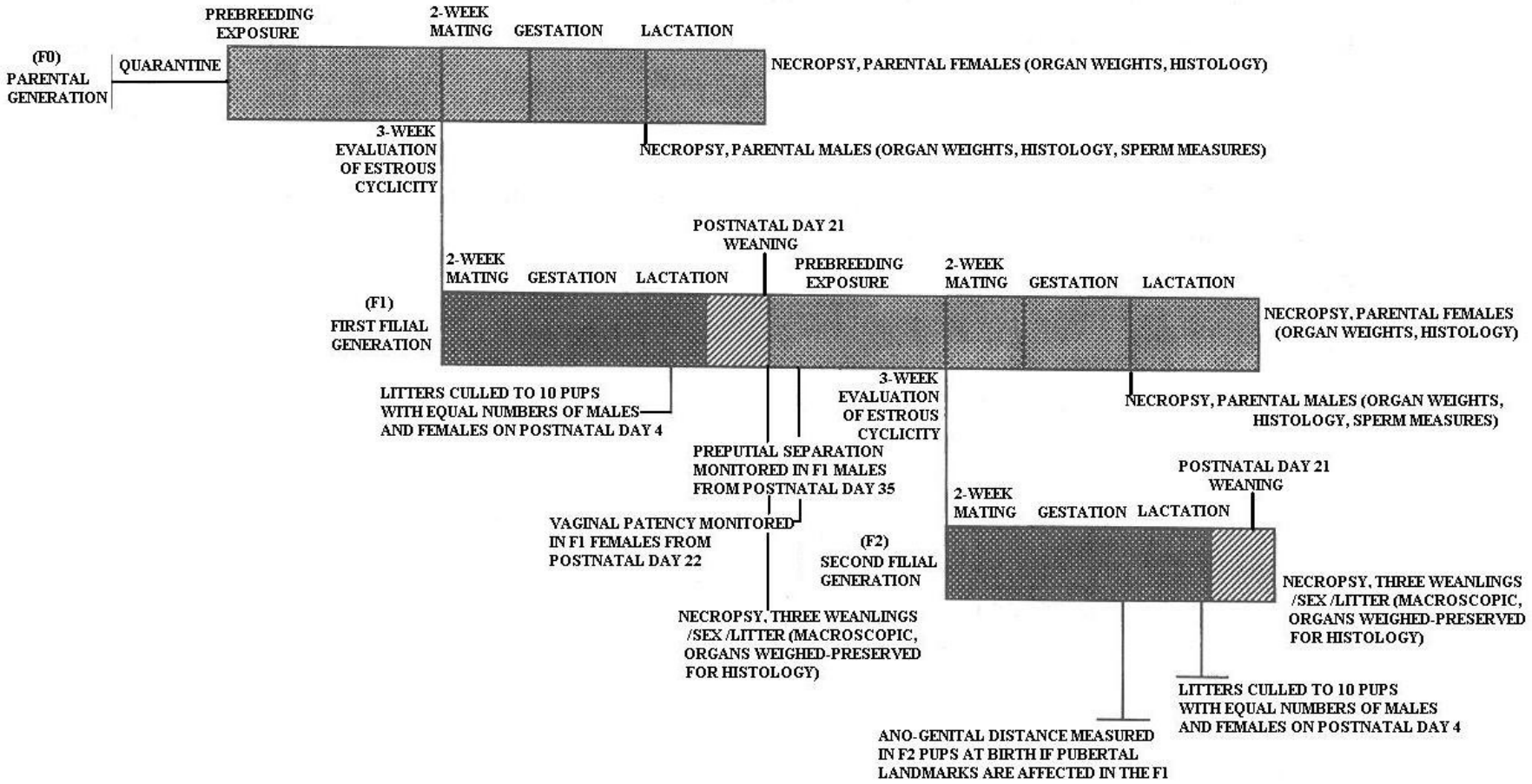
(1) bio-accumulation under sustained exposure,
(2) recessive mutations that may not be apparent in the first generation, and
(3) permanent *epigenetic* changes in the germ line (sperm) that then transmits transgenerationally as adult-onset diseases, even in the absence of any subsequent exposure.



F12.38. Segment I, Segment II and Segment III reproductive studies.
Ecobichon.

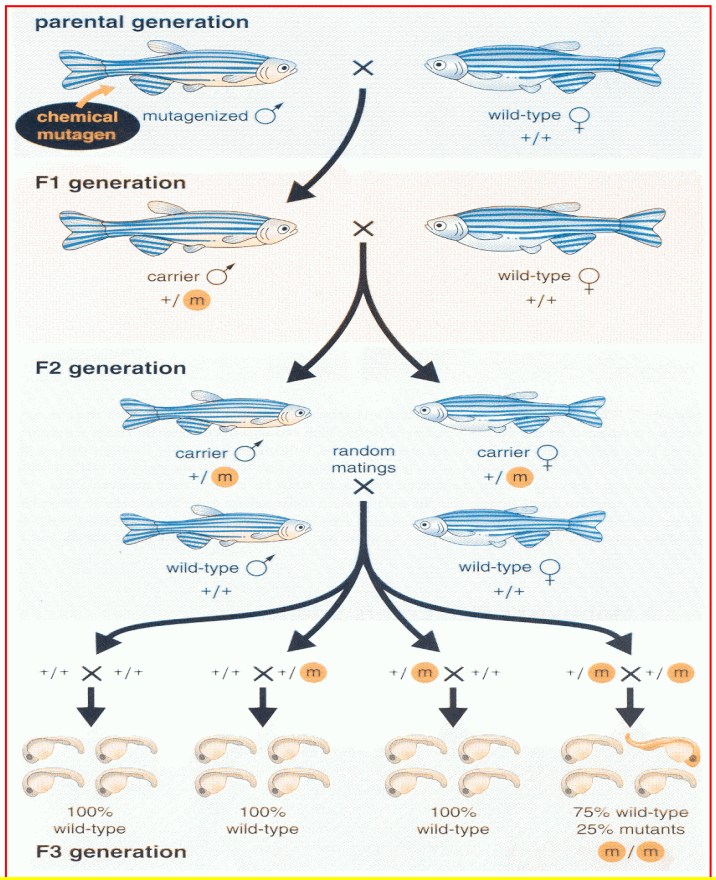
An example of bio-accumulation is the recommendation by the US FDA for studies up to 3 generations in the evaluation of the effects of compounds ingested over long periods of time such as color, food additives and pesticides, which may leave residues in food-producing animals[13]. The details of such a study are illustrated in F12.39.

U.S. EPA HARMONIZED MULTIGENERATION REPRODUCTION TEST



F12.39. Multiple Generation Reproductive Test. In a typical 3-generation study, females ("F0") are bred with males, both treated before and during mating, treatment beginning near puberty. The male and female offspring are further treated with the agent from weaning through the mating period, and bred repeatedly. *Modified from Hayes, 2001*

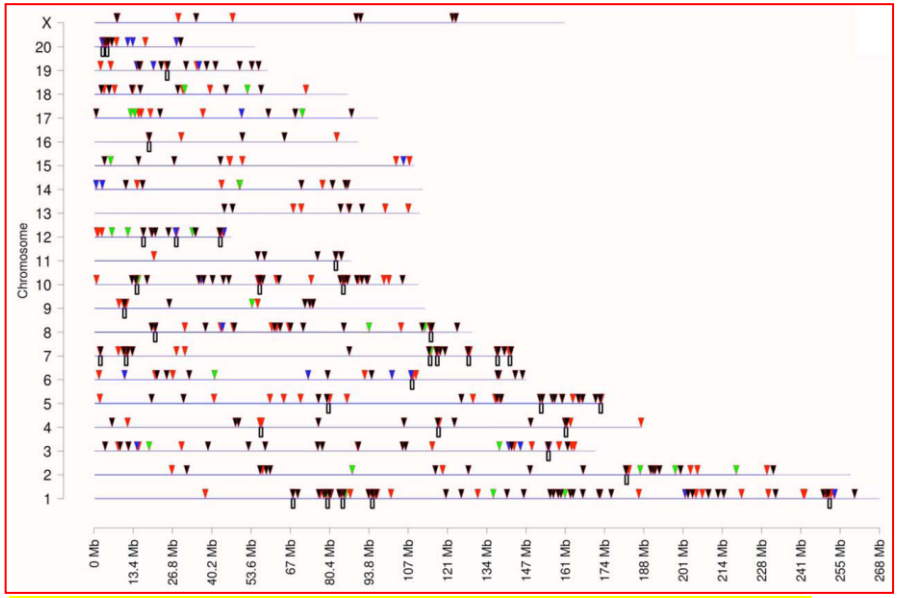
A case of recessive mutations is shown by a multigeneration study in zera fish (F12.40).



F12.40. The need for multi-generation studies. To create mutant zebrafish, a mutagen induces base-pair changes in DNA of developing sperm cells of a male fish (top left). The mutated male breeds with a wild-type (+/+) female (top right) to produce offspring in the F1 generation that carry the mutation (m). Since most mutations are recessive, the carrier (+/m) will be indistinguishable from the wild-type form. The F1 carrier then mates with a homozygous wild-type (+/+) to produce the F2 generation, which will consists of mutation carriers (+/m) and homozygous wild-types (+/+). The F2 generation fish are interbred randomly. Breeding pairs in the F2 generation that consist of two carriers (+/m) will produce one homozygous (m/m) mutant for every three wild-type offspring. All other breeding-pair combinations produce phenotypically wild-type offspring. Scientists must search through all the embryos of the F3 generation to find the mutants. *American Scientist, Sept-Oct 2006.*

An example of epigenetic changes is illustrated below (F12.41). After F0 gestating female rats are transiently exposed to pesticide (permethrin and insect repellant DEET), plastic (bisphenol A and phthalates), hydrocarbon (jet fuel, JP8) mixtures or dioxin (TCDD), the F1, F2 and F3 unexposed generations were assessed for pubertal onset and gonadal function.

The plastics, dioxin and jet fuel were found to promote early-onset female puberty in F3. Spermatogenic cell apoptosis was affected transgenerationally. Ovarian primordial follicle pool size was significantly decreased with all treatments transgenerationally.



F12.41. DNA regions with different methylation were identified for the various agents, and found to be widely distributed in the rat genome. Chromosomal location of the transgenerational DNA Methylation Regions in F3 associated with plastics (red arrow), dioxin (green arrow), hydrocarbon (blue arrow) and pesticide (black arrow). The chromosome number and 2–5 megabase regions size are indicated.

Another examples of such transgenerational transmission is the effect of tributyltin on obesity in the grand-children of exposed mice, in spite of zero subsequent exposure[48].

12.13. Toxicants of Sexual Maturity

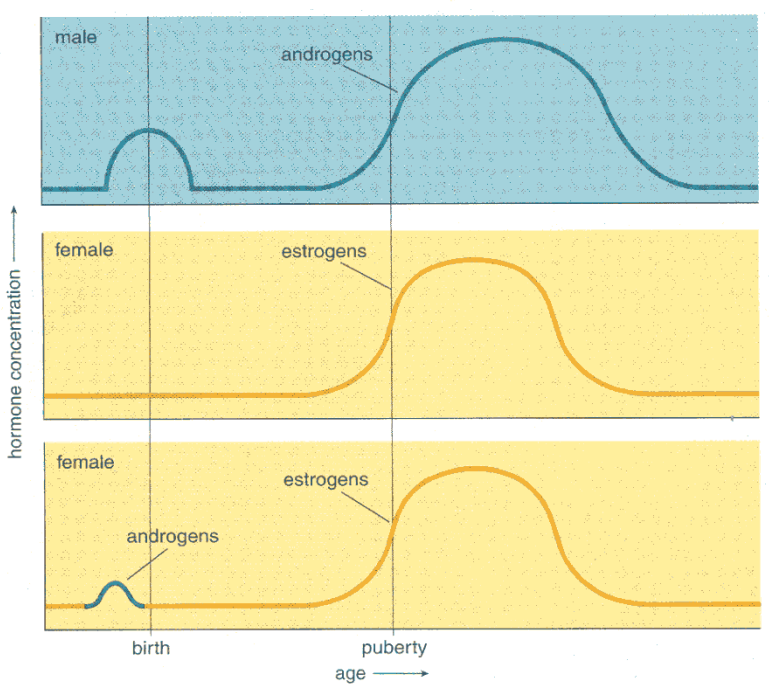
Sexual maturation in girls is delayed by lead concentrations ranging between 2 and 5 µg/dL, although the US limit in the blood is 10 µg/dL[18]. The US Association of Occupational and Environmental Clinics recommends intervention for pregnant women at 5 µg/dL. Because lead crosses the placenta unencumbered, neurobehavioral and cognitive deficits in offspring are likely at 10 µg/dL, as well as preterm birth, decreased gestational maturity, lower birth weight, reduced postnatal growth, increased incidence of minor congenital anomalies, early neurologic or neurobehavioral deficits, and decreased intelligence at ages 3 to 7.

12.14. Alteration of Sexual Identity

The popular weed killer atrazine strips male frogs of a key hormone and turns them into hermaphrodites[10].

12.15. Developmental Testing In Vitro

In vitro techniques evaluate the three components of developmental toxicity: maternal toxicity, embryo toxicity and abnormal development. The maternal component can be measured using a cytotoxicity test (3T3 cells), embryo toxicity using a stem cell test, and abnormal development using measurements of cell differentiation.
However, these tests have not gained wide acceptance because validation work has not been performed. A difficulty with the design of new tests is that the process of embryo development, which involves proliferation, apoptosis and differentiation together, is not well understood at the basic level.



F12.42. Male mice experience two spikes in hormone production in their lifetime. One begins during fetal development and another at puberty (top), The fetal testes begin to produce testosterone 11 or 12 days after conception, and continue to do so until 4 or 5 days after birth.
In contrast, females normally experience only the second, pubertal increase in estrogen concentrations (middle).
However, androgens from male littermates in the womb can diffuse into their sisters' bloodstreams and influence their development (bottom).

12.16. Agents under Investigation

Agents and Substances Reviewed for Reproductive Health Effects by the Office of Technology Assessment
from Chemical Exposures and Toxic Responses

12.16.1. Metals

Lead, Boron, Manganese, Mercury, Cadmium, Arsenic, Antimony.

12.16.2. Chemicals

Agricultural chemicals: Carbaryl, Dibromochloropropane (DBCP), DDT♣, Kepone (Chlordecone), 2,4,5- T Dioxin (TCDD) and Agent Orange, 2,4-D.
Polyhalogenated Biphenyls: Polybrominated biphenyls (PBB), Polychlorinated biphenyls (PBC).
Organic Solvents: Carbon disulfide, Styrene, Benzene, Carbon tetrachloride**,** Trichlorethylene.
Anesthetic Agents: Epichlorohydrin.
Ethylene oxide (EtO)
Formaldehyde
Rubber manufacturing: 1,3-Butadiene, Chloroprene, Ethylene thiourea.
Vinyl halides: Vinyl chloride.
Undefined industrial exposures: Laboratory work, Oil, chemical and atomic work, Pump and paper work, Textile work, Agriculture work.

12.16.3. Physical Factors

Ionizing radiation: X-rays, Gamma rays.
Nonionizing radiation: Ultraviolet radiation, Laser, Visible light, Infrared radiation, Radiofrequency/microwave, Ultrasound, Video display terminals, Magnetic field, Hyperbaric/hypobaric environments, Cold environments, Noise, Vibration.

♣ Each increase in serum DDT of 10 ng/g of serum increases early miscarriage by 17%[25].

12.16.4. Biological Agents

Rubella, Cytomegalovirus, Hepatitis, Other infectious agents, Recombinant DNA.

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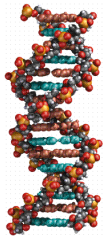
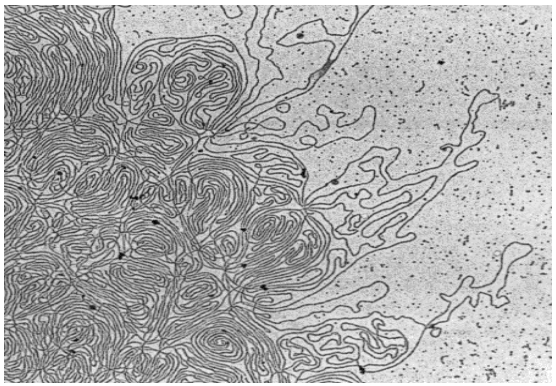
Genotoxicity

13· Genotoxicity

The importance of genetics in the contemporary scientific literature is overplayed, because the field of genetics is young and dynamic. Genes probably have the same importance in living systems that libraries have in society.



Only 5% of the mammalian genome is highly conserved, and therefore of some "important" use. Of the "important" 5%, only 1.5% encodes proteins (*exons*), 3.5% performing other biological functions, such as gene regulation. One famous noncoding RNA, XIST, acts to shut down one of the X chromosomes in females. The expressed 1.5% is made of 23,000 genes. The water flea, *Daphnia pulex*, has 31,000 genes, rice has 37,000. Still, 63% of DNA is copied to RNA[16], but not into proteins, which until now have been viewed as the main actors in biological function. Perhaps this reflects the limits of our understanding of genetics.



- 2-3 pg of DNA in the nucleus
- 2 m in length (unwound, as shown at left)
- 23,000 genes
- 2.5 x 10^9 base pairs
- 8-10% of base pairs are highly repetitive sequences
- 20% are moderately repetitive

Between humans and chimpanzees, genes are the same 96% of the time. Different degrees of gene duplication account for 2.7% of differences, and single pair differences for 1.2%. Most human genes are present in 0 to 5 copies per person, but a few genes may vary between 5 and 368[15].

Individual humans are very similar at the genomic level. *Single Nucleotide Polymorphisms* (SNPs), one nucleotide variations, occur in 0.1% of the genome. *Structural Variations*, involving larger amounts of DNA (repeats, translocations, truncations or deletions) involve 0.5% more of the genome.
The average person has defective copies of 250 to 300 genes, and 75 of those are associated with diseases[15].

Among the *introns* (the 98.5% that is not expressed), 68% has no known function at this time, 28% codes for protein, 5% is untranslated regulatory code and 4% is conserved among vertebrates. Most introns are very old, and they haven't changed very much in slowly-evolving branches of life, such as vertebrates.
Some believe that part of the unexpressed code is involved in the management of embryonic development[8]. Some other segments may control the expression of protein through microRNAs. Still, other unexpressed parts could contain hidden genetic diversity that can erupt from within when conditions change, giving the organism a genetic reserve[12].
Some introns could be functional spacers or inevitable junk left from the processes by which genomes evolve.

The mutation rate of male DNA is twice that of females.
Since 8% of the human genome consists of DNA of viral origin, could viruses be viewed as agents of human evolution?

Genetics has until now concentrated on the structural aspects as opposed to the functional aspects of genes, because structure can be observed more easily. But DNA should not be thought of as a static structure. Genetic code evolves from mutations and from the genetic variations associated with reproduction, but there are other mechanisms of spontaneous genetic evolution.

Since each gene can code for more than one protein, 200,000 to 500,000 different proteins are expected. Post-translational modifications to proteins such as acetylation, glycosylation and phosphorylation can also increase the number of distinct structures. In addition, genomics does not obviously reflect that in order to carry out their cellular roles, many proteins rely on interactions with other proteins or on the formation of complexes.

13.1. Working Structure of Genes

Most of the time, in most cells of the body, 90% of genes are silenced by histone structures, locked away within the compacted but orderly material that makes up chromosomes.

The unicellular *ciliates* have two types of nuclei in the same cell. A small nucleus contains the whole diploid genome. A larger one is copied from the small one, but containing only the 27,000 working genes of the genome, the *exons*, which code for protein. The *introns* are absent from this macronucleus. In the macronucleus, every gene ends up as a separate mini-chromosome, and is copied into about a thousand replicas. Both nuclei divide in mitosis.
Such sophisticated mechanisms to protect the genome are not present in humans.

13.2. Genetic Homogeneity of Organisms

Even healthy people have large gains or losses of DNA. 65 to 80% of people have *copy number variations* that run at least 100,000 bases long. In about 10% of DNA, long stretches can be deleted, duplicated, often many times, or written backwards[17]. Genomic mechanisms may actually predispose us to such changes.

Single organisms can also *express* more than one genotype. They are then called "mozaics". A British geneticist, Mary Lyon, observed that in all females, one of the X chromosomes is inactivated by adhering RNA (from the gene *Xist),* forming the Barr body (this is used to confirm womanhood under the microscope). But how is it possible to inactivate the *same* X chromosome (of the two available) in all cells of the body? Lyon's observation is that the choice for inactivation among the two X chromosomes is actually random, occurring after 9 rounds of mitosis. Females are therefore made of 512 (2^9) different parts, each part being randomly father-X or mother-X: **all females are mozaics**. In human females, this was confirmed by variations in the expression of the G6PD gene (responsible for breaking down of sugars in erythrocytes, in particular).
Mosaicism can be observed by eye in some species when pigmentation is X-chromosome-based. In multi-colored *calico* cats, a gene determining whether fur is orange or black resides on chromosome X. A different gene account for the white areas.

F13.1. Calico Cat.

As if females did not have enough genetic diversity, it has been recently recognized that females often harbor cells from

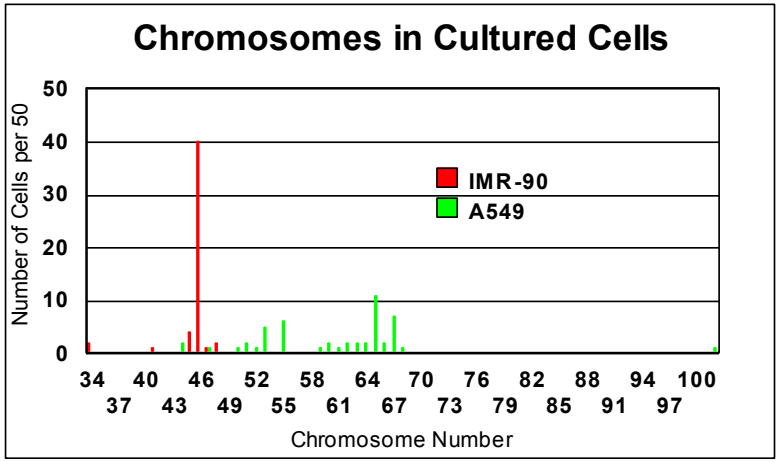
their mothers, as well as from their children (whether they are males or female), possibly adding to immune system confusion in females.

How did the X and Y chromosome configuration come about? Apparently, over evolutionary periods, there was *failure of recombination* between the X and Y genes from spontaneously occurring changes. Some XY crossing over occurs during male meiosis, but 95% of the Y chromosome is on its own, having no code with which to recombine. Presumably, in males, readjustment of X genes expression compensates for having one, rather than two copies. Major segments of the Y chromosome were only sequenced, with great difficulty, in 2003. A gene on the Y chromosome, *Sry*, is important in the regulation of dopamine. It is believed that men and women have distinctive dopamine-regulating systems, perhaps explaining partially psychological differences between the sexes.
At the level of the genes, simple features can account for most of gene-based evolution, even without the creation of new innovative code:

1. *down-regulation* can occur through destructive mutation,
2. *up-regulation* can occur through a stuttering mechanism, repeating the same sequence more than once[13], and
3. *plagiarism*, using the sequences of other life forms.

13.3. Genetic Homogeneity among Cells

In normal cells *in vivo*, the chromosome number is though to be relatively constant, although in certain tissues such as the liver, many cells have two nuclei, and some of the nuclei are tetraploid. When the genome is altered, the cell has a high

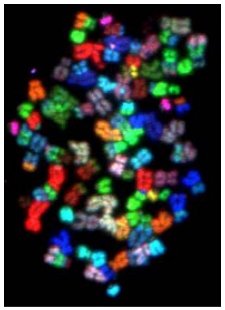


F13.2. Chromosome numbers in human cells grown in culture. IMR-90 is a human lung diploid fibroblast strain, A549 is a human lung carcinoma. Data from ATCC.

probability of dying. *Mutations* occur when the DNA alterations propagate in subsequent generations of cells.

Cells in culture have somewhat less regular chromosome numbers (normal is 46) than body cells, as shown in red in F13.2. These irregularities can be caused by defects in the architecture of the mitotic apparatus, which controls the migration of chromosomes to daughter cells.
Cancer cells, by contrast, most often have a hyperdiploid karyotype (an excess number of chromosomes, green in F13.2). Individual cells of a given cancer cell line differ in properties such as metastatic capacity, transplantability, antigenic makeup, drug sensitivity, growth rate, metabolism, and morphology.
Using transformed Chinese Hamster Embryo cells, it was shown[5] that the number of genes altered at a rate of more than 3% per cell division. It therefore seems that cancer cells have the ability to spontaneously generate genetic variants.

The genetic variations are not limited to chromosome numbers. F13.3 shows an extremely high rate of translocations in cancer cells using the technique called *spectral karyotyping*. This method allows the coloring of chromosomes in individual colors using a combination of fluorescent dyes.
It is believed that this genetic variability may enhance the survivability of tumors in the face of environmental changes, including chemotherapy.



F13.3. Human mitotic chromosomes from a primary tumor are colored with dyes (SKY). The high rate of translocations is indicated by mixed color on the same chromosome. Over 30 translocation events can be detected.[6]

13.4. Karyotype Evaluation

How do we assess a person's general **genetic state**? There are simple laboratory techniques to achieve this (F13.4) using white blood cells from the blood.
Colcemid (F13.4) is a chemical which interferes with the mitotic process, stopping mitosis in metaphase[↑]. Colcemid allows the observation, identification and size measurement of chromosomes, and comparison with known human templates (F13.4).
The chromosomes can be stained for the recognition of bands (F13.6). Further, molecular probes can be mixed with DNA homogenates, attached fluorescent labels becoming visible when bound to matching DNA segments. Because each probe only covers a short DNA segment, a large number of probes is needed for detailed recognition of genetic state.

13.5. Chromosome Nomenclature

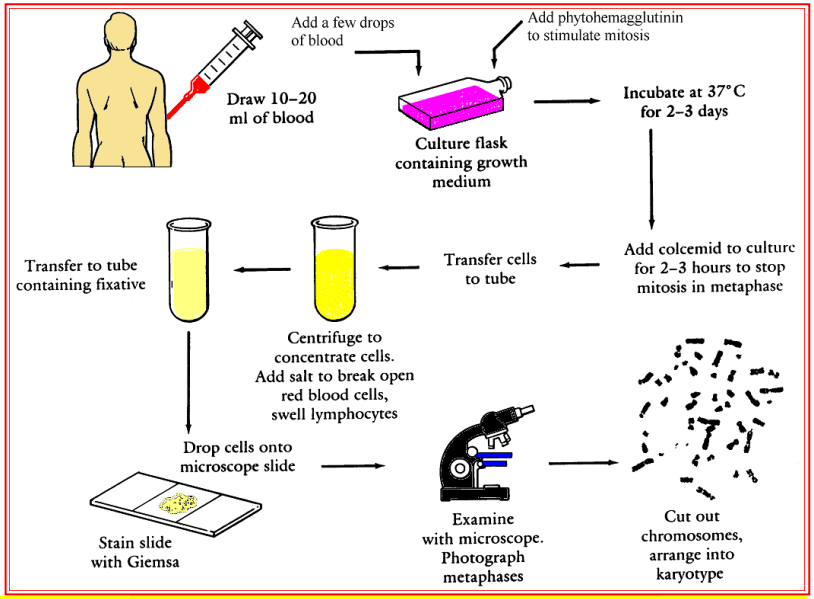
The banding pattern of chromosomes is developed using dyes: Quinacrine or Giemsa are frequently used. The R stain is a fluorescent label, inverse of Q or G.
The labeling nomenclature is illustrated for chromosome 9 (F13.7). The letter "p" is used for the small arm of chromosomes ("petit"). A genome labeled "9p-" would be missing one of the small arms of chromosome 9.
Chromosome numbers are quite different among species, as shown in T13.5. For example, sex is determined among humans according to the formula: XX = female and XY = male. The platypus has 5 pairs of chromosomes that determine its sex ($X_1X_2X_3X_4X_5X_6X_7X_8X_9X_{10}$ = female and $X_1Y_1X_2Y_2X_3Y_3X_4Y_4X_5Y_5$ = male).

13.6. DNA Stability

Evolution put in place mechanisms to stabilize genes and improve the fidelity of genetic inheritance:

- double-stranded DNA as opposed to the older single-stranded RNA,
- error-checking by DNA polymerase: excision and recombination repair, and
- bi-parental inheritance (sex) to provided genetic diversity within meiosis (a method of shuffling genes) without the need for high rates of mutation.

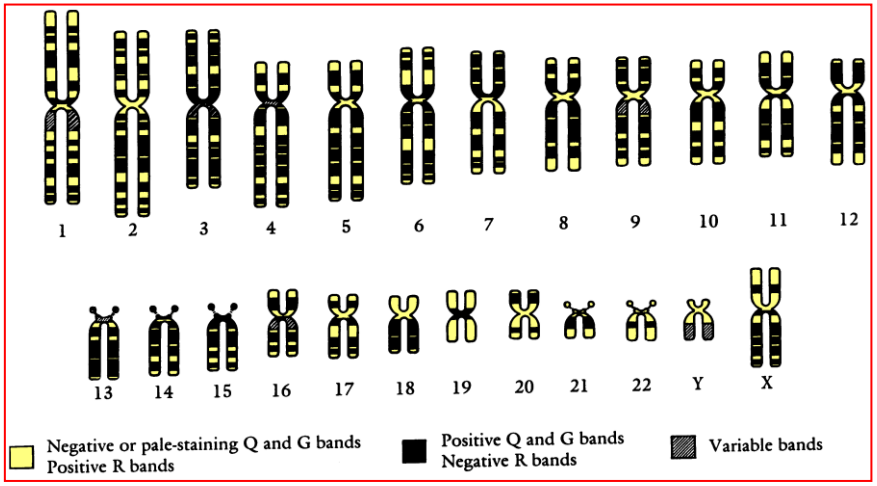
[↑] Any toxic disruption to the mitotic apparatus can lead to gross chromosomal aberrations, for example changes in chromosome numbers (see 13.2).



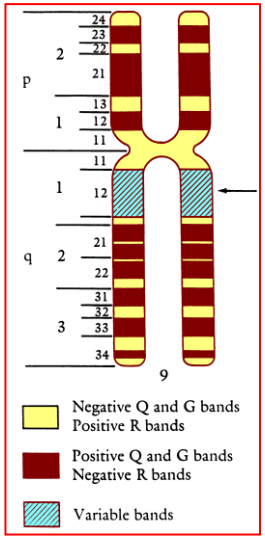
F13.4. Determining Human Heredity. *Cummings.*

T13.5. Chromosome numbers in various species.	
Homo sapiens (human)	46
Mus musculus (house mouse)	40
Zea mays (corn or maize)	20
Drosophila melanogaster (fruit fly)	8
Xenopus laevis (South African clawed frog)	36
Caenorhabditis elegans (microscopic roundworm)	12
Saccharomyces cerevisiae (budding yeast)	32
Canis familiaris (domestic dog)	78
Arabidopsis thaliana (plant in the mustard family)	10
Muntiacus reevesi (Chinese muntjac, a deer)	23
Muntiacus muntjac (its Indian cousin)	6
Myrmecia pilosula (ant)	2
Parascaris equorum var. univalens (parasitic roundworm)	2
Cambarus clarkii (crayfish)	200
Equisetum arvense (field horsetail, a plant)	216
Platypus (duck-billed, egg-laying Australian mammal)	52

There is a correlation between genome size and cell size among various species.



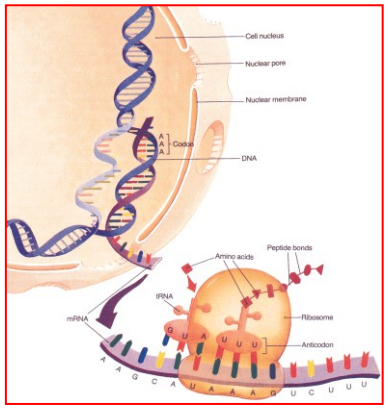
F13.6. Human Chromosomes. *Cummings.*



F13.7. Human Chromosome 9. *Cummings.*

13.6.1. Mutation Resistance of the Genetic Code

It is useful to review the basic genetic mechanisms of transcription and translation (F13.8) and the translation code (F13.9).



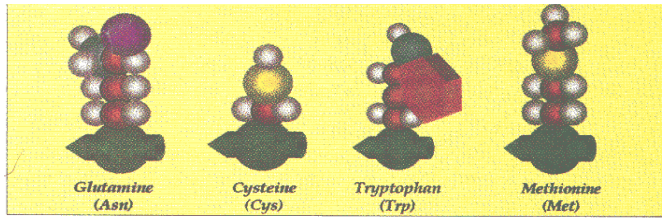
F13.8. Transcription and translation.
Essentials of Environmental Toxicology.

The translation code (below) is slightly different in mitochondria.
Note the apparent redundancy in the code (64 combinations possible, 20 amino acids used). "Synonymous" codons may not be completely equivalent: the choice on one over the other may relate to how common the matching transfer RNA is. If the transfer RNA is rare, the transcription of the codon will be slowed. This pace-setting may affect protein folding.
For example, the mutant codon C3435T of the MDR1 gene codes for isoleucine within P-glycoprotein. Synonymous C3435T codons are associated with above or below average effectiveness in pumping toxicants out of cells. P-glycoprotein is particularly important in determining the drug resistance of cancer cells[14].
Natural selection would favor a genetic code that includes fault tolerance, that is, information can still be accurately recovered in case of error. You will note in T13.9 that similar amino acid names (in blue) are clustered, so that the *third* base is often irrelevant: the same amino acid is coded in spite of third base changes.

T13.9. Translation Code of Bases to Amino Acids

First Base	Second Base				Third Base
	U	C	A	G	
U	UUU Phe	UCU Ser	UAU Tyr	UGU Cys	U
	UUC Phe	UCC Ser	UAC Tyr	UGC Cys	C
	UUA Leu	UCA Ser	UAA STOP	UGA STOP	A
	UUG Leu	UCG Ser	UAG STOP	UGG Trp	G
C	CUU Leu	CCU Pro	CAU His	CGU Arg	U
	CUC Leu	CCC Pro	CAC His	CGC Arg	C
	CUA Leu	CCA Pro	CAA Gln	CGA Arg	A
	CUG Leu	CCG Pro	CAG Gln	CGG Arg	G
A	AUU Ile	ACU Thr	AAU Asn	AGU Ser	U
	AUC Ile	ACC Thr	AAC Asn	AGC Ser	C
	AUA Ile	ACA Thr	AAA Lys	AGA Arg	A
	AUG Met or START	ACG Thr	AAG Lys	AGG Arg	G
G	GUU Val	GCU Ala	GAU Asp	GGU Gly	U
	GUC Val	GCC Ala	GAC Asp	GGC Gly	C
	GUA Val	GCA Ala	GAA Glu	GGA Gly	A
	GUG Val	GCG Ala	GAG Glu	GGG Gly	G

Phenylalanine, Leucine, Isoleucine, Methionine, Valine, Serine, Proline, Threonine, Alanine, Tyrosine, Histidine, Glycine, Asparagine, Lysine, Aspartic acid, Glutamic acid, Cysteine, Tryptophan, Arginine, Serine, Arginine, Glycine.



If we look at the genetic code in detail, we find that whenever a single coding change does *NOT* yield the same amino acid, it still has a good chance of coding for one with *similar hydrophobicity*. All the codons with a middle U correspond to hydrophobic amino acids, which is critical in determining how the protein folds.
Nature "knows" from evolution that level of hydrophobicity is critical, even at the most basic level: at least 2/3 of the time, a

point mutation will either leave the amino acid unchanged or substitute another similarly hydrophobic amino acid. The genetic code itself is designed to protect hydrophobicity information.

The genetic code is optimized to conserve hydrophobicity specification, well ahead of other variables, such as electric charge[11].

If the wrong base is inserted by the molecular machinery (T13.10, top) in the process of copying DNA, errors in protein can result. Some toxicants, like hydroxyurea, can produce errors by producing an imbalance in the pool of intracellular DNA precursors.

A "non-coding" error can also occur when base *analogues*, which cannot serve as a template in replication, are included in DNA strands.

Not all mutations have equivalent effects. T13.10 illustrates various examples of base mutations and their effects on the coded protein.

Changes in the coding of a protein *may* have consequences on the secondary or tertiary structure of a protein, or not at all. A protein product may interact with a single other molecule, or with dozens of them (for example, in a ribosome). It is presumed that molecules that interact with many others have a more critical structure.

But the normal copying, maintenance and transcription of DNA is a fallible process, due to endogenous damaging agents, including metabolites acting as alkylating agents and the ROS (Reactive Oxygen Species) that arise during respiration.

About ten thousand DNA lesions per day per cell are spontaneously produced.Although most of the genetic code in made of non-coding *introns*, consequential alterations to the code are still frequent. Mutation rates vary from about 10^{-4} to 10^{-7}, according to the site. Accurate mutation rates for specific mutations are available from epidemiology.

T13.10. Amino Acid alterations from Codon mutations.

Codon Sequence:	-UUU-	AAG-	UAU-	GGC-	UAA
Amino Acid Sequence:	-Phe-	--Lys-	--Tyr-	--Gly-	--Stop
Alterations: Single Base Substitution (in red)					
No Effect on AA Sequence	UUU Phe	AAG Lys	UA**C** Tyr	GGC Gly	UAA Stop
Missense (change in AA)	UUU Phe	AA**U** **Asn**	UAU Tyr	GGC Gly	UAA Stop
Nonsense (early stop)	UUU Phe	AAG Lys	UA**A** **Stop**	GGC	UAA
Alterations: Base Deletion (of A) or Insertion (of U)					
Frameshift with missense	UUU Phe	A ↑GU **Ser**	AUG **Met**	GCU **Ala**	AA? ???
Frameshift with nonsense	UUU Phe	**U**AA **Stop**	GUA	UGG	CUA
Alterations: Codon Insertion (of GUU) or Deletion (of AAG)					
Insertion	UUU Phe	AAG Lys	**GUU** **Val**	UAU Tyr	GGC Gly
Deletion	UUU Phe	UAU Tyr	GGC Gly	UAA Stop	

The mutations, if not lethal, will be reproduced in downstream cells. Such DNA alterations can give rise to congenital anomalies.

13.7. DNA Damage

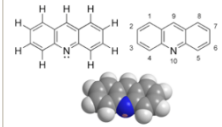
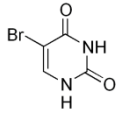
The major forms of DNA damage include SSB (Single-strand Breaks), DSB (Double-strand Breaks), alteration of bases, mispairing in the A-T and G-C bonds, hydrolytic depurination, hydrolytic deamination of cytosine and 5-methylcytosine bases, formation of covalent adducts with DNA, and oxidative damage to bases and to the phosphodiester backbone of DNA.

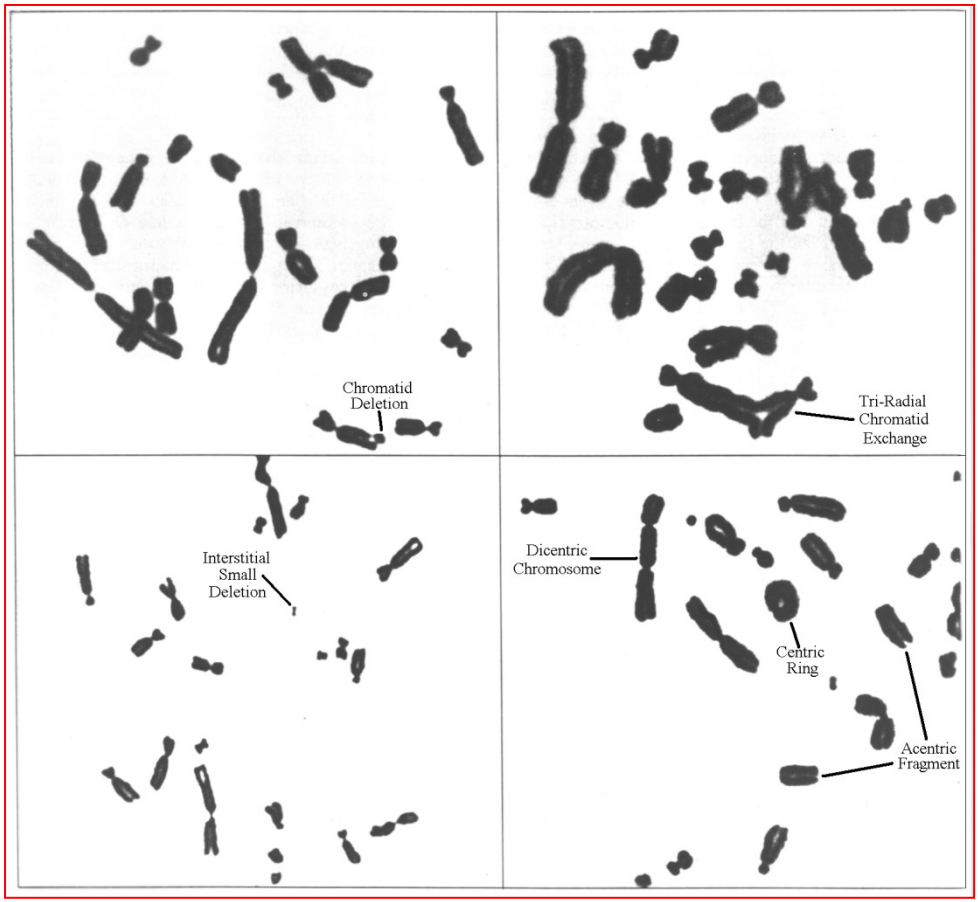
Examples of agents capable of creating a range of lesions are shown in T13.11.
Environmental DNA-damaging agents include UV light and ionizing radiation, as well as a variety of toxicants encountered in foodstuffs, air and water. Electrophyllic molecules in their native form, or from bio-transformation are the most frequent cause of damage. Free radicals and activated molecules result from intoxication as well as normal metabolism. A group of chemicals called base analogues substitute legitimate bases, inducing a coding error.

13.7.1.1. Due to Toxicants

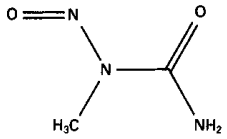
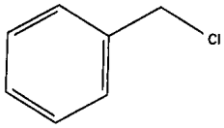
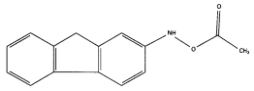
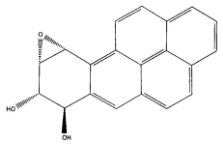
In some cases, the exact site of mutations on the DNA is known for specific toxicants, as shown in the following table.

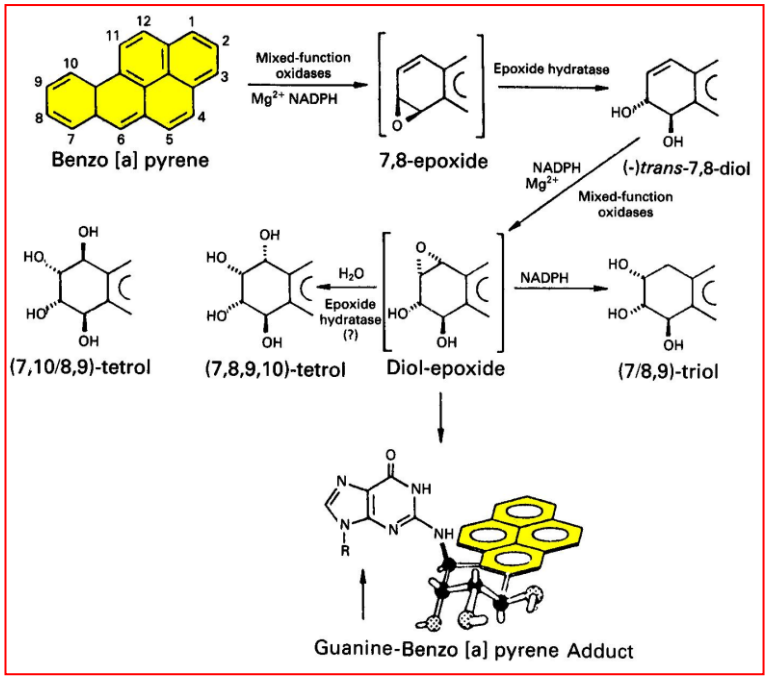
T13.11. The effects of selected physical and chemical mutagens.

Mutagen	Effect on DNA/RNA	Type of mutation
Ultraviolet radiation	C, T, and U dimers that cause base substitutions, deletions, and insertions	No effect, missense, or nonsense
X rays	Breaks in DNA	Chromosomal rearrangements and deletions
Acridines (tricyclic present in dyes) 	Adds or deletes a nucleotide	Missense or nonsense
Alkylating agents	Interferes with specificity of base pairing (e.g., C with T or A, instead of G)	No effect, missense, or nonsense
5-Bromouracil 	Pairs with A and G, replacing AT with GC, or GC with AT	No effect, missense, or nonsense



F13.12. Chromosome breaks (arrows) by X-rays in Chinese Hamster Ovary Cells. *Casarett & Doull.*

T13.13. Toxic Chemicals Reaction Sites on DNA.		
Agent	**Structure**	**Sites**
Methyl Nitroso Urea		N3,N7,O6 of Guanine, N1,N3,N7 of Adenine, N3 of Cytosine, Phosphate
Benzyl Chloride		N7,N2,O6,C5 of Guanine
N-Acetoxy Acetyl-amino Fluorene		C8 of Guanine and Adenine
d-7,8-dihydroxy-9,10-oxy-7,8,9,10-Tetrahydro Benzo(a)pyrene		N2 and O6 of Guanine, N6 of Adenine



F13.14. Formation of a DNA Adduct from a Polycyclic Aromatic Hydrocarbon. *Harris, 1978, Academic Press.*

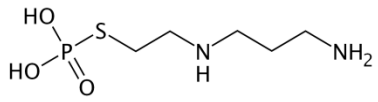
13.7.1.2. DNA Adducts

Exposure to various agents may result in diverse, structurally heterogeneous *adducts* being added to DNA. In this case, the structure of DNA is altered by the covalent addition of molecular species. A subset of these *adducts* can contribute to carcinogenicity.

Some adducts, such as methylation may be reversible and play a part in normal physiology (*gene silencing*). In animals, 2 to 7% of cytosines are normally methylated. Methylation patterns are heritable from generation to generation, and are tissue specific. Modulation within tissues is accomplished by the action of *demethylases*.

13.8. DNA Protection

Many lesions to DNA and other living tissues can be attenuated using antioxidants. Natural antioxidants may be produced endogenously, and also occur in food (vitamins C, E). But very powerful anti-oxidants, such as the injected drug amifostine (WR-2731, shown) are used for protection against medical therapies such as cisplatin and radiation treatment. In the case of this drug, it is believed that it is present in normal cells at a concentration 100 times higher than in cancer cells.
Many of these compounds tend to concentrate in the mitochondria and in the nucleus.



13.9. DNA Repair

13.9.1. DNA Repair Mechanisms

There are more than 100 genes in our genome devoted to detecting and repairing DNA damage. DNA repair enzymes continuously monitor chromosomes to correct damaged nucleotide residues. Failure to repair DNA lesions may result in blockages of transcription and replication, mutagenesis, and cellular cytotoxicity.
The vast majorities of the lesions are repaired by:

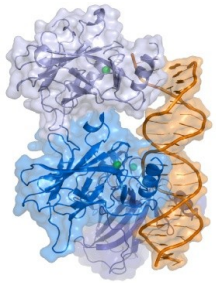
- BER (Base Excision Repair),
- NER (Nucleotide Excision Repair), and
- MMR (Mismatch Repair)

DNA *polymerase* (shown), the enzyme of replication, can move backwards on the DNA strand and perform exonuclease digestion of a sequence surrounding an incorrectly matched base-pair. Exons may be preferentially repaired (by comparison with introns).
For example, in excision repair, thymidine dimers which can be formed by UV exposure are repaired by excision, followed by β-polymerase and finally by DNA *ligase* which seals the gap.

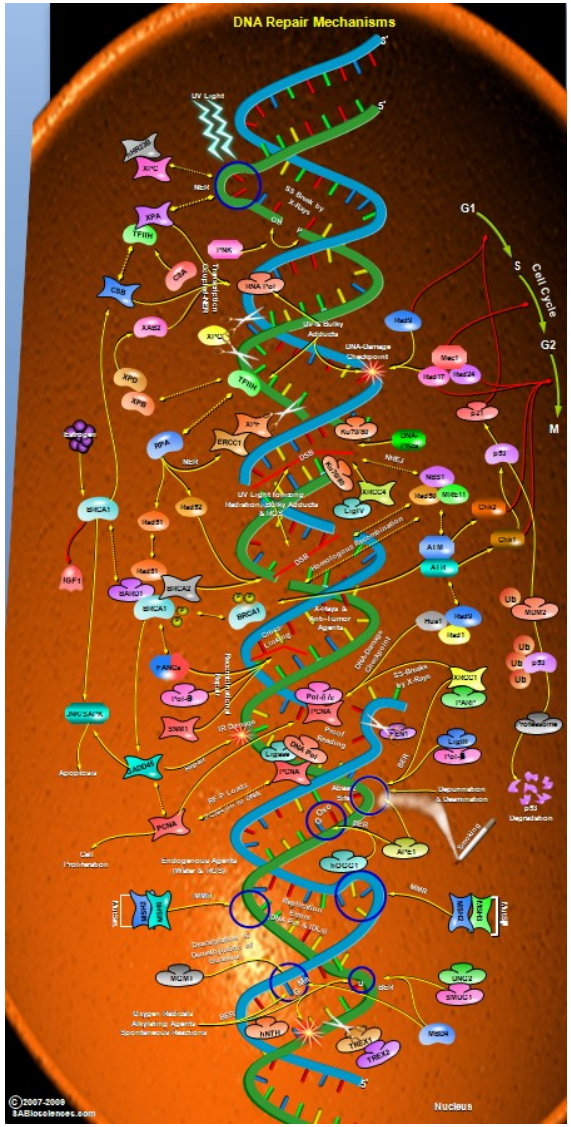
Sunlight-induced *Xeroderma Pigmentosum* is a disease of DNA repair mechanisms (autosomal recessive). Individuals are extremely sensitive to sunlight and usually die early of some form of cancer.
There is a continuous competition between DNA mutation and repair in living organisms.

The process of thymidine dimer repair can be stimulated by skin application of thymidine dinucleotide, a DNA snippet of just two nucleotides. Apparently, there a cellular detection of DNA debris which triggers DNA repair mechanisms[10].

13.9.2. DNA repair: p53



p53 (shown) designates both a universal stress responder gene and a protein product within cells. One of the best known mechanisms known to lead to cancer is damage to the p53 gene and consequently to the supply of p53 protein. The p53 gene is normally silent, but is turned on by DNA damage. When triggered, it halts cell division, making time for repairs. If the repairs are not successful, p53 can also induce *apoptosis*.



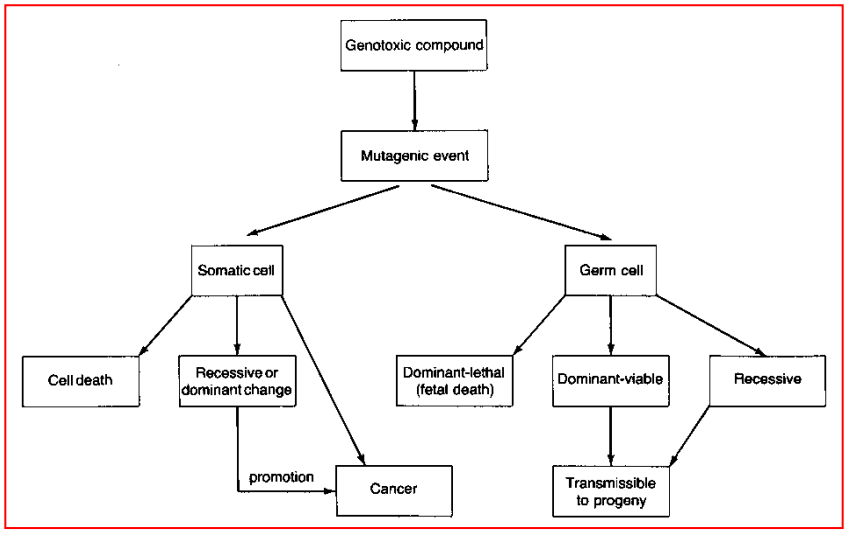
Beyond such natural DNA repair, there is therapeutic repair. When a patient has leukemia, it is possible to remove his bone marrow completely (using radiation and chemicals), and replace it with an autologous graft (the person's own marrow, purified of cancer cells outside the body) or that of a sibling or matched donor (allograft). The modification of hematopoietic stem cells by bone marrow transplant allows the stable genetic modification of an entire organ, the blood.

13.10. Mutagenesis in Somatic and Germ Cells

As illustrated in F13.15, there are two streams of genetic mutation:

13.10.1. Somatic mutation

- is a change from the original parental code which
- is passed on to the **cell**'s offspring.



F13.15. Mutagenic events. *Williams and Burson.*

If the cell divides or proliferates, this creates a sub-group of altered cells. In the case of mutation of *somatic cells*, one cell, and eventually one tissue, and finally the organism can be affected (tumor).

13.10.2. Germinal mutation

- change from the original parental code which
- is passed on to the **organism**'s offspring.

The whole organism is *mutated* (all tissues). Mutation can also occur at the *blastocyst stage* in the course of gestation, affecting part of the cells of an organism. Early in development, germline cells migrate away from the other cells in the embryo, and follow a special "program". Later, they migrate back into the location in the embryo where the sex organs will form.

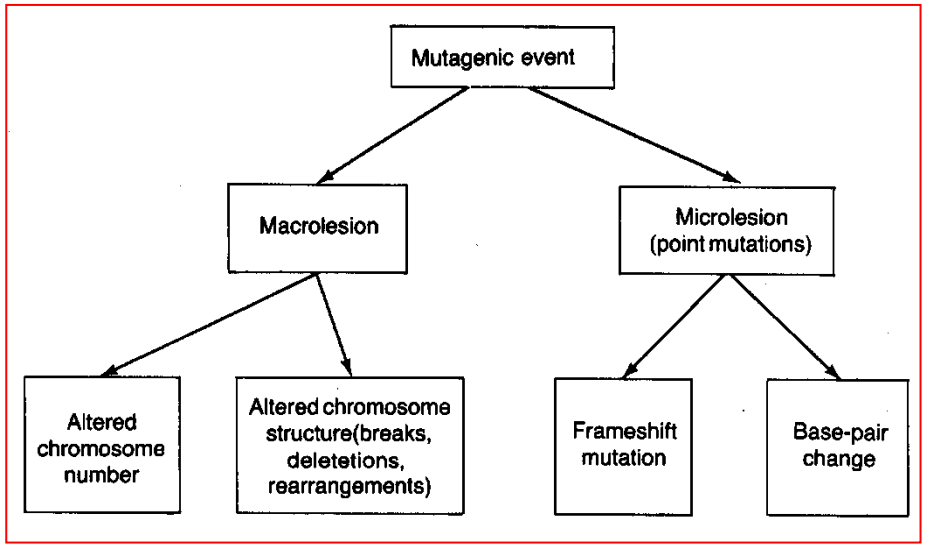
13.11. Consequences of Mutagenic Events

F13.16 shows the scenarios resulting from mutations. There may be changes in the number of chromosomes, chromosome breakages (shortened or lengthened), including exchanges between segments of chromosomes (which may not lead to functional trouble), and micro lesions: code mutations where the base-pairs are altered.

Note: the term *clastogen* refers to a chemical with the ability to break chromosomes apart.

Radiation is the best studied example of a dose-dependent mutagen.

Generally, any *free radical* has an unpaired electron with the ability to create chemical instability. This instability is well correlated with mutagenic and carcinogenic potency. Cancer originates, so to speak, from a *quantum mechanical* need for an extra electron.



F13.16. Consequences of Mutagenic events. *Williams and Burson*.

About 5000 diseases in humans are presently known to be caused by defective genes (T13.17). Defective genes account for 20% of infant mortalities, half of miscarriages and 80% of mental retardation cases.

T13.17. MUTATIONS IN GERM CELLS

Genetic Disease or Condition	Cases in the US
Dyslexia	15,000,000
Manic depression	2,000,000
Schizophrenia	1,500,000
Juvenile diabetes	1,000,000
Adult polycystic kidney	500,000
Familial Alzheimer's disease	250,000
Multiple sclerosis	250,000
AAT deficiency (emphysema)	120,000
Myotonic muscular dystrophy	100,000
Fragile X chromosome	100,000
Sickle cell anemia	65,000
Duchenne muscular dystrophy	32,000
Cystic fibrosis	30,000
Huntington's chorea	25,000
Hemophilia	20,000
Phenylketonuria	16,000
Retinoblastoma (childhood eye cancer)	10,000

vanDelft, Mutation Research, 1998

13.11.1. Macro-Lesions

Those are large scale changes in chromosome number or structure. Many cancer cells thought to have a normal genotype have in fact genes where large parts have been recombined.

13.11.1.1. In Normal Reproduction

Meiosis, in which the number of chromosomes is halved, involves a random distribution of chromosomes pairs into zygotes, as well as *recombination*[♣] between chromosome pairs (2^{23} possibilities among whole chromosome combinations seems insufficient to satisfy evolutionary needs). There is intimate twisting of homologous chromosomes in meiosis. If the process is completed without a hitch, no mutations occur, just homologous code exchange. When something goes wrong, the fertilized egg often dies.

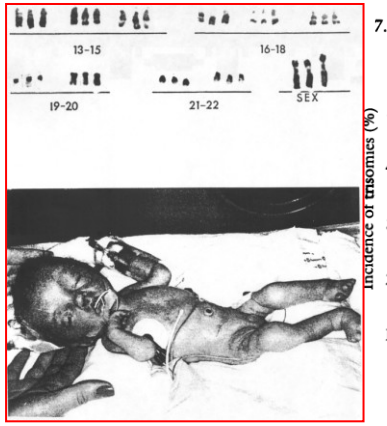
13.11.1.2. In Abnormal Reproduction

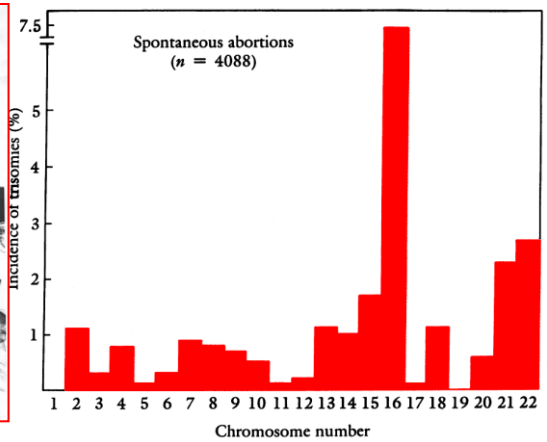
Cell nuclei with altered chromosome numbers can be *poly*ploid or *aneu*ploid.

[♣] Recombination between chromosome pairs allows segments of maternal and paternal chromosomes to be exchanged.

13.11.1.2.1. Polyploidy

Polyploidies (= many copies of all genes) that are possible in humans are triploid and tetraploid. Triploid occurs when two sperm make it at the same time to the egg (F13.18).





F13.18. Triploid human infant. Lethal, enlarged head.

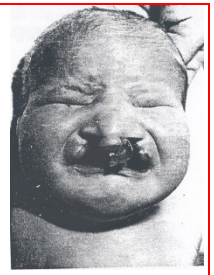
13.19. Lost trisomies in humans.. *Cummings.*

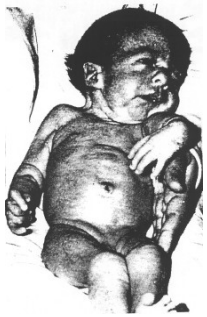
13.11.1.2.2. Aneuploidy♥: monosomy and trisomy

Monosomies (a single chromosome rather than two) are lost very early. A few trisomies (three chromosome rather than two) are survivable, as long as not all chromosomes are involved, but most trisomies result in abortions (F13.19).
Most frequent are Patau syndrome (trisomy 13, F13.20), Edwards syndrome (trisomy 18, F13.21) and Down syndrome (trisomy 21, F13.22).
Anomalies are also possible in the sex chromosomes. Klinefelter syndrome is rather frequent (1 in 1000 male births) and characterized by minor breast development and sagging

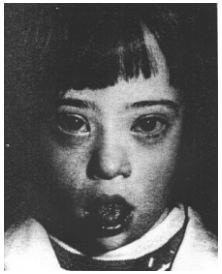
♥ Changes in the normal number of single chromosomes.

F13.20. Trisomy 13 or Patau syndrome. Lethal, cleft lip and palate, small eyes, mean survival 6 months. *Cummings.*





F13.21. Trisomy 18 or Edwards syndrome. Lethal, means survival 3 months. *Cummings.*



F13.22. Trisomy 21 or Down syndrome. Straight hair, flattened face, open mouth with large tongue, upward slanting eyes with epicanthal folds. Few reach 50 years of age. *Gelerhter.*

abdominal wall, with a variable chromosome number: 47-XXY or XXX, 48-XXYY, XXXY, 49-XXXXY. Do you know who is the well known person (F13.23) probably afflicted by the syndrome?

F13.23. From 1350 BC, Klinefelter syndrome. *Cummings.*

13.11.1.2.3. Structural chromosome aberrations

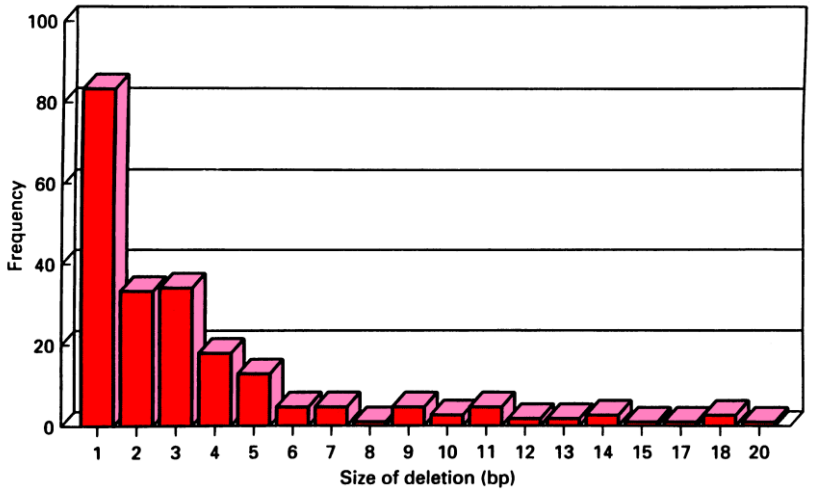
Chromatid breaks, deletions and rearrangements also occur. Breaks and most deletions are lethal.

Viable **deletions**:

- Cri-du-chat: 5p-. Infants have catlike cry, facial anomalies, severe mental retardation.
- Retinoblastoma: 13q-. Cancer of the eye.

Rearrangements are part of the normal process of meiosis, and are often not deleterious, since no genetic material is lost. But note that 5% of Down syndrome cases involve *genetic translocations*.

13.11.2. Micro-Lesions



F13.24. Probability of base pair deletions. *Vogel.*

Smaller mutations to the base pairs tend to be more frequent than larger ones (F13.24). Most of these mutations are recessive. The rates of the small mutations can be used as a clock to analyze evolutionary divergence in the chromosomes of cells or mitochondria (where the mutation rate is higher).

13.11.3. Examples of Micro-Lesions

13.11.3.1. Occurring Naturally

- A single point mutation with a frequency of 5×10^{-6} is Marfan's syndrome, a dominant mutation of the gene for collagen with variable penetration. A classic sign is arachnidodactyly, and frequent aneurysm of the aorta.

- A point mutation is believed responsible for cystic fibrosis. The mutation alters the coding of a functional part of a cell receptor for chlorine in lung tissue.

F13.25. Testicular feminization victim. *Cummings.*

- *Testicular feminization* is an X-chromosome-linked recessive disorder which causes XY individuals to develop into phenotypic (very attractive) females because of insensitivity to testosterone: they have a defective nuclear testosterone receptor♦. Subjects usually have testes hidden in the inguinal region, lack pubic hair and do not menstruate. They progressively turn into males over time (F13.25).

- **Methylation**. The term methylation refers to the addition of a methyl group to the cyclic carbon 5 of a cytosine nucleotide. A family of conserved DNA methyltransferases catalyzes this reaction. Basically, the methyl group tags a gene so it is turned off, and an unnecessary protein product is not produced in a particular cell. For instance, one of the two X chromosomes in female mammals is inactivated by methylation.

♦ Generally, receptors for water-soluble substances are on the cell membrane, whereas receptors for fat-soluble substances are in the cytosol or in the nuclear membrane.

13.11.4. Genetic Therapy

While it would seem like a good idea in medical therapy to correct genetic deficiencies in subjects by inserting the correct gene and deleting the bad one, the cells of the body are not as easily accessible as the code on a computer hard disk.
Although we have been successful at *reading* the genome, effectively *fixing* it in adults has remained a difficult problem.
1150 human disease genes have been discovered, but only a few discoveries have led to a cure. Only *one*, for Chronic Myeloid Leukemia, has reached the market.
Viral vectors or electroporation techniques for achieving this delivery have various problems. Until now, very modest successes have been possible correcting genetic diseases in tissues of the body that proliferate continuously. French workers reported curing 9 infants of severe combined immune deficiency in 2000, but two of them developed leukemia 2 years later[9]. *Unexpected results have been frequent in the few attempts made at genetic therapy*.

13.12. Risks of Recessive Mutations

Recessive mutations pose the greatest risk and are of the greatest concern to geneticists.
Redundancy in safety systems poses a problem if there is no warning of its loss. *In genotoxicity, how are recessive mutations detected?* They will only be detected when some other agent produces a dominant gene function loss or when an individual becomes *homozygous* for the recessive trait.
Hemophilia, color-blindness and hereditary baldness are all caused by recessive alleles on the X chromosome, of which there are two in women but only one in men.. This means that an agent producing recessive mutations on X in women could only be uncovered by testing in combination with another agent capable of disabling the dominant gene or by insuring that test subjects are homozygous for that gene (the same is true for all autosomes). The presence of a deleterious recessive mutation would be detected as an increased rate of disease from an agent affecting the dominant gene. *Because of this mechanism, there is potential for an unrecognized degeneration of the gene pool from apparently inoffensive toxicants.*
Preservation of the genome depends on our understanding of it. Because **ex**ons are actually **ex**pressed, at least some of their mutations would be detected. Since introns are processed out, how do we detect mutations in introns, and can we guarantee that they have no subtle role in species-level health?
Is the redundancy of the genetic code a true redundancy?
If we have difficulties quantifying the effects of *one agent*, how good are we at detecting the effects of *agent combinations*, which appears necessary to detect recessive mutations?

13.13. Mutagenicity Testing

In vivo toxicity experiments often pinpoint the target organ, but not the mechanism by which the agent produces the effect.

In vitro models that are fast and inexpensive have become dominant in mutagenicity testing (T13.26). Even when animals are used for mutagenicity testing, the detection procedure is generally performed *in vitro*, excluding only the exposure segment.

Two large classes of models are available: cells without nuclei and cells who have them. Cells without nuclei are inherently more vulnerable to attack because their

genes are not segregated in a nucleus, and also because they are not diploid.
Furthermore, the bacteria used in genotoxicity tests carry an *rfa* mutation leading to a defective polysaccharide coat, making the cells more permeable to large molecules. Even further, the *uvrB* mutation impairs DNA repair by excision.

13.13.1. Testing with Prokaryotic Cells

T13.26. *In vitro* Mutagenicity Testing systems.	
Bacteria	Salmonella typhimurium
	Escherichia coli
Fungi	Saccharomyces cerevisiae (yeast)
	Neurospora crassa (fungus)
High life forms	Plants: tradescantia, corn, allium, beans…
	Coelenterates - Hydra attenuata
	Insects - Drosophila melanogaster
Mammalian cells	Mouse lymphoma cells
	Chinese hamster ovary cells (CHO)
	Hamster pulmonary cells (V79)
	Rodent bone marrow cells
	Human lymphocytes

It has been know for a long time (the work of Beadle and Tatum) that mutants of molds, for example, develop nutritional dependencies after being mutated. Ames developed a bacterial test systems using *mutant* organisms with a highly specific defect in a genetic locus, which cannot grow unless some vital nutrient (amino acid, sugar) is included in the growth medium. When the vital nutrient is deliberately withheld from the medium, the test organism cannot grow. But if the organism mutates, *reverting by chance* to the normal "wild-type" organism, it grows on the medium.
Having spread organisms on nutrient agar, Petri dishes (F13.27) are incubated at a suitable temperature for 24 to 48 hours, by which time any colonies are visible to the eye. The rapid rate of cell division typical of bacteria is good for the test, since the DNA is then most vulnerable.
The mutant organism, supported by the inclusion of a complete nutrient, is mixed with the test agent, incubated, and then decanted onto an agar plate which contains only a minute amount of the nutrient (to get growth started). Continued growth and the development of visible colonies will require that the test agent be mutagenic, causing some reaction with the DNA to result in a reversal of the genetic material back to a "wild-type" organism.

The basis of the bacterial tests involve either:

(1) a reverse-mutation from a nutrient-dependent organism to a "wild-type" capable of sustaining itself on minimal medium, or

(2) a forward-mutation where additional, chemical-induced changes in the genetics result in a new, easily identified phenotype.

Forward mutations present a larger genetic target for the chemical, with mutations occurring at several loci within one gene or being spread over several genes. Reverse-mutation assays, using organisms with mutations at an easily detected locus, provide small, specific, and selective targets.

13.13.1.1. Ames Test (prokaryotes in vitro)

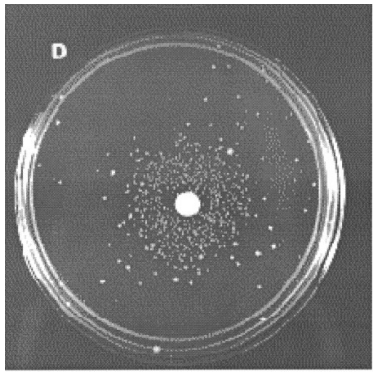
The Ames Test was the forerunner in this field, using a number of mutants of *Salmonella typhimurium* that were usually defective for histidine. However, two problems became obvious with widespread use of this assay system (below).

Genotoxicity tests such as the Ames test measure a compound's ability to induce DNA damage, a process *associated* with

carcinogenesis. Developed in the 1970s, it measures the reversion of a mutant *his* gene in *Salmonella typhimurium*. Although some substances that cause cancer in laboratory animals (dioxin, for example) do not give a positive Ames test (and vice-versa), the ease and low cost of the test make it invaluable for *carcinogenicity screening*.

The bacterium used in the test is a strain of *Salmonella typhimurium* that caries a defective (mutant) gene making it unable to synthesize the amino acid histidine (*his*) from the ingredients in its culture medium. However, some types of mutations can be reversed, a *back mutation*, with the gene regaining its function. These revertants are able to grow on a medium lacking histidine.

F13.27. Ames Test.



F13.27 shows a qualitative version of the Ames test. A suspension of a histidine-requiring *his*- strain of *Salmonella typhimurium* has been plated with a mixture of rat liver enzymes on agar lacking histidine. The disk of filter paper has been impregnated with 10 µg of 2-aminofluorine, a known carcinogen.

The mutagenic effect of the chemical has caused many bacteria to regain the ability to grow without histidine, forming the *his*+ colonies seen around the white disk. The scattered colonies near the margin of the disk represent *spontaneous revertants*. The strain contains a number of other alterations that dispose it to mutagenesis studies, including a deletion in the excision repair gene *uvrB* and addition of genes that enhance UV and chemical mutagenesis. The low rate of spontaneous reversion and the high rate of chemically induced reversion seen with the test strain make this assay the industry standard for assessing genotoxicity.

13.13.1.2. Problems of the Ames Test

The problems associated with the Ames test are:

(1) Not all mutagens cause mutations in the test, since some require biotransformation into active intermediates. This difficulty is overcome by the inclusion of rodent liver homogenate ("S9") to provide the necessary enzymes during incubation to activate promutagenic agents to mutagens. The homogenate is however toxic, so that such tests cannot be done long-term.

(2) The Ames Test is super-sensitive: increased reversion to "wild-type" organisms occurs even when a test tube of the organisms is shaken up. This over-sensitivity is overcome by using other cell systems (E. coli, yeasts, fungi, etc.) and developing a *test battery* rather than relying on a single test.

The Ames Test is actually a test of *mutagenic action on bacteria, rather than carcinogenicity*. When many chemicals with a good safety record, synthetic and natural, are tested, many record positive in the Ames test. This proves that bacteria are exceptionally sensitive to chemicals. But to assess carcinogenic risks to humans, it is necessary to introduce a *battery* of tests of mutagenicity (see 13.11). Decisions for the further evaluation of a chemical rest on the outcome of the results of these numerous tests.
An investigator can use any biological fluid (sputum, urine, bronchial lavage, etc.) from exposed individuals and incorporate it into the standard Ames Test[2].

T13.28. Foods found to have Mutagenic Components in the Ames Assay
Coffee
Tea
Broiled beef and pork
Broiled fish
Pickled vegetables (Japanese)
Flavonoids in many edible plants
Mushrooms (Agaricus bisporus)
Salted fish (Chinese)
Caramelized sugars (glucose / fructose)
Pyrolysates of onion and garlic
Aflatoxin and other mycotoxins (food contamination)
Safrole

13.13.2. Testing with Eukaryotic Cells

Bacterial metabolism and human metabolism are not the same, so investigators began to use mammalian and human cells for testing. Some of these cells are capable of biotransformation of the agent, eliminating the need for agent activation by rodent liver homogenates.

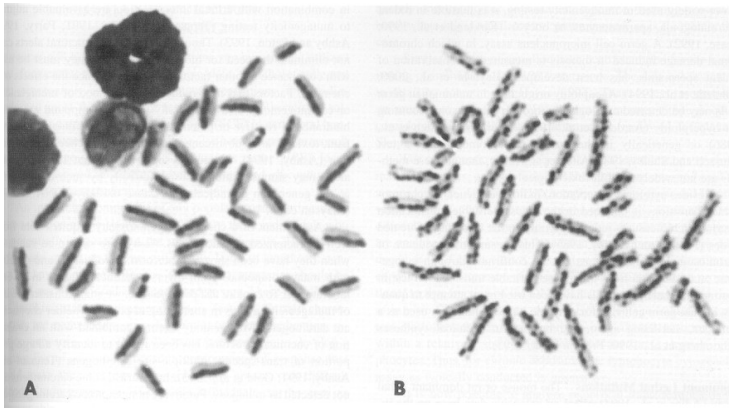
Although one can usually obtain and grow cells of human origin *in vitro* for the purposes of experimentation, the process of cell culture itself may not allow transmission of essential characteristics. By DNA methylation or acetylation of histones, genes can be imprinted for suppression or expression. Erasure of imprinting is normal in conception (but may not be total), where the imprints of gametes are lost and then re-established. This also happens in the adult body, where maternal or paternal alleles are suppressed, affecting the nature and amount of protein produced by cells. The number of epigenetic differences in 50-year old twins is more than triple that in 3-year old twins.

Imprinting present in an animal or human can be erased by various procedures, leading to changing characteristics or even abnormalities. But cell culture corrupts imprinting, especially over time, because cultured cells optimize themselves to survive under culture conditions, leading to alterations in their epi-genetic features.

The endpoints measured for mutation vary considerably and these tests are used extensively: DNA damage/repair assays, unscheduled DNA synthesis, enzymatic changes, morphological aberrations (sister chromatid exchanges, micronucleus formation, chromosomal breaks - clastogenesis).

13.13.3. Sister Chromatid Exchange (mammalian cells in vitro)

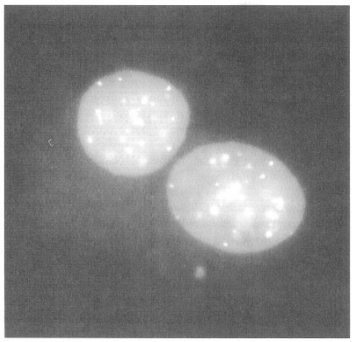
In this test, chromosomes are marked by growing cells in the presence of a thymidine analog, bromodeoxyuridine (BrdU). As cells continue to grow in the presence of the agent, breakages in the chromosome may occur and be repaired by cellular machinery. But because of the marking, the fixes will be visible as *harlequin* chromosomes. (F13.29)



F13.29. A: Untreated human lymphocyte. B: Exposed to ethyl carbamate. *Casarett & Doull.*

13.13.4. Micronuclei (eukaryotes in vivo)

A "micronucleus" is literally a small nucleus. During cell division, the genetic material should spread equally between the two daughter cells. If chromosomes are broken, pieces of chromosomes or whole chromosomes may fail to be included in either of the two daughter nuclei because they lack a centromere and are not pulled to the appropriate pole of the spindle. The material may form its own "micronucleus", which is clearly visible with a microscope. If a treated group of animals shows significantly higher frequencies of micronucleated cells than do the untreated control animals, then the chemical is considered to be capable of inducing chromosomal damage[3].



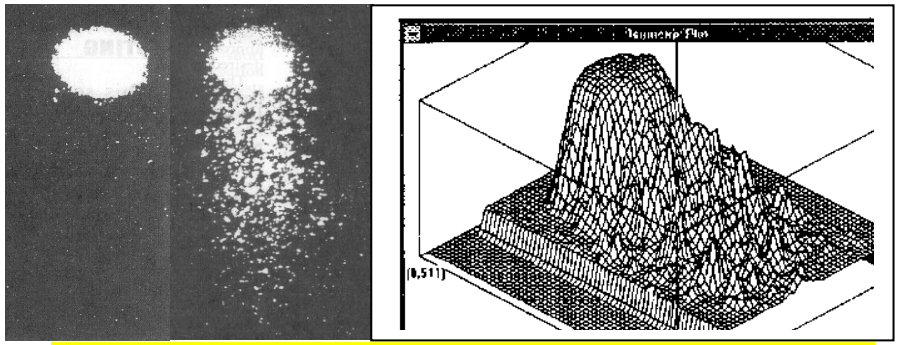
F13.30. Micronuclei in human lymphocyte. *Casarett & Doull.*

13.13.5. Comet Test (in vivo)

This test, also called the single cell gel assay**,** is a very important alternative to the cytogenetic tests. It is less labour intensive, more rapid and less expensive. There are no restrictions on the source of the cells, which cells may be exposed either in vivo or in vitro. After exposure, cells are obtained as a suspension and laid down on a slide in a thin agarose gel. Once on the slide, the lysed cells are subjected to electrophoresis in neutral or basic medium. The pH, which influences the level of DNA uncoiling, determines in part whether single strand or double-strand DNA breaks are detected by the method. The nuclear material can be stained after electrophoresis, and breaks in DNA appear graphically as a tail from the round location of the nucleus, thus the name "Comet Test". Study of the length of these tails is indicative of DNA damage.

The comet assay is exquisitely sensitive to the detection of DNA damage in individual cells, and it is becoming an influential tool in the measurement of genotoxicity.

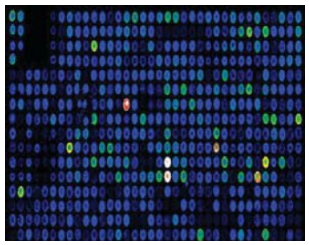
It was used initially to assess sulfuric acid-potassium permanganate mixtures in coordination with the Ames test. Applications followed with the insecticide lindane, organic mercury compounds, l-chloro-methyl-pyrene, as well as documenting the antigenotoxic activities of lactic acid bacteria.



F13.31. Computerized view of a Comet tail. *Lai.*

The test is able to provide information on individual cells and both on damage to DNA and on alteration of the ability of cells to repair DNA.

13.13.6. Genetic MicroArray Testing



Genetic MicroArrays can be used to assess cellular responses to ionizing radiation, for example, and may be used to determine individual genetic sensitivity to radiation in the future. It is known from micronuclei formation in T lymphocytes that individuals vary

in their responses, probably because of variations in DNA repair mechanisms. Specific genetic responses are triggered in cultured primary keratinocytes from adult skin at high (2 Gy) or low (10 mGy) doses of gamma rays. Experiments with DNA microarrays (10,500 gene probes) show that among 853 modulated probes, the expression of 214 are specifically modulated by low-dose (10 mGy) and 370 genes by high-dose (2Gy) exposure. Low-dose-specific genes (140 known genes) include mostly genes of homeostasis, cell communication, signaling, membrane, cytoskeleton, RNA and protein synthesis, chromatin, energy metabolism, stress, cell death and transport but rarely DNA repair genes[18]. The conclusion is that radiation response at low doses is quite specific and different from that obtained at high doses.

T13.32. Comparative Mutagenicity of Various Compounds. N = no; Y = yes; 0 = not tested. *Williams and Burson.*

Compound	Established Human Carcinogen	Bacteria	Yeast	Drosophila	Mammalian Cells	Human Cells
Epichlorohydrin	**N**	**N**	**0**	**Y**	**Y**	**Y**
Ethyleneimine	**N**	**0**	**Y**	**Y**	**Y**	**Y**
Trimethyl phosphate	**0**	**Y**	**0**	**Y**	**Y**	**Y**
Tris	**N**	**Y**	**0**	**Y**	**Y**	**Y**
Ethylene dibromide	**N**	**Y**	**Y**	**Y**	**Y**	**N**
Vinyl chloride	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**
Chloroprene	**Y**	**Y**	**0**	**0**	**0**	**Y**
Urethane	**N**	**Y**	**Y**	**Y**	**Y**	**0**

13.14. Test Batteries in Mutagenic Testing

With many genotoxicity methods available in many models, the results do not always corroborate one another (T13.32).

To improve on the accuracy of a single test, standard three-test *batteries* for genotoxicicy are used. A common triad is:

(i) an *in vitro* test in bacteria;
(ii) an *in vitro* test in mammalian cells with cytogenetic evaluation of chromosomal damage and/or a test that detects gene mutations;
(iii) an *in vivo* test for chromosomal damage using rodent hematopoietic cells.

13.15. Cancer and Mutation

It is generally assumed that genotoxicity underlies carcinogenicity, and this is mostly true. However, because organisms have DNA repair mechanisms as well as cell population management mechanisms, actual causes of carcinogenicity can be quite complex.
It is conceptually possible for a single molecular hit to create a cancer, but a more likely cause is a mutation which disables a physiological system. Down the road, this impairment leads to cancer. This is why tumors often take some time to appear, together with the fact that they need some time to grow.

13.16. Genotoxicity to Carcinogenicity

Somatic cells can mutate directly to forms expressing rapid division and de-differentiation, resulting in cancerous tissue (F13.15). An example illustrating the link between cancer and DNA damage comes from lung cancer. It was found that lung cancer patients are more likely (41%) than the general population (4%) to be low in the DNA repair enzyme 8-oxoguanine DNA N-glycosylase (OGG). This enzyme repairs DNA damaged by oxidation[7]. Smoking boosts lung cancer risk by 10 to 20-fold. If further you have low OGG, the boost is 100-fold.

When somatic cells mutate directly to forms expressing rapid division and de-differentiation, we usually obtain cancerous tissue (F13.15).
There are specific mechanisms known to lead to cancer. One of the best known is damage to the p53 gene and consequently to the supply of p53 protein. The p53 gene is normally silent, but is turned on by DNA damage. When triggered, it halts cell division, making time for repairs. If the repairs are not successful, it can also induce *apoptosis*.

However, the path to cancer can be more complex than a specific mutation, and can involve more indirect mechanisms (such as promotion) based on normal physiological maintenance. Because of this, the connection between mutagenicity and carcinogenicity is not simple (F13.33).

F13.33. All carcinogens are not mutagens. *Richards, 2008.*

Known Human Carcinogens	Mutagen	Suspected Human Carcinogens	Mutagen
Aflatoxins	Yes	Acrylamide	Yes
Arsenic and ar.compounds	No	Benz[a]anthracene	Yes
Asbestos	No	Benzidine-based dyes	Yes
Azathioprine	No	Benzo[a]pyrene	Yes
Benzene	No	Carbazole	No
Benzidine	Yes	Chlordane	No
Beryllium	Yes	Ethylene dibromide	Yes
Cadmium compounds	Yes	Glycidol	No
Chromium [VI] compounds	Yes	Lead	Yes
Cyclophosphamide	No	Styrene-7,S-oxide	No
Diethylstilbestrol	Yes	Tetrachloroethylene	No
Ethylene oxide	Yes	Benzofuran	No
Formaldehyde	Yes	Tris(2,3-dibromopropyl) phosphate	No
Gallium arsenide	Yes	Vinyl bromide	Yes
Vinyl chloride	Yes	Vinyl fluoride	Yes

13.17. Apoptosis

F13.34 Processes of Cell Death

Necrosis: the process of elimination of dead material.
Apoptosis: programmed cell death in which caspases are activated and apoptosomes are formed.
Paraptosis: cell swells but also forms vacuoles. Can be triggered by withholding an hormone or protein on which the cell has become dependant.
Autoschizis: the nucleus departs the cytoplasm. Can be triggered by oxidative attack.
Oncosis: cell swells to death from oxygen starvation, ATP depletion, excess sodium and water, protein denaturation and intracellular calcium excess.

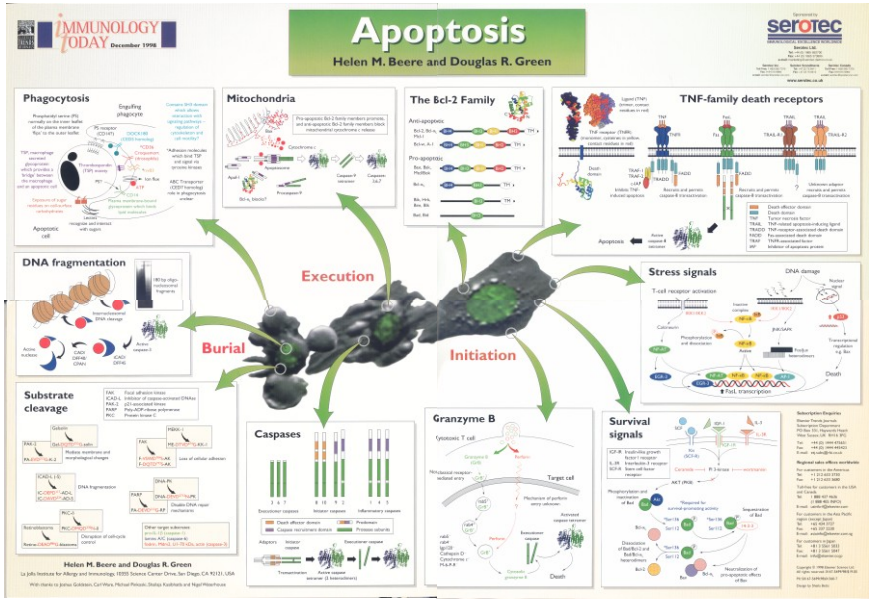
Short-term death in cells (F13.34) can result from necrosis or *apoptosis,* an endogenous death mechanism. Whether cells die by one mechanism or the other often depends on how quick and severe the damage is. High intensity stresses, for example, large concentrations of a toxicant, kill by necrosis, while milder stresses may induce *apoptosis*.
Apoptosis (F13.35) destroys cells without inflammation after some dangerous damage has been inflicted on them. Apoptosis is predictive of epigenetic carcinogenicity.
There are at least two pathways leading to apoptosis, an "Extrinsic" and an "Intrinsic" Pathway. Both activate a family of Cys (Cysteine) Proteases, named Caspases that act in a proteolytic cascade to dismantle and remove the dying cell.

One of the extrinsic pathways is the the stimulation of tumor necrosis factor receptors such as CD95 (or Fas). This leads to activation of the p53 gene, which triggers decay of the mitochondria. The mitochondria in turn release cytochrome C (an energy enzyme) into the cytoplasm of the cell, resulting in full-fledged apoptosis.
Any damage to p53 will inhibit this self-destruction mechanism, which rids the body of defective cells. Many human cancers have been found to have alterations to p53. p53

knockout mice are used as an animal model to assess recessive mutations.

Apoptosis is important in many cases of chronic toxicity if the dissolved cells are not completely replaced. On the other hand, caloric deprivation slows aging by activating sirtuins, a family of deacetylase enzymes that reduce apoptosis by protecting cells against reactive oxygen species and DNA damage.

Another self-regeneration mechanism is *autophagy*, by which whole organelles, particularly mitochondria, are catabolized. Mitochondria in non-proliferating tissues like brain, heart and liver are replaced every 10-25 days. The rate of replacement is enhanced by nutrient deprivation and glucagon.



F13.35. Mechanisms of Apoptosis.

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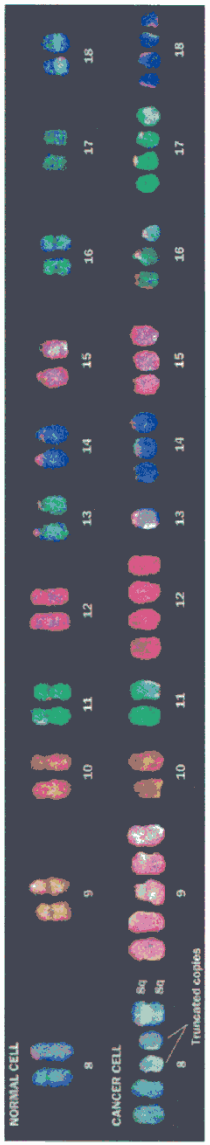
Carcinogenicity

14· Carcinogenicity



In 1914 Theodor Boveri wrote that cancer cells could be distinguished from normal ones in that they had abnormal numbers of chromosomes (see inset at left). Cells can actually sometimes be cancerous without being aneuploid. The leading hypothesis on the nature of cancer suggests that a variety of gene changes can induce *genetic instability*, and that subsequent changes result in cancer, that frequently show aneuploidy. The conventional view is therefore that cancer cells are genetically "abnormal".

Given a system as complex as the genome, the "normal" cells of the body may be more remarkable than the cancer cells. To a point, stable genomes sacrifice adaptability and variability, a hallmark of bacteria, for the benefit of building coherent multi-cellular organisms, presumably an advantage to establish immunity.

Cancer itself is enormously complex, even if all tumors share some characteristics in common: runaway cell division and hypoxia.

It would be expected that different cancer types show different genetic changes, but it is more surprising that even among people diagnosed with the same type of cancer, there are individual patterns of genetic changes.

Therapies for cancer would gain from being highly individualized.

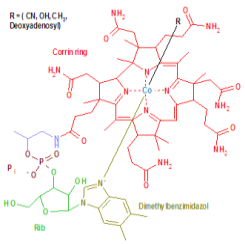
14.1. Causes of Cancer

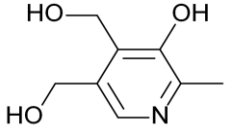
A brief view of cancer *causes* (F14.1) is that one-third is from smoking, one-third from diet and one-third from environmental and occupational causes. Obesity contributes 10% of all cancer cases[10].

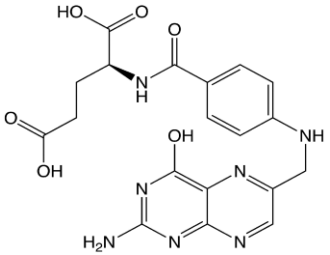
14.1.1. Molecular Basis

Although the environment can be controlled to reduce cancer rates, there will probably always be a basal cancer rate resulting from genetic structure. This basal cancer rate can only be changed by altering the genome itself towards increased stability. Therefore, cancer is a disease that needs to be fought ongoingly in species that show genomic adaptability.

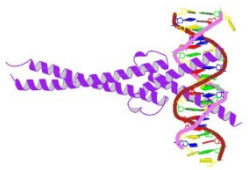
Cancer can happen in a wide variety of ways, some almost trivial. Ames has shown the deficiencies in vitamins B_{12}, B_6 or





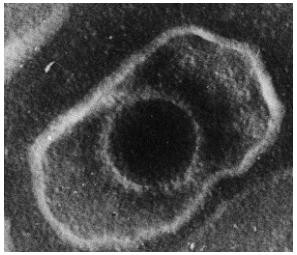


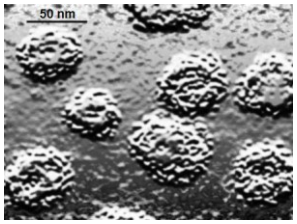
folic acid (shown in sequence) cause replicating DNA to incorporate uracil instead of thymine. When two such events occur closely on opposite strands, repair can yield double-stranded nicks which break chromosomes, causing cancer[5].



The *myc* protein (shown), when enhanced, spurs liver tumor growth from apparently normal tissue. Stopping *myc* manufacture causes the cancer cells to differentiate back into apparently normal liver cells. However, their cancerous character can be reawakened by the return of *myc* synthesis[7].

The cancer pathways are extremely numerous. The retinoblastoma[α] protein, for example, is central to the process of cell proliferation, activating the genes for cell division. An interesting property of this protein is that it is active in a wide variety of cellular processes, and interacts with more than 100 other proteins.



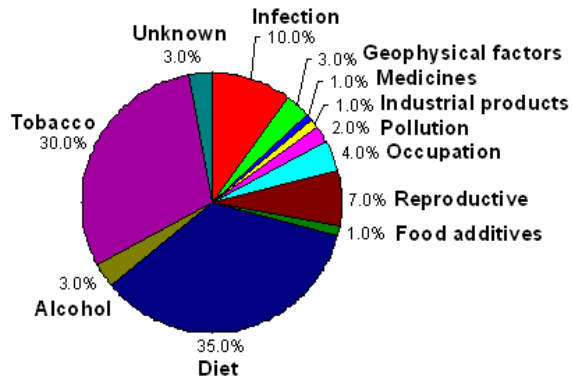


Several viruses have been linked to cancer. Epstein-Barr virus (shown) to Burkitt's lymphoma, Hepatitis B/C viruses to liver cancer, HTLV-I to T-cell leukemia and Papilloma viruses (shown) to cervical cancer. In rare cases in animals (canine transmissible venereal sarcoma), it can even be transmitted through cell exchange.

14.1.2. Environmental Cause of Cancer

Cancer is feared. Information that a chemical causes cancer sets the media into a frenzy of reporting to avid readers or listeners, and governments into regulatory responses.
For example, a statement many years ago by John Higginson that 80% of all cancers could be attributed to the "environment" was interpreted by the press and the reading public to mean that things outside their control were the root causes of cancer. Higginson meant "environment" to include lifestyles, diet, drugs and workplace exposures in addition to contaminants in the air, soil, and water (F14.1). This statement is supported by international observations on cancer incidence in migrants. National differences in the incidence of cancer are obvious from F14.7, but for virtually all cancers, with the passage of time, or in succeeding generations, rates tend to approach those of the native-born in the country of adoption, pointing to the importance of environmentally determined hormonal or dietary causes.
Some controllable causes of cancer are smoking and drinking alcohol. In the case of alcohol, it is known that alcohol consumption induces cytochrome P450 2El enzymes. The enzyme also activates procarcinogens such as nitrosamines. Genetic variants of the CYP 2E1 gene were previously reported to be risk factors for various cancers. It is estimated that 30% of cancers could be eliminated through dietary intervention.

F14.1. Cancer Deaths due to "Environmental" Factors



Avoidable Cancer Risk Factors
causing more than 30% of cancers

1. Overweight
2. Low fruit and vegetable intake
3. Physical inactivity
4. Smoking
5. Alcohol consumption
6. Unsafe sex
7. Urban air pollution
8. Indoor smoke from coal
9. Contaminated injections from health care

[α] childhood eye cancer arising from immature cells in the retina.

14.1.3. Occupational Causes of Cancer

T14.2. Cancers and Metals.				
A. Metals Causing Human Cancer				
Arsenic				
Cu refinery	Pulmonary carcinoma			
As pesticides	Lymphoma, leukemia			
Chemical plants	Dermal carcinoma			
Drinking water (oral) Cigarette smoke	Hepatic angiosarcoma			
Cadmium				
Cd refinery	Pulmonary carcinoma, Prostatic carcinoma			
Chromium				
Cr refinery	Pulmonary carcinoma			
Chrome plating, Chromate pigments	Gastrointestinal carcinoma			
Nickel				
Ni refinery	Pulmonary carcinoma, Nasolaryngeal carcinoma, Gastric and renal carcinoma, Sarcoma (?)			
B. Metals causing Animal Cancers				
METALS	**ANIMALS**	**TUMOR**	**SITE**	**ROUTE**
Beryllium	Mice, rats, monkeys	Osteosarcoma Carcinoma	Bone Lung	IV, INH
Cadmium	Mice, rats, chickens	Sarcoma Teratoma	Injection site Testes	IM, SC, ITS
Cobalt	Rats, rabbits	Sarcoma	Injection site	IM, SC
Chromium	Mice, rats, rabbits	Sarcoma Carcinoma	Injection site Lung	IM, SC, IP, INH
Iron	Hamsters, mice, rats, rabbits	Sarcoma	Injection site	IM, IP, SC
Nickel	Mice, rats, cats, hamsters, rabbits Guinea pigs, rats	Sarcoma Carcinoma Carcinoma	Injection site Lung Kidney	IM, ITS, SC INH, IP, IR
Lead	Mice, rats	Carcinoma	Kidney	IP, PO, SC
Titanium	Rats	Sarcoma	Injection site	IM
Zinc	Chickens, rats, hamsters	Carcinoma Teratoma	Testes Testes	ITS
IV = intravenous; INH = inhalation; IM = intramuscular; SC = subcutaneous; ITS = intratesticular; IP = intraperitoneal; IR = intrarenal; PO = per os. SOURCE: Sky-Peck (1986).				

Despite efforts at controlling exposure to carcinogens, occupational cancer is still prevalent among the workforce, particularly in the older individuals whose exposure predated many regulations lowering the toxicant levels.

The first association between cancer and an industrial chemical was found by Percival Potts, a surgeon, in 1775 (T14.3). Subjects were usually in their early 20s and had been employed as young boys to climb up into large chimneys and scrape down the accumulated hydrocarbons (polycyclic aromatics, PAHs), metals (lead, arsenic), and carbon. Potts was busily removing diseased testicles from these people. Their scrotal cancer related to soot from the soft coal burned in the fireplaces. The scrotum is an area where chemicals can be efficiently absorbed. Not surprisingly, PAHs and arsenic caused a local irritation, changes in DNA and, eventually, cancer.

14.2. History

T14.3. Historical Development of Cancer Science.	
1761	Increased incidence of nasal cancer among snuff users.
1775	Increased incidence of scrotal cancer among chimney sweeps.
1843	Scrotal cancer in smelters due to arsenic.
1881	Lung malignancy in uranium miners.
1893	Bladder cancer due to aromatic amines.
1901	Lung fibrosis.
1914	*Mutagenesis and Somatic Mutation Theory* suggests that cancer is related to chromosome abnormalities and alteration in genetic material.
1918	First Animal Data: multiple applications of coal tar to rabbit ears produces skin carcinomas.
1930s	Isolation of a single active carcinogenic chemical from coal tar (Benzo-a-Pyrene).
1934	Isolation of single cell clones from a tumor and finding that injection of these cells into a healthy host reproduces the disease, demonstrating that cancer is a stable, heritable cellular alteration.
1940	Radium brushes and lip cancer in dial painters.
1951	Cancer related to asbestos exposure.

Animal experiments and occupational workers exposed to benzene, chromates, tars, coal-tar dye intermediates, radiation and asbestos were the first to associate substance use with cancer.

14.3. Operational Definition of a Carcinogen

A carcinogen is an agent having the ability to *change the tumor incidence normally observed* in control subjects by:

1. *decreasing the delay* in the appearance of the naturally observed tumors,
2. *increasing the frequency* of the normally observed tumors in a population,
3. increasing the *number of tumors per individual*,
4. producing *new tumor types*,
5. increasing the *growth rate of tumors*.

Carcinogens are recognized by their ability to create tumors because diagnosing cancer from blood tests is unreliable, largely due to the fact that the malignant phenotype is derived from normal cellular functions. Of course, by the time a cancer produces clinical symptoms, the window of curative possibilities has largely passed.
The operational definition of a carcinogen refers to changing "the tumor incidence normally observed" because the incidence of tumors in humans and in animal models is not zero, even under the best conditions (T14.5). To adequately detect changes in tumor incidence, the species should not have a natural rate that is too low or too high, to insure sensitivity and specificity. For more details on cancer rates for mice and rats, see the files "Cancer Rate Mouse Data.pdf" and "Cancer Rate Rat Data.pdf".

T14.4. Regulatory Classification of Carcinogens.

Organization	Rating	Description
Environmental Protection Agency (EPA)	A	Sufficient evidence from epidemiologic studies to support a causal association
	B1 (probable)	Limited evidence in humans from epidemiologic studies
	B2 (probable)	Sufficient evidence from animal studies but limited evidence in humans
	C (possible)	Limited or equivocal evidence from animal studies and inadequate or no data in humans
	D	Inadequate evidence in animals
	E	No evidence of carcinogenicity in at least two adequate animal tests in different species
International Agency for Research on Cancer (IARC)	1	Sufficient epidemiological evidence for carcinogenicity in humans
	2A	Probably carcinogenic in humans based on limited evidence in humans and sufficient evidence in animals
	2B	Possibly carcinogenic in humans based on sufficient evidence in animals but inadequate evidence in humans or limited evidence in humans with insufficient evidence in animals
	3	Not classifiable
	4	Not carcinogenic

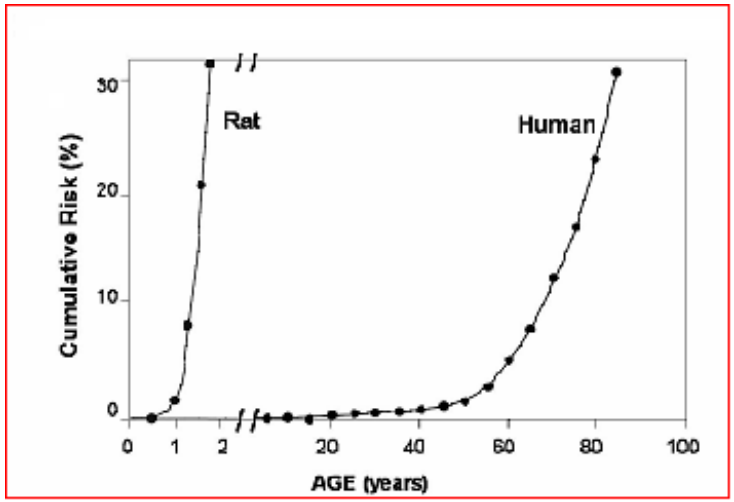
For evidence of carcinogenicity, production of benign neoplasms is accepted, as no chemical has been found that produces them exclusively. Regulatory agencies (IARC) accept that benign neoplasms represent the first stage of the cancer process. The conventional classification of carcinogens based

on the "weight of evidence" approach is presented in T14.4. These ratings are mentioned in the MSDS sheets. Carcinogens are often potent toxicants. If cancer is avoided, it is rather unlikely that other toxicities will occur.

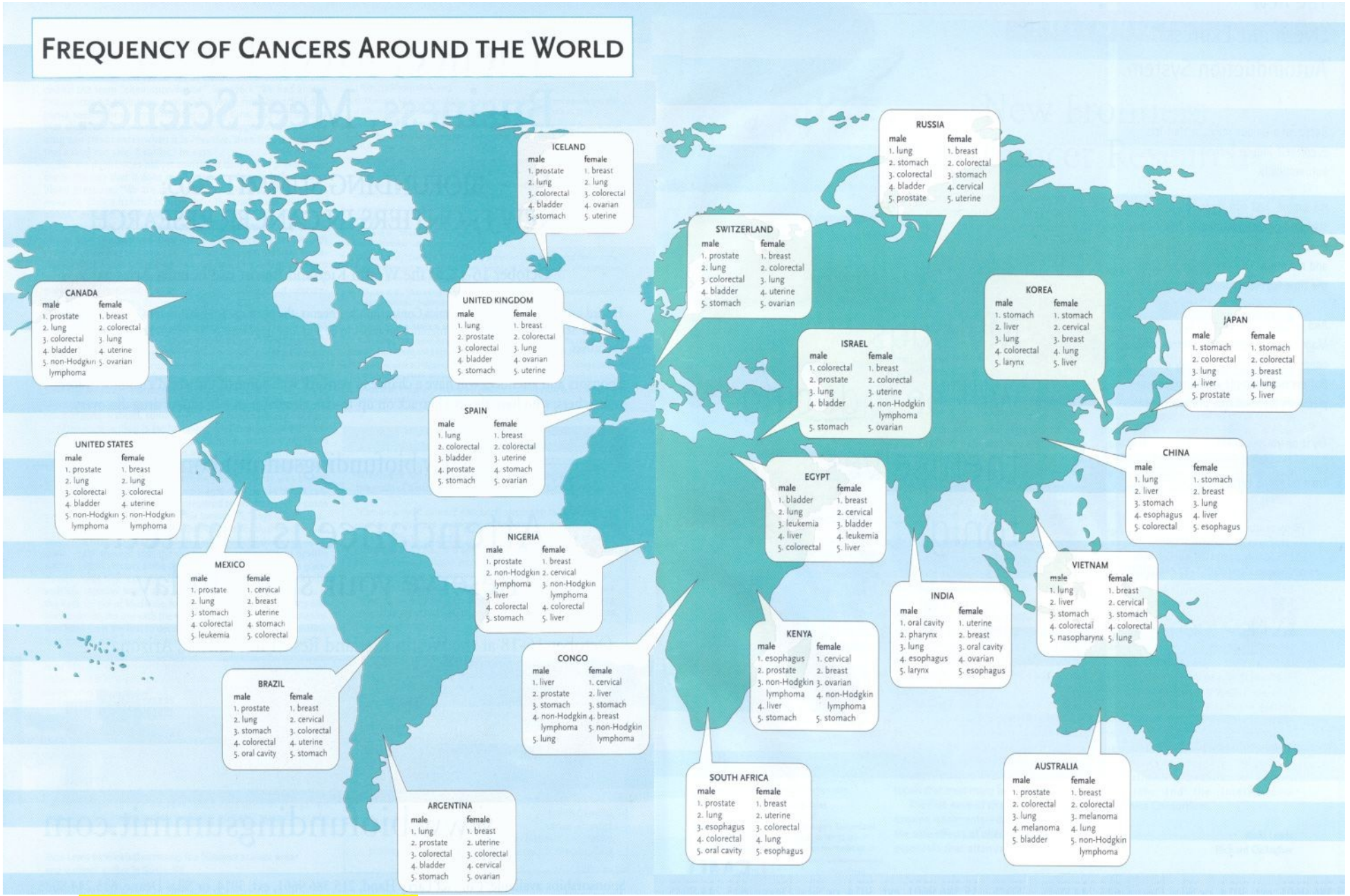
T14.5. Spontaneous Tumor Incidence (%, benign and malignant) by Site and Sex in B6C3Fl Mice and F344 Rats.

	B6C3F1 MICE		F344 RATS	
Site	**Male**	**Female**	**Male**	**Female**
Liver Adenoma	10.3	4.0	3.4	3.0
Liver Carcinoma	21.3	4.1	0.8	0.2
Pituitary	0.7	8.3	24.7	47.5
Adrenal	3.8	1.0	19.4	8.0
Thyroid	1.3	2.1	10.7	9.3
Hematopoietic	12.7	27.2	30.1	18.9
Mammary gland	0	1.9	2.5	26.1
Lung	17.1	7.5	2.4	1.2

From Goodman et al. (1979) and Chandra and Frith (1992).



F14.6. Cumulative Risk of Death from Cancer for Rat and Human.



F14.7. Cancer frequency around the world.

14.4. Tumor Types

14.4.1. Carcinoma

A malignant new growth of epithelial cells that gives rise to metastases. A carcinoma is a malignant growth that arises from the *epithelium*♦. Carcinomas tend to spread (metastasis) through the blood vessels, lymph channels or spinal fluid to other organs such as the bone, liver, lung or the brain. At least 80% of all cancers are carcinomas.

14.4.2. Sarcoma

An often highly malignant tumor made of tissue similar to normal "connective tissues". It is derived from the mesoderm: fat, muscle, blood vessels, deep skin tissues, nerves, bones, and cartilage.
In spite of these broad classifications, it is probable that each patient's cancer has a unique profile of genetic alterations.

14.5. Carcinogens

14.5.1. List of Carcinogens

Although many chemicals have shown to induce tumors in animals, remarkably few have been proven to cause cancer in humans. Carcinogenicity is easy to prove if, as in the case of vinyl chloride, there is strong evidence from epidemiological studies of an association or causal relationship with an exceedingly rare carcinoma, angiosarcoma[1]. However, one generally encounters the situation where the human data is weak. Reliance must then be placed on the weight and strength of animal studies.
The courts are not terribly sophisticated in their approach to science, so they tend to bias strongly the acceptable evidence for causation of cancer on epidemiology. Researchers and regulators, by contrast, frequently make judgments from animal or *in vitro* experiments. Nearly 50% of *probable human carcinogens* and a few *know human carcinogens* are considered such by IARC without epidemiological evidence (based on animal and *in vitro* studies).
Agencies such as the U.S. EPA or the International Agency for Research on Cancer (IARC) in Lyon, France, have developed lists of carcinogens, but not all agencies list the same chemicals.
Generally, about 45 to 190 chemicals, of which only half are industrial chemicals (the others being drugs), are recognized.
See the lists of recognized and suspected carcinogens at the end of this chapter.
Lists of *recognized* and *suspected* carcinogens found in the workplace are given in T14.8-9.

14.5.2. Carcinogen Types

In the early days, a number of compounds were isolated from coal tar that could induce cancer at the point of application (F14.11). They were polycyclic hydrocarbons, containing virtually nothing but carbon and hydrogen. Some years later, chemicals (F14.12) applied to animals were found to induce cancers at remote sites (liver, kidneys, etc).

As knowledge improved, it was determined that some carcinogens needed biological activation from *pro*carcinogen to *proximate* carcinogen to *ultimate* carcinogen (F14.13) by Phase I enzymes or by conjugation (F14.14) with Phase II enzymes.

♦ The *epithelium* is the covering of internal and external surfaces of the body, including the lining of vessels and other small cavities. The epithelium also includes the skin and lining of the organs such as breast, prostate, lung, stomach or bowel.

T14.8. Industries-Trades with Proven Excess Cancers		
AGENT	**INDUSTRIES AND TRADES**	**PRIMARY AFFECTED SITE**
Para-aminodiphenyl	Chemical manufacturing	Urinary bladder
Asbestos	Construction, asbestos mining and milling, production of friction products and cement	Pleura, peritoneum, bronchus
Arsenic	Copper mining and smelting	Skin, bronchus, liver
Alkylating agents (mechloro ethamine hydrochloride and bis[chloromethyl]ether)	Chemical manufacturing	Bronchus
Benzene	Chemical and rubber manufacturing, petroleum refining	Bone marrow
Benzidine, beta-naphthylamine, and derived dyes	Dye and textile production	Urinary bladder
Chromium and chromates	Tanning, pigment making	Nasal sinus, bronchus
Isopropyl alcohol manufacture	Chemical manufacturing	Cancer of paranasal sinuses
Nickel	Nickel refining	Nasal sinus, bronchus
Polynuclear aromatic hydro-carbons from coke, coal tar, shale, mineral oils, and creosote	Steel making, roofing, chimney cleaning	Skin, scrotum, bronchus
Vinyl chloride monomer	Chemical manufacturing	Liver
Wood dust	Cabinetmaking, carpentry	Nasal sinus

14.5.2.1. Genotoxic, Initiator or Ultimate

These carcinogens act directly on DNA to form strong covalent bonds.

14.5.2.1.1. Direct Carcinogens

Direct Carcinogens are intrinsically reactive compounds that do not require metabolic activation by cellular enzymes to interact with DNA. Irreversible DNA damage can occur through alkylation or oxidation. For example, radiation can ionize DNA, as well as ionize water to produce reactive oxygen species that can alter DNA. Inorganic Agents can generate free radicals or interfere with enzymes associated with replication of DNA, altering fidelity of replication.

T14.9. Industries and Trades with Suspected Excess Cancers *Casarett and Doull.*		
AGENT	**INDUSTRIES AND TRADES**	**SUSPECTED HUMAN SITES**
Acrylonitrile	Chemical and plastics	Lung, colon, prostate
Beryllium	Beryllium processing, aircraft manufacturing, electronics, secondary smelting	Bronchus
Cadmium	Smelting, battery making Welding	Bronchus
Ethylene oxide	Hospitals, production of hospital supplies	Bone marrow
Formaldehyde	Plastic, textile, and chemical production; health care	Nasal sinus, bronchus
Synthetic mineral fibers (e.g., fibrous glass)	Manufacturing, insulation	Bronchus
Phenoxyacetic acid	Farming, herbicide application	Soft tissue sarcoma
Polychlorinated biphenyls	Electrical equipment production and maintenance	Liver
Organochlorine pesticides (e.g., chlordane, dieldrin)	Pesticide manufacture and application, agriculture	Bone marrow
Silica	Casting, mining refracting	Bronchus

14.5.2.1.2. Procarcinogens

Procarcinogens require biotransformation to reactive intermediates, or to *proximate carcinogens* capable of reacting with DNA. Most chemical carcinogens belong to this second group.

14.5.2.1. Epigenetic or Promoter

Epigenetic or Promoter carcinogens are agents that do not alter the base sequences of DNA, but modify cell behavior by the way of non-heritable molecular modifications.
These molecular changes may enhance absorption, reduce elimination or increase-decrease biotransformation.

- DNA methylation results in "epi-genetic" inactivation of genes.

Newly synthesized strands of DNA are not methylated, but over time, 3-5% of cytosine-guanine pairs become methylated, silencing transcription of the methylated segments. These components often cluster near the beginning of a gene, where protein attach to turn genes on. So, the presence of methyl groups turns genes off.

T14.10. Types of Carcinogens with examples.

Genotoxic ("Initiators")	Direct	Nitrosomethylurea (NMU), N-methyl-N'-nitro-N-Nitrosoguanidine, Nuclear radiation, Electromagnetic radiation, Arsenic, Chromium, Nickel.
	Procarcinogens	Benzo[a]pyrene (BaP), BP-7,8-diol-9,10-epoxide.
Epigenetic ("Promoters")	Alter expression of genes, proliferation and differentiation.	12-0-tetradecanoyl phorbol-13-acetate (TPA).

Epigenetic marker patterns are programmed long before an animal's birth. All cells types have a distinct pattern of methylation and histone modification that prepare a cell to do its specific job in the body. As a consequence of enzyme activity, epigenetic modifications are passed down as cells divide.

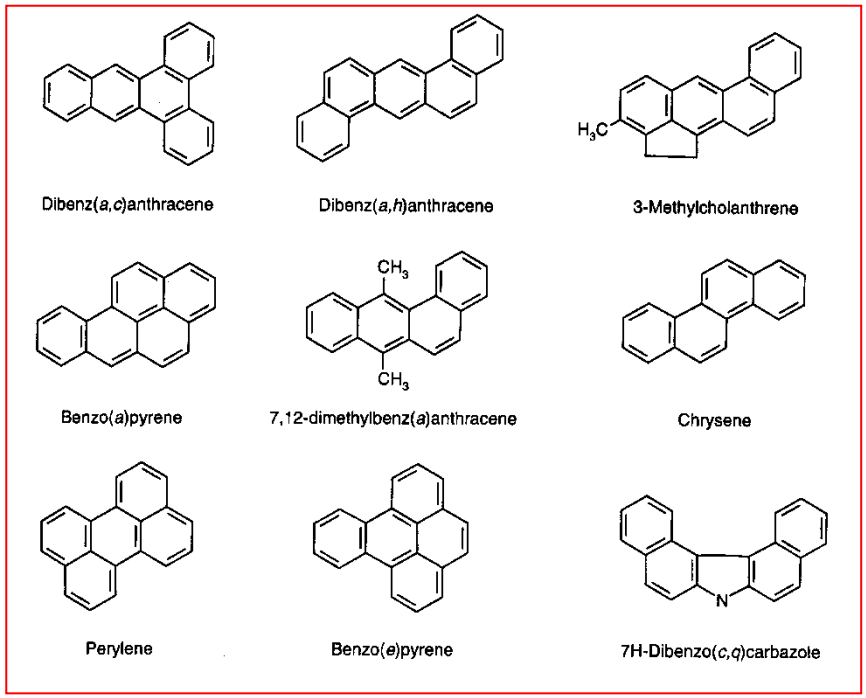
So, there are different epigenetic modifications in every tissue, and even in every person.
However, identical twins who start out with nearly identical methylation patterns grow apart epigenetically as they age.
In cancer, it is often found that protective protein have been turned off by epigenetic modifications. Therefore, drugs such as decitabine which demythylate DNA may have a role in cancer therapy.

- Histone modifications: the protein that act as support to DNA can bear a "histone code", sometimes referred to as "peri-genetic" changes,
- or RNA interference (RNAi): alteration of translation, sometimes referred to as "extra-genetic" changes.

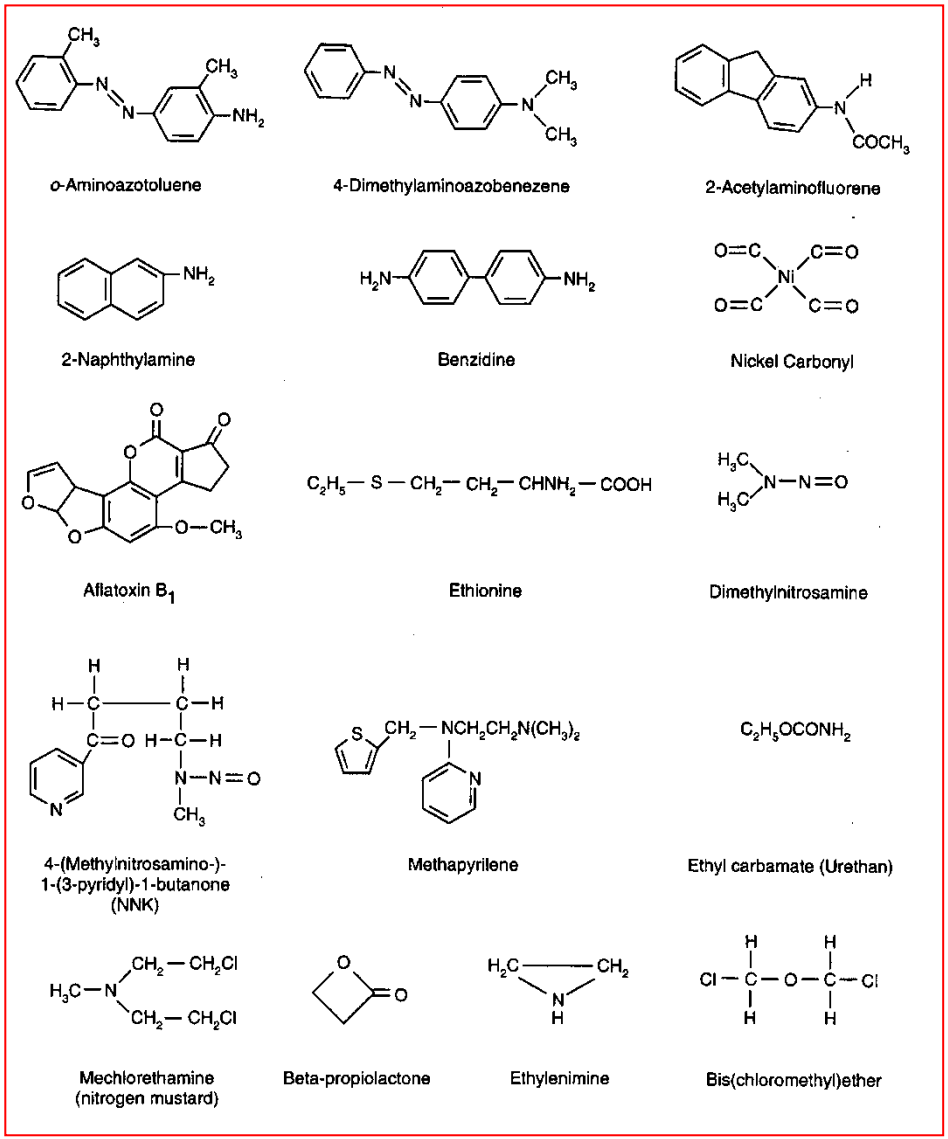
Other mechanisms are:

- Stimulation of proliferation
- Inhibition of apoptosis
- Cytotoxicity followed by compensatory regeneration
- Modification of endocrine function
- Immunosuppression
- Activation of specific receptors (ex., PPARα)

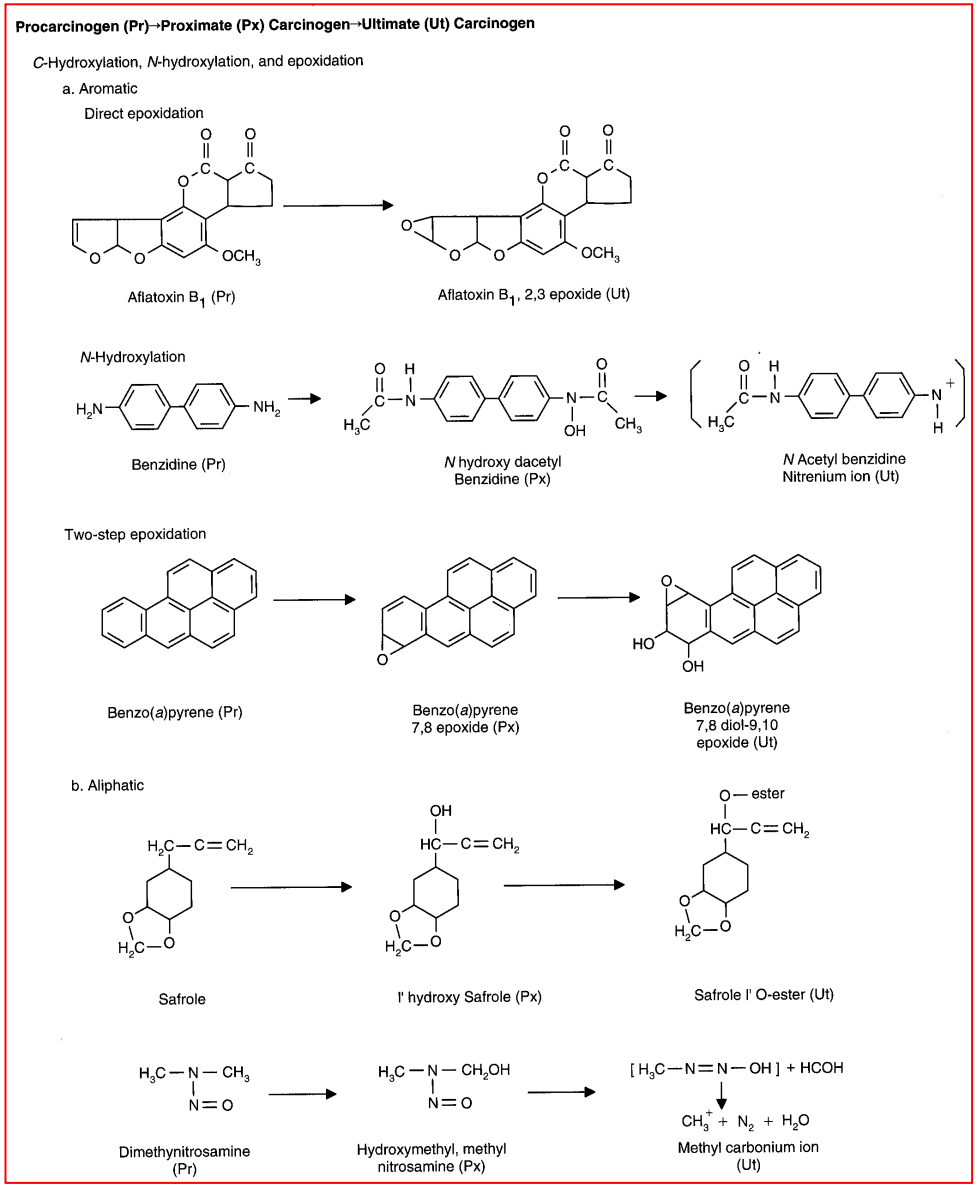
There are no pure promoters. If you give enough of a promoter, even without an initiator, cancer will occur. Probably, resident initiators are always present at a low level.
A chemical able to induce cancer by itself is called a **complete carcinogen**.



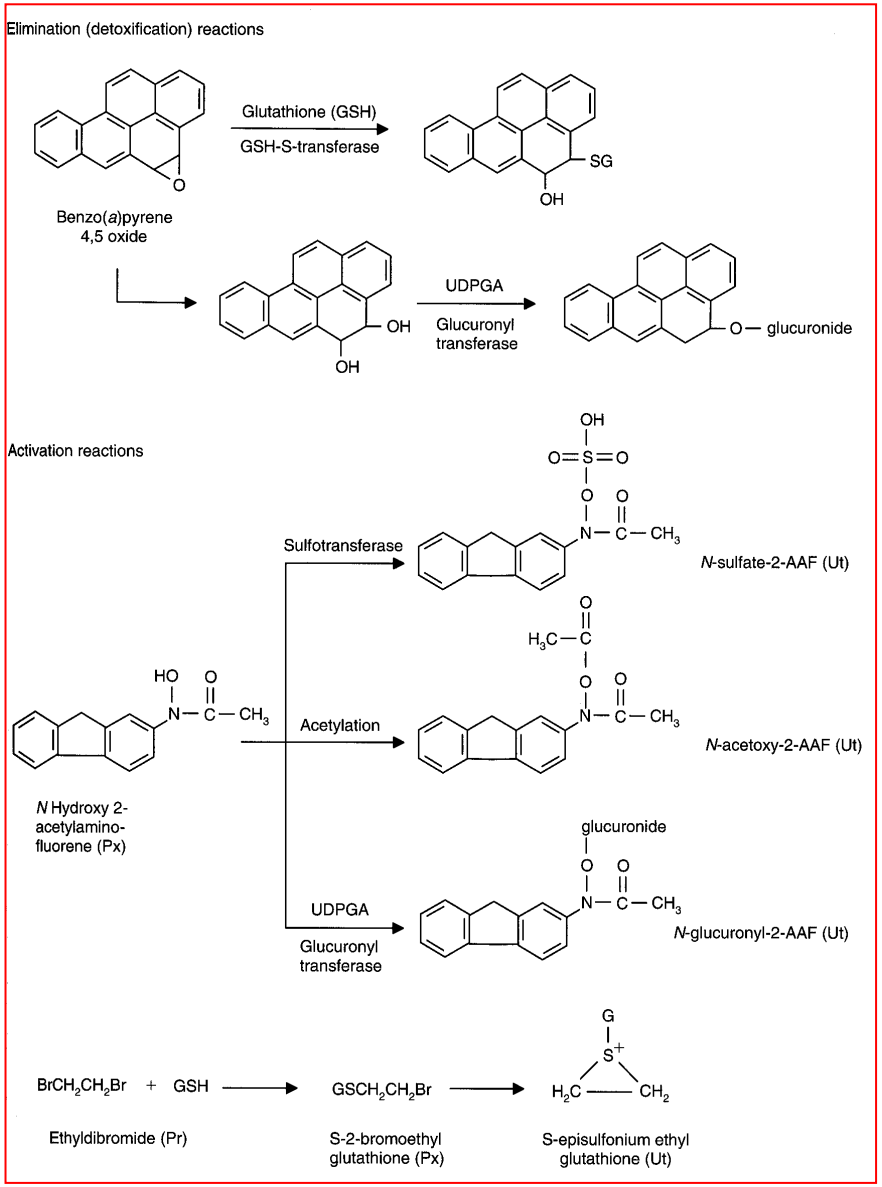
F14.11 Topical Carcinogens.



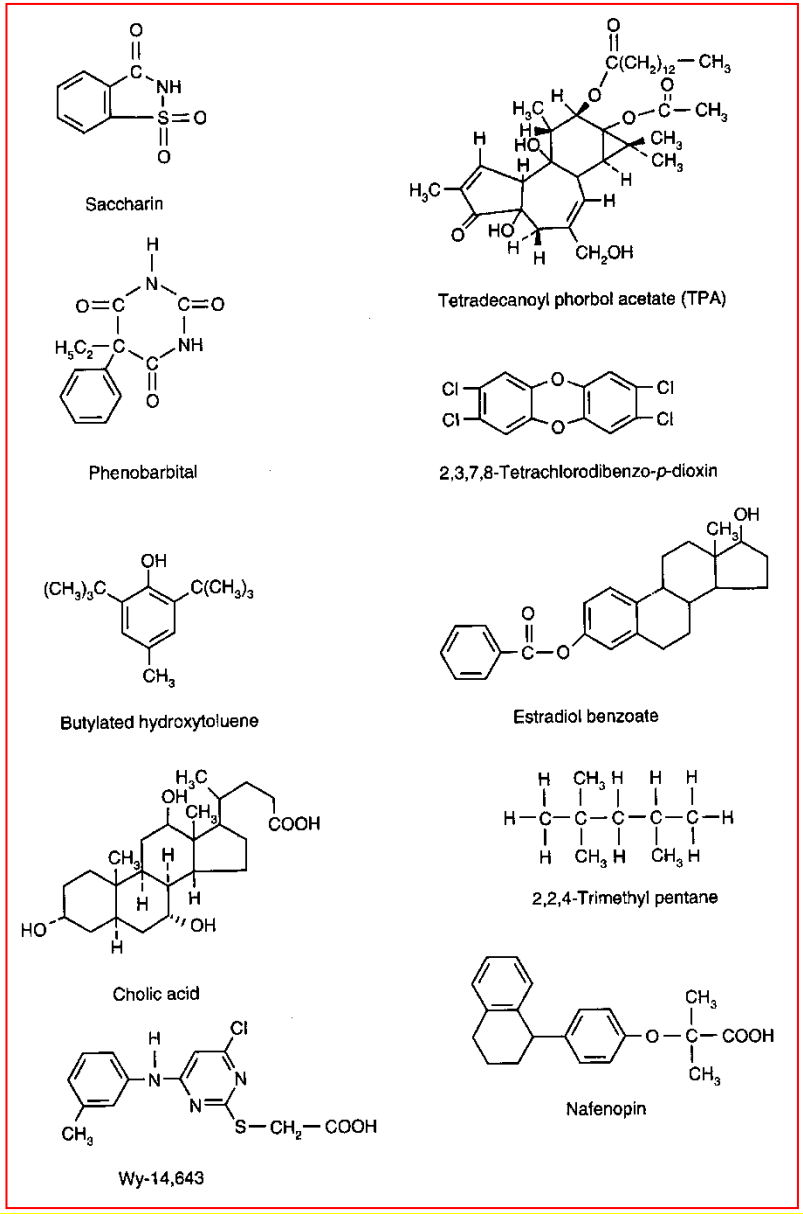
F14.12 Remote Carcinogens.



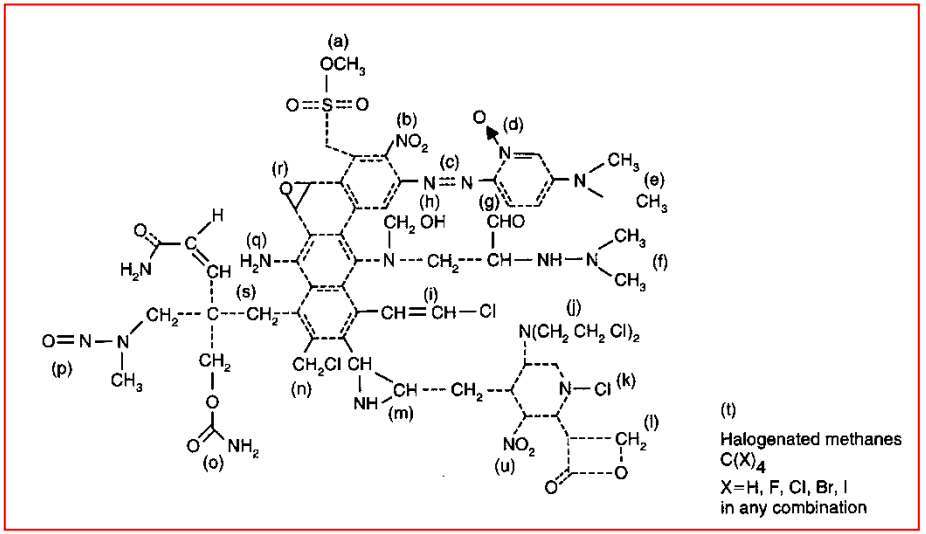
F14.13. Metabolic activation of carcinogenicity by Phase I enzymes.
Casarett & Doull.



14.14. Metabolic activation of carcinogenicity by Phase II enzymes.
Casarett & Doull.



F14.15. Representative Promoters.

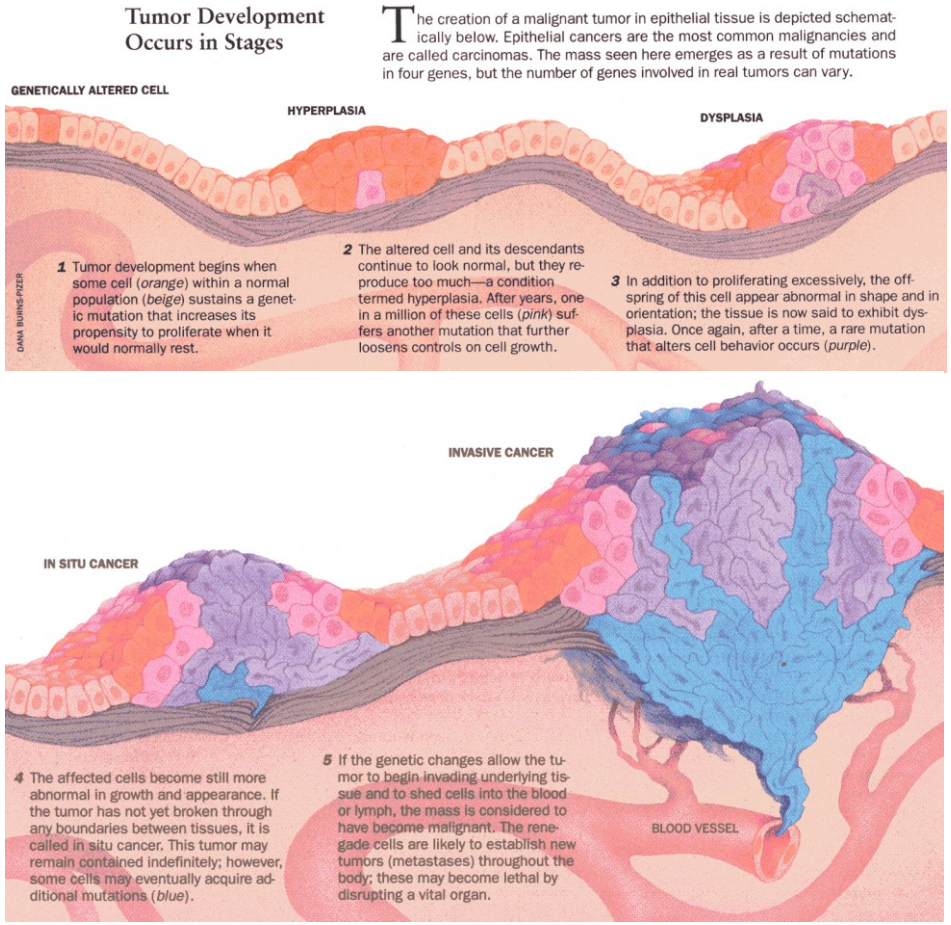


F14.16. Structural alerts (identified by letters) for mutagenicity.
Casarett & Doull.

Promoters can be very variable in structure (F14.15), probably as a reflection of the wide variety of physiological effects they can address.

Co-carcinogens involve hormonal action on cell proliferation, inhibition of intercellular communication and immunosuppression. These substances favor the proliferation of cells with an altered genotype (cells containing a mutation) as well as proliferative and differentiated phenotypes, thereby enhancing tumor growth.

Finally, there is some predictability in carcinogenic potential of molecules which can be summarized in the *ultimate carcinogenic molecule* (F14.16), compacting all alterations known to favor the expression of mutagenicity.



F14.17. Phenomenological Development of Cancer.
Scientific American.

14.6. Classical Mechanism of Tumor Formation

Cancer is uncontrolled cell growth, differentiation, and development. A phenomenological description of cancer development in epithelial tissue is shown in F14.17.

14.6.1. Metastasis

Metastasis is the spread of a disease (usually cancer) from an original site to other parts of the body. This usually happens when cancer cells break off from the original tumor and travel through the blood vessels to a new site. This kind of cancer is called malignant, meaning that it is life-threatening and usually fatal.

A *Primitive Tumor* is determined to have originated in the tissue itself (not a metastasis). When metastasis occurs, it localizes as a result of circulation patterns (F14.18) or of cellular affinity to specific tissues. If cells from human breast tumors that have metastasized to the lungs are injected intravenously into mice, we observe implantation of the cells into the lungs of the animals[8].

Most neoplasms show chromosomal aberrations, as the cause or result of the cancerous activity. This suggests that carcinogenic agents interact with DNA before the carcinogenic process begins.

Because many cancers take decades to develop, it is probable that the process typically occurs in steps (F14.19).

There is a practical distinction between *initiation* (involving a genetic damage) and *promotion-progression*, which may act by non-genetic means:

1. action of non-mutagens should be required for a longer period of time than initiators, and
2. action of non-mutagens should be reversible at some point.

A normal cell is "converted" into a preneoplastic cell, this altered cell eventually becoming a neoplastic cell. The multi-step hypothesis finds support in the existence *in vitro* of *transformed cells*. These cells have a different appearance, survive without growth factors and do not need to be anchored to a solid support (can grow in soft agar). A test called “soft agar cloning”, the ability of cells to invade gels, is a major test for carcinogenicity at the cellular level.
Most human trials monitoring cancer follow the relatively rapid development of precancerous biomarkers such as elevated concentrations of enzymes, cell changes or activation of certain genes.
In experimental animal models, carcinogenesis is divided into at least three stages:

14.6.2. Initiation

An irreversible genotoxic event resulting in alteration of the primary sequence of DNA, rather than cell morphology. Permanent, heritable change (Somatic Mutation Theory), possibly resulting from a single bolus.

14.6.3. Promotion

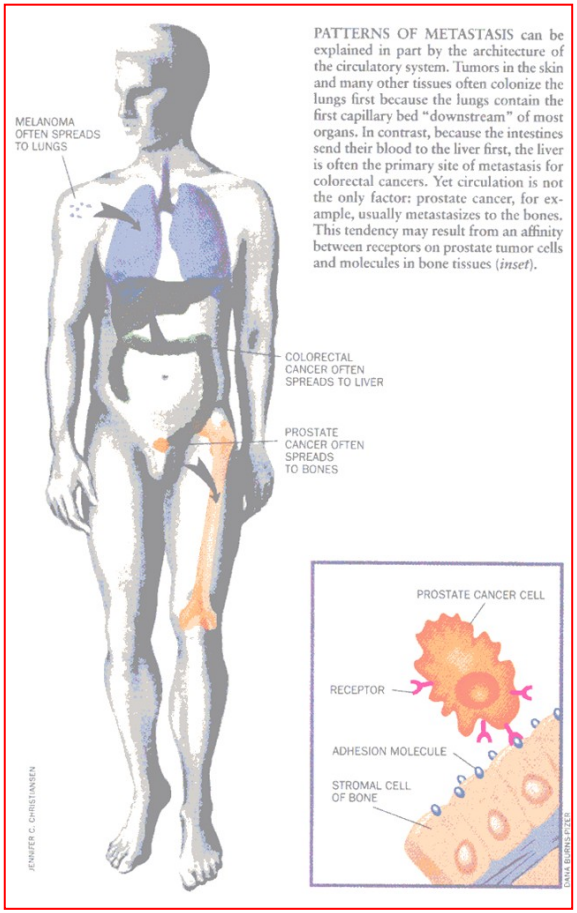
A reversible epigenetic event affecting an initiated cell. Promoters must be administered in a sustained way to be effective. The initiated (mutant) cell may remain dormant until exposed to a *tumor promoter*, which allows the initiated cell to expand clonally and eventually produces a tumor. Promoters influence growth hormone production, regulate gene expression (stimulation or suppression) and interfere with cell differentiation (proliferation of colonies of immature cells).

Promotion is probably intimately related to apoptosis, and many suppressors of apoptosis are cancer promoters.

14.6.4. Progression

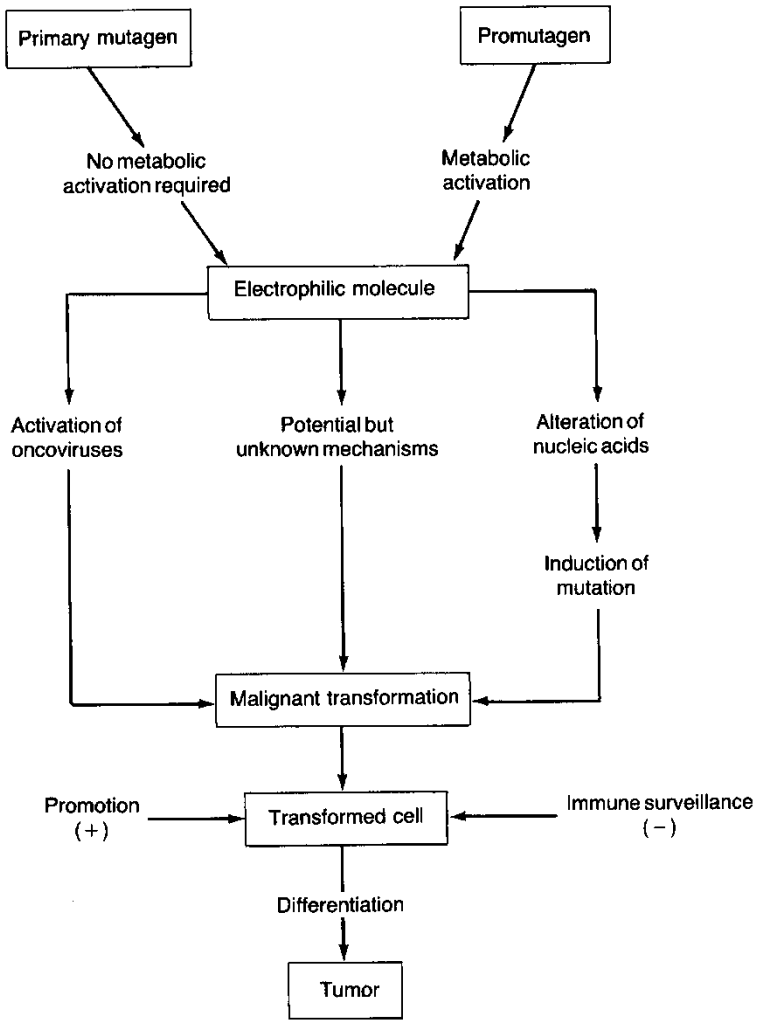
Irreversible conversion of a benign tumor to a malignant tumor. Involve further genetic changes (karyotype instability), it is characterized by changes in the phenotypic appearance of the cells (size, shape), in growth rates. Progression is brought about either by the activation of proto-oncogenes or by the inactivation of tumor suppressor genes.

F14.18. Circulatory Patterns of Metastasis.
Scientific American.



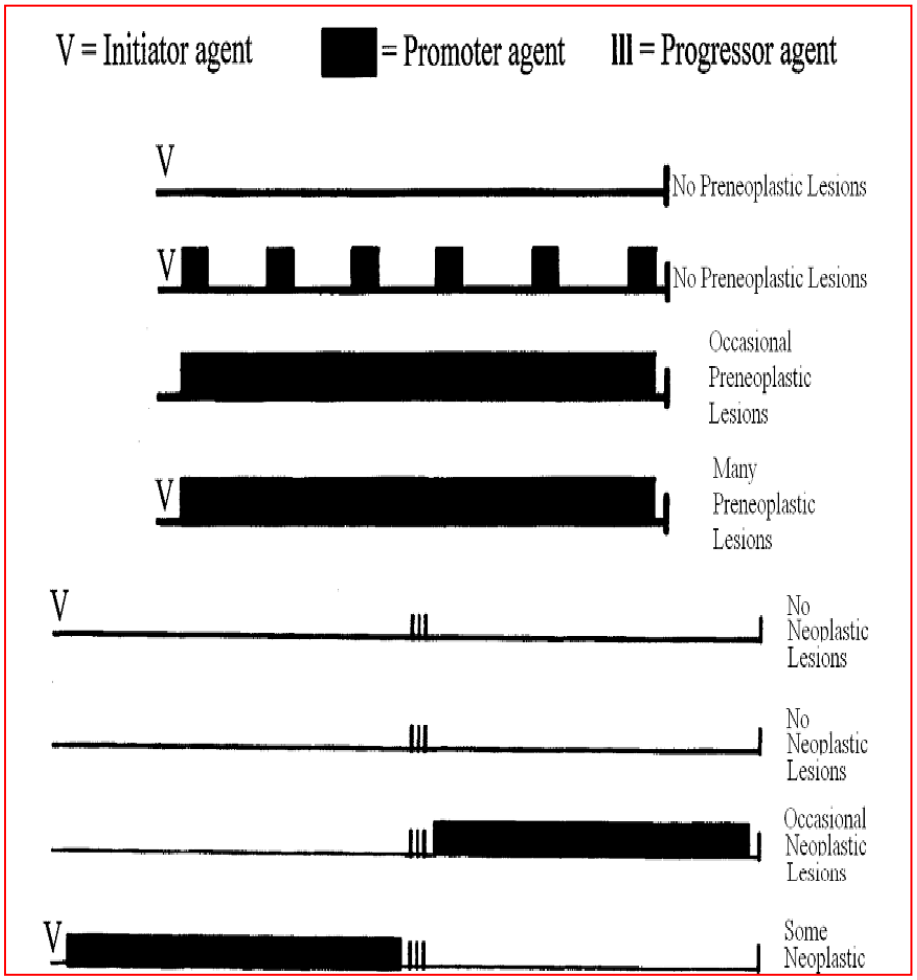
MINIMUM CONDITIONS FOR CANCER

A long-standing question has been whether it is possible to generate a cancer from a single event (single hit) or whether multiple events are necessary (multi-hit). These two alternatives model to a different dose-response.
If a single initiating event triggered cancer, the risk would be expected to rise continuously as the radiation, for example, increased. Any type of more intricate physiology in carcinogenesis could produce more complex curves, perhaps with breaks in the slope as one increases dose. Dose-responses should also ideally include background cancer rates that happen spontaneously as a result of the presence of proto-oncogenes, for example.
Until recently, it was thought that in humans, four to six independent events were necessary for tumorigenesis.
It is possible to trigger cancer in the **mouse** with just 2 genetic changes, the addition of 2 oncogenes.
It has been possible recently to create a human cancer cell purely from genetic manipulations. In **humans** it is necessary to have 2 oncogenes (*ras*, SV40 large T antigen which inhibits p53 and retinoblastoma protein) plus telomerase (hTERT).
Inhibitors of **telomerase** can remortalize human cancer cells, ultimately leading to death of transformed human breast epithelial cells and human prostate cancer cells in culture (Geron). Telomerase activity is detectable in over 30 different human cancer types and is the *primary* mechanism for maintaining telomere length in human cancers.



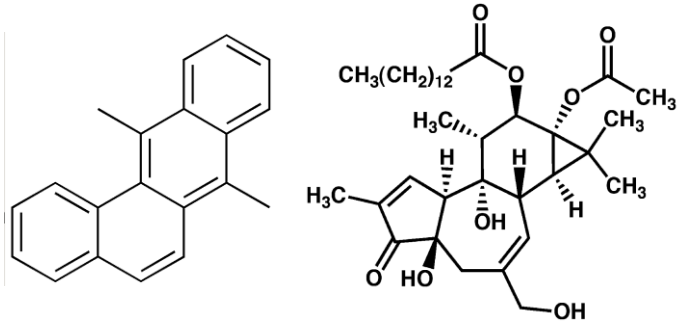
F14.19. Steps in Cancer Development. *Williams and Burson.*

14.6.5. Initiation-Promotion-Progression



F14.20. Interaction of Initiators, Promoters and Progressors.

The Classical Initiation-Promotion model is DMBA-TPA (shown).



Initiator: DMBA = 7, 12-dimethylbenz[a]anthracene,
Promoter: TPA = 12-0-tetradecanoyl phorbol-13-acetate.
A short course of an initiator is followed by sustained administration of a promoter (F14.20). For example, DMBA for 2 weeks, TPA 2 times per week for 6-30 weeks.
If repeated exposures are required to manifest the carcinogenic potential of a substance, the distinction between initiator and promoter can become problematic.

14.6.6. Genetic View of Cancer

When studied at the level of genes, one obvious characteristic is that tumors have altered numbers of chromosomes (sidebar on page 14-2). Genetic alterations that are lethal at the level of the whole organism are acceptable if the only function of the cell is to proliferate. Those alterations may be effects rather than causes.

14.6.6.1. Oncogenes

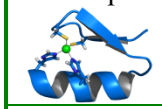
On the other hand, certain genes are known, **oncogenes,** that can cause cancer when mutated or inappropriately expressed. These genes are normal components of cellular machinery which are over- or under-expressed.

Typically, the gene encodes a protein that promotes growth (cell division): if normal control of growth is lost, a normal cell transforms into a malignant (tumor) cell.
Whether or not an oncogene causes cancer depends on: genetic makeup and environmental variables (vitamin deficiency or smoking habits).
If the oncogene is introduced in a mouse model by genetic engineering, it typically causes cancer in only a few tissues, even if the gene is expressed in all.
A protein expressed in a variety of cells may only induce cancer in some tissues, and then only at a specific times in development. Highly specific conditions allow the cancer to develop under the influence of the protein product.
Oncogenes explain why certain strains of animals appear to have a higher than "normal" incidence of certain tumor types. Probably, these animals have not only been already initiated (cellular genome has been altered), but they contain oncogenes or proto-oncogenes that only require a promoting agent.
This would mean that most of the agents that we have identified as carcinogenic are actually promoters.

14.6.6.2. Tumor Suppressors

Are negative regulators of cell growth. Damage to a tumor suppressor (p53, retinoblastoma gene) can also trigger malignant transformation.
p53 holds the record for being mutated in the largest percentage of tumors relative to any other tumor suppressor.
The complexity of suppressor biology should not be under-estimated. For example, knocking out the retinoblastoma gene in a mouse model can cause severe abnormalities and embryonic death. But the same knockout in another mouse strain has no effect.
Animals from closed colonies (no new arrivals introduced into the strain) obtained from brother-sister matings over 20 or

T14.21. Some Oncogenes.

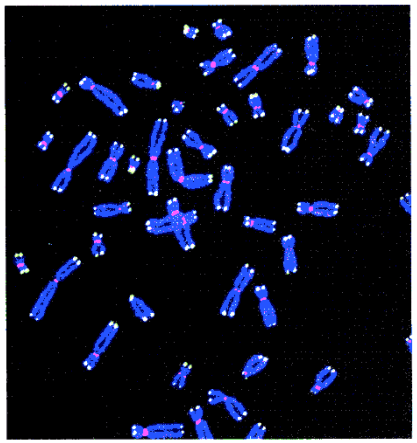
Function of Product	Gene	Localization
Growth factors	sis, fgf	Extracellular
Receptor/protein tyrosine kinases	met, neu	Extra cell/cell membrane
Protein tyrosine kinases	src, ret	Cell membrane/ cytoplasmic
Membrane-associated G proteins	ras, gip-2	Cell membrane/ cytoplasmic
Cytoplasmic protein serine kinases	raf, pim-l	Cytoplasmic
Nuclear transcription factors	myc, fos, jun	Nuclear
Unknown, undetermined	bcl-2, crk	Mitochondrial, cytoplasmic
Some Tumor Suppressor Genes		
GTPase activation	NF1	Cell membrane/ cytoplasmic
Cell cycle-regulated nuclear transcriptional repressor	RB-l	Nuclear
Cell cycle-regulated nuclear transcription factor	p53	Nuclear
Zinc finger• transcription factor 	WTl	Nuclear
Mismatch DNA repair	hMLHl	Nuclear
Zinc finger transcription factor (?)	BRCA1	Unknown

• Zinc fingers are protein, stabilized with a zinc atom, that can bind to DNA, regulating gene activity.

more generations become vulnerable to non-pathogenic viruses[◊], setting up these strains with a higher-than-normal incidence of certain tumors (mouse lymphoma, rat mammary tumor). Cervical cancer can be induced in humans by the Human Papilloma Virus, although the induction takes a long time.
Humans also harbor *proto-oncogenes*[1]. This suggests that some humans are already primed for cancer and are only waiting for the proper agent. This would explain ethnic and regional differences in both incidence rate and type of tumor.

14.6.6.3. Telomeres

The shortening of the chromosomes' telomeres at each cell division ultimately prevent the cells from dividing and is believed to contribute to tissue aging, but in humans it is also observed that telomere shortening usually precedes and contributes to cancer of the prostate and pancreas. This occurs presumably when old cells are not destroyed by apoptosis.
The erosion of telomeres might drive a genetic instability (the ends of chromosomes become "sticky") required for cells to become malignant[6]. In many tissues that need sustained division (blood, intestinal tissues), the enzyme telomerase maintains telomere length. In cancerous tissues, telomerase often keeps telomeres from shrinking (anti-telomerase drugs, like GRN163L, are being developed to fight cancer).
Cell loss as a result of apoptosis may be compensated by stem cells within a tissue.



T14.22. Telomeres (light green) and centromeres (red) on human chromosomes (blue).

Consequently, aging is not an inevitable feature of all tissues or organisms. For example, planaria worms are practically immortal.
Aging cells in the worm are constantly replaced using pluripotent stem cells[9].

14.6.7. Cell Proliferation

It is believed that chemicals able to increase cell proliferation in normal cells (T14.25) constitute a cancer risk, because, among other reasons, the genetic material is more vulnerable while cells are dividing (T14.23). For example, cell proliferation is a very powerful predictor of epigenetic bladder cancer.
Non-genotoxic mechanisms producing cancer are diverse, but many act on cell proliferation (T14.24). Many epigenetic cancer induction studies use cell proliferation or apoptosis as central variables (T14.27). Definite evidence for the effect of proliferation on cancer is available for a certain number of substances (T14.28).
A trauma such as a cut has been identified as a required element in initiating Kaposi's sarcoma, a common cancer in AIDS patients[3]. Because of this promotion and progression physiology, tests that measure increased cell proliferation are important in carcinogenicity determinations *in vitro* (T14.27). Even extracellular medium composition may have an effect on cancer expression.

[◊] In virus-induced cancers, chromosomes continue to mutate after infection, evolving into numerous chromosomal rearrangements.

[1] A normal gene which, when altered by mutation, becomes an oncogene that can contribute to cancer.

T14.23. Why Mitogenesis is Mutagenic.

1. Shortened cell interphase, leaving less time for cell repair.
2. Dividing DNA is less stable, as separated from histones.
3. Mitosis is a dangerous event (delicate mitotic apparatus).
4. Rise in the mutated population of cells.

T14.24. Actions on Non-Genotoxic Carcinogens.

1. action on cellular receptor or growth factor
2. action on a hormonal receptor
3. action on organ size
4. mitosis for replacement of dead cells at relatively low concentration
5. sustained cytotoxicity-coordinated hyperplasia with subsequent regenerative growth
6. interruption of cell-cell communication
7. inhibition of apoptosis
8. inflammation
9. simple tissue destruction
10. cell proliferation together with oxidative damage
11. biological stimulation of proliferation

T14.25. Chemicals that Produce Hyperplasia in Humans.

Agent	Target Tissue	Reference
Arsenicals	Epidermis	U.S. EPA (1984)
Anthralin	Epidermis	Bock (1963)
Halogenated hydrocarbons (dioxin)	Epidermis	Moses (1985) cutaneous study in workers
Formaldehyde	Respiratory epithelium	Ura (1989) Klein-Szanto (1989)
Tobacco	Respiratory epithelium	Auerbach (1961)
Phenatecin	Urothelium	Schmahl (1977) Iatrogenic
Hydantoin	Gingiva-Lymphoid Tissues	Schmahl (1977)

T14.26. Non-genotoxic carcinogens increasing the labeling index *in vivo*.

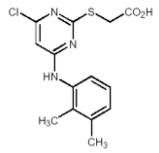
Compound	Proliferative Increase
Wy-14643 (shown at left[2])	x 10 male rat liver
Unleaded Gasoline	x 5 female mouse liver
Furans (F8.21)	x 10 male rat liver

T14.27. Tests based on Increased Cell Proliferation.

Benzoyl peroxide	Invasiveness-Proliferation/ Rodent epidermal carcinoma	Bonfil (1989)
TPA	Invasiveness-Proliferation/Human immortalized epithelium	Aufferman (1985)
TPA	Invasiveness-Proliferation/Human bronchial epithelium BEAS-2B	Bonfil (1989)

The first stage of cancer, hyperplasia, is induced by a variety of carcinogens (T14.25), and some non-genotoxic carcinogens are notorious for producing large increases in the labeling index *in vivo* (T14.26).

[2] Wy-14643 is a peroxisome proliferation activator, a controversial toxicity indicator.



T14.28. Proliferation Agents associated with Human Cancers. *modified from Preston-Martin et al*	
Agent of Cell Proliferation	**Cancer Site**
Hormones	
Estrogen	Endometrium
Estrogen and progesterone	Breast
Ovulation	Ovary
Testosterone	Prostate
Drugs	
Oral contraceptives	Liver
Anabolic steroids	Liver
Infectious agents (through cell destruction)	
Hepatitis B virus, genotype C (hepadnavirus)	Liver
Schistosoma hematobium	Bladder
Schistosoma japonicum	Colon
Clonorchis siniensis	Biliary tract
Opisthorchis viverrini	Biliary tract
Tuberculosis	Lung
Epstein-Barr virus	Burkitt's lymphoma, AIDS
Chemical agents	
Tobacco	Oral cavity, lung, pharynx
Salt	Stomach
Bile and pancreatic juice	Small intestine
Betel nut, lime	Oral cavity
Physical or mechanical trauma	
Asbestos	Mesothelioma
Chronic impulse noise	Acoustic neuroma
Other chronic irritations	
Tropical ulcers	Skin

14.7. Testing for Cancer

Animal carcinogenicity is usually determined in 2-year rodent (rats, mice) studies costing 1 million $ or more. These are labor-intensive, requiring a minimum of 50 animals of each sex per dosage level, with extensive morphological examination and identification of tumor types (benign, malignant). The results hopefully produce a dose-effect relationship for determination of a Safe Human Dose.

Animal models of carcinogenicity do not always seem to extrapolate to humans, as inspection of T14.29 will show. It is difficult to know whether an absence of correlation between human cancer incidence and animals models is due to misleading epidemiological data or to inappropriate animal models. A good case in point is the animal carcinogenicity models for arsenic, which show no effect, while human studies do.

In spite of this, cancer bioassays in animals have long been recognized and accepted since the 1960s and early 1970s as valid predictors of human cancer hazards. The tests expose animals from several weeks after birth to 2 years.

The sensitivity of carcinogenesis bioassays may be improved by exposing rodents earlier in utero, and continuing for 2.5 years or even until natural death of the animals (about 3 years)[11].

14.8. Toxicity of Cancer Treatments

For the top 12 types of cancer, therapy is still today based on radiation, chemotherapy and surgery[4]. Of these, chemotherapy is the least discriminating, killing dividing cells everywhere in the body. The only logic is that cells dividing frequently should suffer the most, and this works well in leukemias, but poorly in

tumors that grow slowly. The hair and lining of the gut are affected, causing hair loss and diarrhea.

Induction of Cancer by Exposure to Specific Metals

A. Metals Causally Associated with Human Cancer

METAL AND SOURCE	MALIGNANCY
Arsenic	
Cu refinery	Pulmonary carcinoma
As pesticides	Lymphoma, leukemia
Chemical plants	Dermal carcinoma
Drinking water (oral)	Hepatic angiosarcoma
Cigarette smoke	
Cadmium	
Cd refinery	Pulmonary carcinoma
	Prostatic carcinoma
Chromium	
Cr refinery	Pulmonary carcinoma
Chrome plating	Gastrointestinal carcinoma
Chromate pigments	
Nickel	
Ni refinery	Pulmonary carcinoma
	Nasolaryngeal carcinoma
	Gastric and renal carcinoma
	Sarcoma (?)

B. Carcinogenicity of Metals in Experimental Animals

METALS	ANIMALS	TUMOR	SITE	ROUTE
Beryllium	Mice, rats, monkeys	Osteosarcoma	Bone	IV, INH
		Carcinoma	Lung	
Cadmium	Mice, rats, chickens	Sarcoma	Injection site	IM, SC, ITS
		Teratoma	Testes	
Cobalt	Rats, rabbits	Sarcoma	Injection site	IM, SC
Chromium	Mice, rats, rabbits	Sarcoma	Injection site	IM, SC, IP,
		Carcinoma	Lung	INH
Iron	Hamsters, mice, rats, rabbits	Sarcoma	Injection site	IM, IP, SC
Nickel	Mice, rats, cats, hamsters, rabbits	Sarcoma	Injection site	IM, ITS, SC
	Guinea pigs, rats	Carcinoma	Lung	INH, IP, IR
		Carcinoma	Kidney	
Lead	Mice, rats	Carcinoma	Kidney	IP, PO, SC
Titanium	Rats	Sarcoma	Injection site	IM
Zinc	Chickens, rats, hamsters	Carcinoma	Testes	ITS
		Teratoma	Testes	

IV = intravenous; INH = inhalation; IM = intramuscular; SC = subcutaneous; ITS = intratesticular; IP = intraperitoneal; IR = intrarenal; PO = per os.
SOURCE: From Sky-Peck (1986).

T14.29. Carcinogenicity of Metals in Humans and Animal Models.

Immune strategies for treating cancer use antibodies binding to antigens expressed specifically by cancer cells.
Vascular strategies attempt to curb the tumor's blood supply.
Oncological hyperthermia takes advantage of a tumor's increased susceptibility to heat, while **radiation therapy** further damages cancer cells.

Cell proliferation assessment is important in cancer genesis, but also in the evaluation of the effectiveness of anti-cancer drugs. Cancer cells (tumors), because of their high proliferation rate, can be preferentially killed by drugs that are **cytotoxic,** but almost all cancer drugs are carcinogenic.

Some tumors such as **mesothelioma** are very difficult to treat. So, many different drugs are tried **alone** in the treatment of mesothelioma: 5-Fluorouracil, Cisplatin, Cyclophosphamide, Detorubicin, Doxorubicin, Hypericin, Ifosfamide, Mechlorethamine, Melphalan, Methotrexate, Mitomycin-C, Paclitaxel, Procarbazine, m-tetrahydroxyphenylchlorin, Thio-tepa, Vinorelbine.
Many drugs are also given in **combination**. Drugs combined with Doxorubicin: 5-Azacytidine, Actinomycin-D, Cisplatin, Cyclophosphamide, Dacarbazine, Razoxane, Vincristine, Cisplatin Mitomycin-C, Cyclophosphamide Cisplatin, Cyclophosphamide Vincristine, Dacarbazine Cyclophosphamide, Dacarbazine Vincristine, Dacarbazine Vincristine Cyclophosphamide, Vincristine Methotrexate Folinic-acid, Cyclophosphamide Methotrexate Etoposide Vincristine, Dacarbazine Vincristine, Cyclophosphamide, Actinomycin-D.

Progress in molecular biology has brought very little improvement in cancer treatment. In fact, the rate of introduction of truly new drugs has slowed.
There is a line of thinking among oncologists promoting the *control* of tumors, considered more feasible than their *eradication*. And there are many remaining mysteries.

Taxol (Paclitaxel), which kills cancer cells by destabilizing cytoskeletal microtubules and inhibiting cell division should work against many more tumors than it does. Methotrexate and

5-Fluorouracil should also be more effective. Why are the vinca alkaloids (Vinblastine, Vincristine, Vindesine) ineffective against breast cancer, while effective against leukemia and lymphoma?

A deep puzzle is drug resistance and tumor adaptation over time. Relapses occur because drugs have selected resistant variants of tumor cells. Without drug resistance, most cancers would be curable right now. P-glycoprotein causes multi-drug resistance not only in leukemia, but also in breast, lymphatic, colon, prostate, renal and liver cancers.
Cancer cells develop the ability to expel a wide variety of anticancer drugs (from the anti-folate family, for example). Cancer cells also create "waste baskets" into which anti-cancer drugs (mitoxantrone, for example) are deposited. These vesicles enlarge and inflate until the drug concentration is a thousand times greater than in the surrounding cell culture.

Chemotherapy can also stimulate cancer cells to grow at certain non-therapeutic concentrations. The common chemotherapy agent doxorubicin actually encourages the growth of ovarian cancer stem cells[12]. The immature cells make up less than 1 percent of an ovarian cancer, but just a few left behind after surgery can reestablish a tumor.

14.8.1. NIH List of Carcinogens

http://ehp.niehs.nih.gov/roc/toc10.html.

Aflatoxins
Alcoholic Beverage Consumption
4-Aminobiphenyl
Analgesic Mixtures Containing Phenacetin (See Phenacetin and Analgesic Mixtures Containing Phenacetin)
Arsenic Compounds, Inorganic
Asbestos
Azathioprine
Benzene
Benzidine (See Benzidine and Dyes Metabolized to Benzidine)
Beryllium and Beryllium Compounds
1,3-Butadiene
1,4-Butanediol Dimethylsulfonate (Myleran®)
Cadmium and Cadmium Compounds
Chlorambucil
1-(2-Chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea (MeCCNU)
bis(Chloromethyl) Ether and Technical-Grade Chloromethyl Methyl Ether
Chromium Hexavalent Compounds
Coal Tar Pitches (See Coal Tars and Coal Tar Pitches)
Coal Tars (See Coal Tars and Coal Tar Pitches)
Coke Oven Emissions
Cyclophosphamide
Cyclosporin A (Ciclosporin)
Diethylstilbestrol
Dyes Metabolized to Benzidine (See Benzidine and Dyes Metabolized to Benzidine)
Environmental Tobacco Smoke (See Tobacco Related Exposures)
Erionite
Estrogens, Steroidal
Ethylene Oxide
Melphalan
Methoxsalen with Ultraviolet A Therapy (PUVA)
Mineral Oils (Untreated and Mildly Treated)
Mustard Gas
2-Naphthylamine
Nickel Compounds (See Metallic Nickel and Nickel Compounds)
Radon
Silica, Crystalline (Respirable Size)
Smokeless Tobacco (See Tobacco Related Exposures)
Solar Radiation (See Ultraviolet Radiation Related Exposures)
Soots
Strong Inorganic Acid Mists Containing Sulfuric Acid
Sunlamps or Sunbeds, Exposure to (See Ultraviolet Radiation Related Exposures)
Tamoxifen
2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD); “Dioxin”
Thiotepa
Thorium Dioxide
Tobacco Smoking (See Tobacco Related Exposures)
Vinyl Chloride
Ultraviolet Radiation, Broad Spectrum UV Radiation (See Ultraviolet Radiation Related Exposures)
Wood Dust

14.8.2. NIH List of Probable Carcinogens

Acetaldehyde
2-Acetylaminofluorene
Acrylamide
Acrylonitrile
Adriamycin® (Doxorubicin Hydrochloride)
2-Aminoanthraquinone
o-Aminoazotoluene
1-Amino-2-methylanthraquinone

2-Amino-3-methylimidazo[4,5-*f*]quinoline (IQ)
Amitrole
o-Anisidine Hydrochloride
Azacitidine (5-Azacytidine®, 5-AzaC)
Benz[*a*]anthracene (See Polycyclic Aromatic Hydrocarbons)
Benzo[*b*]fluoranthene (See Polycyclic Aromatic Hydrocarbons)
Benzo[*j*]fluoranthene (See Polycyclic Aromatic Hydrocarbons)
Benzo[*k*]fluoranthene (See Polycyclic Aromatic Hydrocarbons)
Benzo[*a*]pyrene (See Polycyclic Aromatic Hydrocarbons)
Benzotrichloride
Bromodichloromethane
2,2-bis-(Bromoethyl)-1,3-propanediol (Technical Grade)
Butylated Hydroxyanisole (BHA)
Carbon Tetrachloride
Ceramic Fibers (Respirable Size)
Chloramphenicol
Chlorendic Acid
Chlorinated Paraffins (C_{12}, 60% Chlorine)
1-(2-Chloroethyl)-3-cyclohexyl-1-nitrosourea
bis(Chloroethyl) nitrosourea
Chloroform
3-Chloro-2-methylpropene
4-Chloro-*o*-phenylenediamine
Chloroprene
p-Chloro-*o*-toluidine and *p*-Chloro-*o*-toluidine Hydrochloride (See *p*-Chloro-*o*-toluidine and *p*-Chloro-*o*-toluidine Hydrochloride)
Chlorozotocin
C.I. Basic Red 9 Monohydrochloride
Cisplatin
p-Cresidine
Cupferron
Dacarbazine
Danthron (1,8-Dihydroxyanthraquinone)
2,4-Diaminoanisole Sulfate
2,4-Diaminotoluene
Dibenz[*a,h*]acridine (See Polycyclic Aromatic Hydrocarbons)
Dibenz[*a,j*]acridine (See Polycyclic Aromatic Hydrocarbons)
Dibenz[*a,h*]anthracene (See Polycyclic Aromatic Hydrocarbons)
7*H*-Dibenzo[*c,g*]carbazole (See Polycyclic Aromatic Hydrocarbons)
Dibenzo[*a,e*]pyrene (See Polycyclic Aromatic Hydrocarbons)
Dibenzo[*a,h*]pyrene (See Polycyclic Aromatic Hydrocarbons)
Dibenzo[*a,i*]pyrene (See Polycyclic Aromatic Hydrocarbons)
Dibenzo[*a,l*]pyrene (See Polycyclic Aromatic Hydrocarbons)
1,2-Dibromo-3-chloropropane
1,2-Dibromoethane (Ethylene Dibromide)
2,3-Dibromo-1-propanol
tris(2,3-Dibromopropyl) Phosphate
1,4-Dichlorobenzene
3,3'-Dichlorobenzidine and 3,3'-Dichlorobenzidine Dihydrochloride (See 3,3'-Dichlorobenzidine and 3,3'-Dichlorobenzidine Dihydrochloride)
Dichlorodiphenyltrichloroethane (DDT)
1,2-Dichloroethane (Ethylene Dichloride)
Dichloromethane (Methylene Chloride)
1,3-Dichloropropene (Technical Grade)
Diepoxybutane
Diesel Exhaust Particulates
Diethyl Sulfate
Diglycidyl Resorcinol Ether
3,3'-Dimethoxybenzidine (See 3,3'-Dimethoxybenzidine and Dyes Metabolized to 3,3'-Dimethoxybenzidine)
4-Dimethylaminoazobenzene
3,3'-Dimethylbenzidine (See 3,3'-Dimethylbenzidine and Dyes Metabolized to 3,3'-Dimethylbenzidine)
Dimethylcarbamoyl Chloride
1,1-Dimethylhydrazine
Dimethyl Sulfate
Dimethylvinyl Chloride
1,6-Dinitropyrene (See Nitroarenes (selected))
1,8-Dinitropyrene (See Nitroarenes (selected))
1,4-Dioxane
Disperse Blue 1
Dyes Metabolized to 3,3'-Dimethoxybenzidine (See 3,3'-Dimethoxybenzidine and Dyes Metabolized to 3,3'-Dimethoxybenzidine)
Dyes Metabolized to 3,3'-Dimethylbenzidine (See 3,3'-Dimethylbenzidine and Dyes Metabolized to 3,3'-Dimethylbenzidine)
Epichlorohydrin
Ethylene Thiourea
di(2-Ethylhexyl) Phthalate
Ethyl Methanesulfonate
Formaldehyde (Gas)
Furan
Glasswool (Respirable Size)
Glycidol
Hexachlorobenzene
Hexachlorocyclohexane Isomoers
Hexachloroethane
Hexamethylphosphoramide
Hydrazine and Hydrazine Sulfate (See Hydrazine and Hydrazine Sulfate)
Hydrazobenzene
Indeno[1,2,3-*cd*]pyrene (See Polycyclic Aromatic Hydrocarbons)
Iron Dextran Complex
Isoprene
Kepone® (Chlordecone)
Lead Acetate (See Lead Acetate and Lead Phosphate)
Lead Phosphate (See Lead Acetate and Lead Phosphate)
Lindane and Other Hexachlorocyclohexane Isomers
2-Methylaziridine (Propylenimine)
5-Methylchrysene (See Polycyclic Aromatic Hydrocarbons)
4,4'-Methylenebis(2-chloroaniline)
4-4'-Methylenebis(*N,N*-dimethyl)benzenamine
4,4'-Methylenedianiline and 4,4'-Methylenedianiline Dihydrochloride (See 4,4'-Methylenedianiline and its Dihydrochloride Salt)
Methyleugenol
Methyl Methanesulfonate

N-Methyl-*N*'-nitro-*N*-nitrosoguanidine
Metronidazole
Michler's Ketone [4,4'-(Dimethylamino)benzophenone]
Mirex
Nickel (Metallic) (See Nickel and Nickel Compounds)
Nitrilotriacetic Acid
o-Nitroanisole
6-Nitrochrysene (See Nitroarenes (selected))
Nitrofen (2,4-Dichlorophenyl-*p*-nitrophenyl ether)
Nitrogen Mustard Hydrochloride
2-Nitropropane
1-Nitropyrene (See Nitroarenes (selected))
4-Nitropyrene (See Nitroarenes (selected))
N-Nitrosodi-*n*-butylamine
N-Nitrosodiethanolamine
N-Nitrosodiethylamine
N-Nitrosodimethylamine
N-Nitrosodi-*n*-propylamine
N-Nitroso-*N*-ethylurea
4-(*N*-Nitrosomethylamino)-1-(3-pyridyl)-1-butanone
N-Nitroso-*N*-methylurea
N-Nitrosomethylvinylamine
N-Nitrosomorpholine
N-Nitrosonornicotine
N-Nitrosopiperidine
N-Nitrosopyrrolidine
N-Nitrososarcosine
Norethisterone
Ochratoxin A
4,4'-Oxydianiline
Oxymetholone
Phenacetin (See Phenacetin and Analgesic Mixtures Containing Phenacetin)
Phenazopyridine Hydrochloride
Phenolphthalein
Phenoxybenzamine Hydrochloride
Phenytoin
Polybrominated Biphenyls (PBBs)
Polychlorinated Biphenyls (PCBs)
Polycyclic Aromatic Hydrocarbons (PAHs)
Procarbazine Hydrochloride
Progesterone
1,3-Propane Sultone
β-Propiolactone
Propylene Oxide
Propylthiouracil
Reserpine
Safrole
Selenium Sulfide
Streptozotocin
Styrene-7,8-oxide
Sulfallate
Tetrachloroethylene (Perchloroethylene)
Tetrafluoroethylene
Tetranitromethane
Thioacetamide
Thiourea
Toluene Diisocyanate
o-Toluidine and *o*-Toluidine Hydrochloride(See *o*-Toluidine and *o*-Toluidine Hydrochloride)
Toxaphene
Trichloroethylene
2,4,6-Trichlorophenol
1,2,3-Trichloropropane
Ultraviolet A Radiation (See Ultraviolet Radiation Related Exposure)
Ultraviolet B Radiation (See Ultraviolet Radiation Related Exposure)
Ultraviolet C Radiation (See Ultraviolet Radiation Related Exposure)
Urethane
Vinyl Bromide
4-Vinyl-1-cyclohexene Diepoxide
Vinyl Fluoride

14.9. Case Study: Tetrachlorodibenzodioxin Contamination

(modified from Williams & Burson)

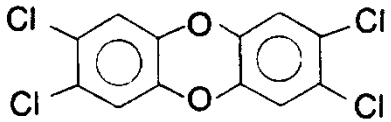
The child, a girl, had a bleeding bladder (hemorrhagic cystitis). She lived near a riding arena where a number of horses had become ill; some had, in fact, died. The physicians felt that her illness might somehow be related to the illness in the animals. At first an infectious disease, perhaps a viral infection, was considered. An epidemiologist surveyed the riding arena. He collected soil samples and examined some of the sick horses. The epidemiologist was told that after the riding arena had been sprayed for dust control, birds and insects near the arena began to die. Perhaps something had been volatilized from the soil that was killing these animals; this might also have affected the little girl.

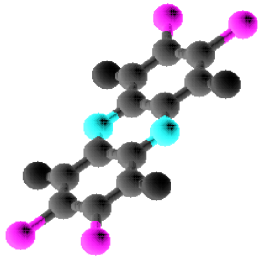
Because of this possibility, the chemists in the laboratory started looking for volatile substances, but were unable to come up with anything. Indeed, the search did not produce results until two years later, when one of the chemists, while trying to distill chemicals from the soil, obtained crystals that condensed on a cold finger of his distillation apparatus. He identified the crystals as 2,4,5-trichlorophenol. The chemist concluded that the illness of the girl might have been caused by 2,4,5-trichlorophenol.

However, 2,4,5-trichlorophenol is not extremely toxic; on the other hand, it is known that 2,4,5-trichlorophenol can be contaminated with an extremely toxic substance, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), which is accidentally manufactured during production of 2,4,5-trichiorophenol. It was therefore decided to

perform rabbit ear tests in order to establish whether the soil was also contaminated with TCDD; possibly TCDD might account for the illness.
The rabbit ear tests were done with an extract from the soil. Some of the rabbits died within a week and all rabbits developed hyperkeratosis on their ears, a typical reaction to TCDD. It could therefore be established that TCDD was most likely present, and additional chemical analysis confirmed this. A reinvestigation of the case was begun.
By now, three years had elapsed; the riding arena had been excavated twice and topsoil had been removed, because the soil caused continual problems. The removed soil had been used as fill beneath a road, in a back yard, and in various other locations.
Not only one riding arena had been affected, but three. One of the riding arenas stabled 125 horses, of which 65 had eventually died. Other animals also died. All horses exposed repeatedly to the contaminated arena surfaces suffered severe weight loss before they died. Investigation also revealed that not very many people had been consistently exposed to the soil of the riding arenas. Primarily. children showed most of the effects probably because they played in the soil and had more opportunities for contact with it.
Symptoms in the index patient -the six-year-old girl who had been hospitalized- were hemorrhagic cystitis, headaches, and diarrhea. A ten-year-old girl had nosebleeding and headaches. People exposed to TCDD often complain of joint pains and pleuritic pains. Also, skin lesions were noticed. We assumed that the other children affected-two three-year-old boys-at one point had chloracne, which had subsided by the time the CDC reinvestigated the case.
The next question we attempted to answer in the investigation was where the TCDD came from and how it got onto the riding arena surface. We learned of an oil dealer who collected used oil and then sold it to refineries to be recycled. He had been hired to spray the riding arenas with oil for dust control, which is a customary procedure. We wondered if somehow he had picked up the material from a firm that made 2,4,5-trichlorophenol, the starting material for (2,4,5-trichlorophenoxy)acetic acid (known simply as 2,4,5-T) and for hexachlorophene.
The oil dealer had a very poor memory about where he collected the TCDD. What we then had to do was to find a company that was making either 2,4,5-T or 2,4,5-trichiorophenol. During the Vietnam War a number of factories produced 2,4,5-T as an ingredient for Agent Orange. Agent Orange was a mixture of 2,4,5-T and 2,4,-D used as a defoliant in Vietnam. Therefore we contacted the Department of Defense and asked them to review their records of all producers of 2,4,5-T. They identified a company located in the area of interest to us. This particular company had stopped its production of 2,4,5-T in 1969, before the contamination at the riding arenas had occurred. It had, however, subleased part of its facility to another company, which had made 2,4,5-trichlorophenol for the production of hexachlorophene. It was the second company that had produced the waste material. When 2,4,5-trichlorophenol is made, TCDD forms as a contaminant. When the company's factory was visited, a tank was found on the premises that was still partially filled with waste material containing TCDD.
The company's operators explained that at first they had sent the TCDD containing waste material to a chemical disposal company; this had been expensive, and they later subcontracted disposal to the oil dealer we had interviewed earlier. The oil dealer had indeed mixed the waste with salvaged oil, and had sprayed the riding arenas with this mixture for dust control.
One of the oil dealer's drivers admitted, on questioning, that while hauling oil on one occasion, he found his truck was overloaded when he passed through a weighing station. Before reaching the next weighing station, he unloaded some of the excess material at a farm owned by the oil dealer. Chickens on that farm subsequently died. We later took samples on the farm and were able to find TCDD in soil there.
After establishing these facts, CDC personnel visited the other riding arenas and other locations and took samples for TCDD analysis. It was established that the other arenas were similarly contaminated. The oil dealer had several times picked up material from the plant where the TCDD-containing chemical tank was found and had also collected polychlorinated biphenyls from other companies; these were then mixed with the TCDD and the oil.
Recently it has been established that contamination by TCDD is much more extensive than was earlier thought and that the waste-oil dealer sprayed many more

2,3,7,8-Tetrachlorodibenzo-p-dioxin





sites with TCDD than those identified in 1974. The contaminated sites at present number 37; some are rural, others are urban, commercial, or residential. Many sites have soil levels above 100 µg/kg soil, and in a few areas the concentrations are above 1 mg/kg soil. The Environmental Protection Agency learned about these sites from former drivers employed by the waste-oil dealer and from the owner of one of the riding arenas who followed the trucks to their source. Human health and environmental studies are continuing in Missouri.

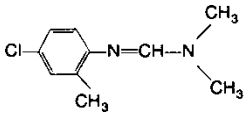
Dioxin is extremely toxic. In animals it causes severe weight loss, affects reproduction, causes liver damage and skin lesions in some species, depresses the cell-mediated immunity, and has been shown to be carcinogenic in rodents. Human illnesses following exposure to TCDD have not been well delineated. Chloracne, porphyria, cutanea tarda, sensory neuropathy, elevated serum cholesterol, and a neurasthenic syndrome have been described. Most chemicals that we are familiar with are toxic in the milligram or gram range. For example, parathion, which is extremely toxic, has a LD_{50} in rats of a few milligrams per kilogram body weight,

but TCDD has an LD_{50} far lower than that: in rats, the oral LD_{50} is between 22 and 44 µg/kg. In addition to being acutely very toxic, TCDD also produces chronic toxic effects at much lower dosage levels. It accumulates in tissues and is persistent. Other extremely toxic compounds, such as some of the chemical warfare gases, break down very rapidly and the body is able to excrete them. TCDD is not easily eliminated from the body.

14.10. Case Study: Industrial Exposure to Chlordimeform

(modified from Williams & Burson)

This case occurred in Tennessee at a pesticide packaging plant. Apparently a group of people who were packaging a chemical called chlordimeform developed hematuria. The state health department in Tennessee was contacted first, and somebody from that department contacted the CDC.



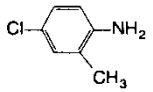
Chlordimeform

A number of chlorinated aniline-type derivatives are currently used as pesticides. Many of them have not been studied very well; we know very little about their health effects, and little published information exists on animal toxicology data.

The medical problems among the workers were first discovered when they started going to physicians. Different people went to different physicians and it was not recognized at first that all workers had an occupational disease in common. It is at times difficult to recognize occupational illnesses, particularly if they resemble diseases common in the general population. One patient, for instance, was treated for gonorrhea, and another worker was treated for inflammation of the bladder. When three people from the pesticide packaging plant went to the same physician, however, this physician began to question whether or not the problems his patients were having might be associated with their work environment.

Questioning revealed that a similar outbreak of illness had occurred at the plant a year before when chlordimeform was being packaged. The affected workers displayed a number of symptoms at that time, including increased urinary frequency. Primarily, they all noticed that their urine was bloody. They also had such symptoms as skin rash and dizziness.

Limited animal studies had been conducted with chlordimeform, and CDC personnel reviewed these. There was no information on human exposures or toxic effects in humans. Dog, rat, and rabbit studies conducted with chlordimeform did not suggest that this material caused hematuria. However, the animal studies showed that chlordimeform is metabolized, and one of the compounds it is metabolized to is 2-methyl-4-chloroaniline. Sometimes a metabolite-not the parent compound-will cause toxic effects by the removal of a side chain:



2-Methyl-Chloroaniline

A literature search concerning 2-methyl-4-chloroaniline produced several articles from Germany and England from the early 1930s. It was reported in these articles that workers who had worked with this material had developed hematuria. One of the authors had also studied 2-methyl-4-chloroaniline in cats. It is known that cats respond more like humans than do other animal species if they are exposed to anilines and chloroanilines.

At the CDC we performed limited animal experiments by exposing cats to chlordimeform and 2-methyl-4-chloroaniline, and found that toxic effects could indeed be caused in the bladder of the cat, though these effects were not as pronounced as those that had been observed in the workers. Additionally, urine was collected from the workers to determine whether it contained the 2-methyl-4-chloroaniline, and whether chlordimeform could be identified.

The pesticide packaging plant was characterized by very poor work practices: workers were essentially "bathing" in chlordimeform. There were no facilities where the workers could change clothes or wash. The packaging process was a very dusty operation; we thought at first that the problem was simply very heavy overexposure in the packaging plant. But when we examined some farmers who used the material, we found that they also had red blood cells in their urine, though they did not have pronounced hematuria, and their symptoms subsided several weeks after they stopped using this particular pesticide.

Chlordimeform was subsequently taken off the market for a while. At present, the rules and regulations for using and packaging this material are much more stringent than they were, so there is no longer as much exposure to it. But marketing of the material is once again allowed and the pesticide is presently being used in the United States and other parts of the world.

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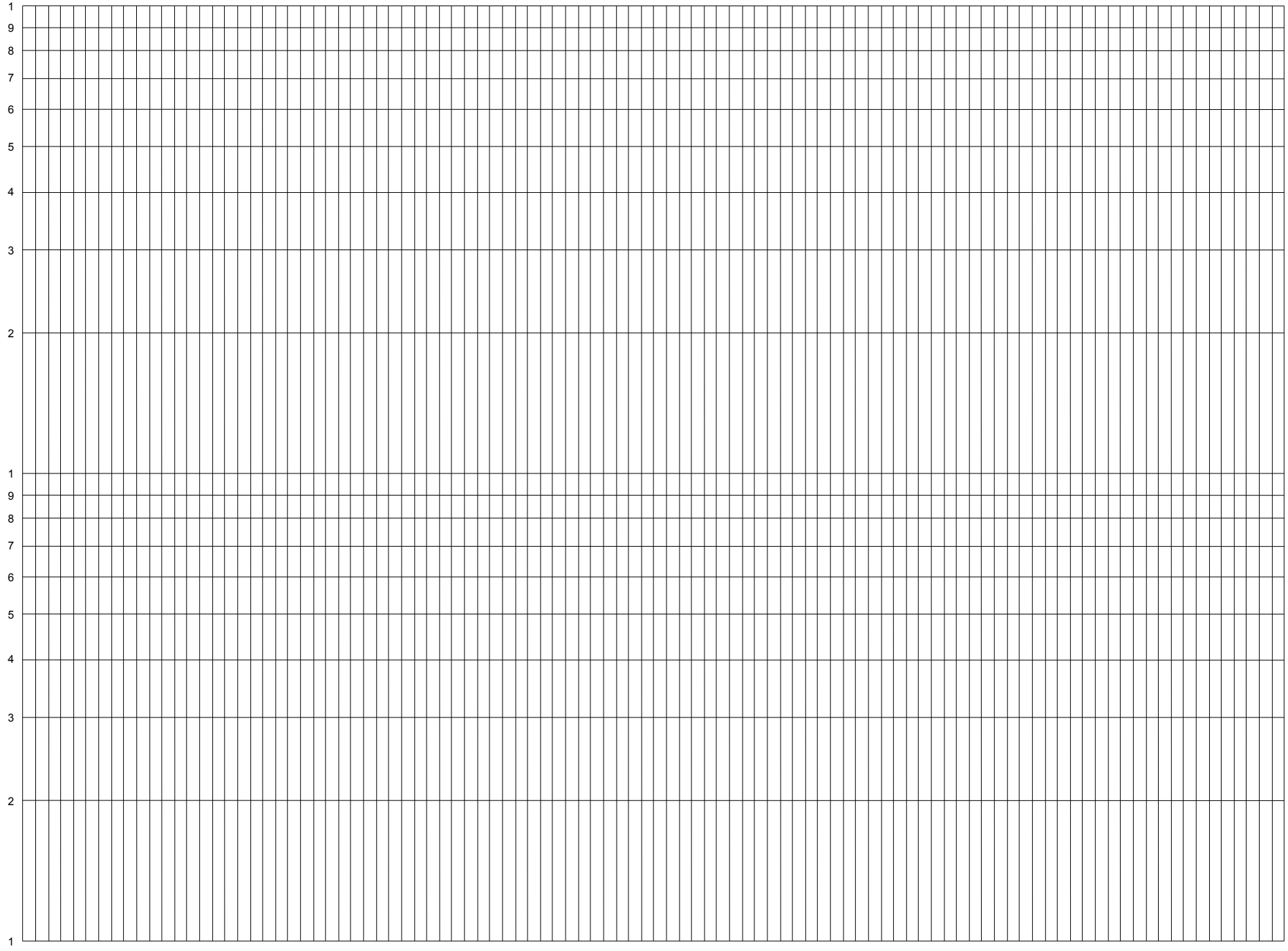
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