CHAPTER 4

Chemical Factors That Influence Toxicity

The physical membranous barriers to translocation of a chemical in an organism are barriers only to compounds possessing specific chemical properties. Nonpolar compounds, as exemplified by ethyl alcohol, appear to pass readily across all biologic membranes in the living organism. The degree of ionization of a chemical in solution is a determinant of the ability of compounds to traverse membranes. Likewise, the solubility of the compounds in lipid material is an important factor with respect to transfer of chemicals across membranes. The chemical structure of a compound determines the ability of the compound to have a biologic action, and around this fact is built the science of structure–activity relationship (SAR).

Since all living tissues or cells are capable of carrying on metabolic processes, such cells possess the ability to alter (biotransform) many normally existing compounds as well as some foreign compounds with which the organism may come in contact. The biotransformation mechanisms are in many cases the result of enzymatic reactions which generate not only energy and products from nutrient chemicals, but also products of many foreign chemicals to which the tissue or organism may be exposed. Furthermore, certain laboratory animals as well as humans possess additional enzyme systems which may exist solely for the purpose of altering the structure of foreign chemicals. Therefore, the chemical factors that influence toxicity fall into two categories. The first category is composed of those chemical and physicochemical properties of compounds which individually and collectively determine the ability of the compounds to pass across biologic membranes. Such properties are important because they regulate the translocation of the chemical throughout the biologic tissue.

The second category comprises the chemical structure of compounds which enables them to produce specific actions on the tissues and to be susceptible to transformation by mechanisms present in the biologic specimen. Such biotransformation mechanisms are important because they may result in the formation of products possessing less toxicity than the parent compound or in the production within the organism of products possessing greater toxicity than the parent compound.

NONSPECIFIC CHEMICAL ACTION

Prior considerations of dose (or concentration)–response relationships indicate that all chemicals are potentially capable of producing harmful effects on living tissue. The mechanisms by which harmful effects are produced vary from a generalized destruction of protein to specific action on single-enzyme systems. Thus, strong acids or bases in high concentrations produce generalized destruction of all living cells, probably by precipitation of proteins with the consequent denaturation of the proteins and disruption of the integrity of the cell membranes. This nonspecific action is induced by concentrated solutions of all caustic or corrosive chemicals and involves partial to complete indiscriminate destruction of all parts of biologic cells.

A generalized overwhelming action of this type is no different from that which results from "cooking" or "burning" the tissue, so that such chemically induced effects are commonly referred to as "chemical burns." Such effects are produced not only by strong acids or bases applied in unphysiologic concentrations, but also by exposure to concentrated solutions of organic solvents, such as ether, chloroform, or carbon tetrachloride. The intensity of such nonspecific toxicity is directly related to the concentration of the chemical which comes in contact with the biologic cell. Generalized destruction of cells can be produced by any chemical that is sufficiently soluble in tissue fluids to gain access to the cells in high concentrations. In humans, these actions usually are limited to readily accessible tissues such as the skin, eyes, mouth, nasal membranes, and pulmonary airways.

SELECTIVE CHEMICAL ACTION

In contrast to the nonspecific, chemically induced destruction of cells from unreasonable exposure to chemicals, the majority of chemicals of interest in toxicology and pharmacology are sufficiently selective in their action that they produce harmful effects at specific sites in biologic specimens in concentrations far below those necessary to produce overwhelming destruction of cells.

Target and Receptor Concepts

Selectivity of action of chemicals signifies that within the biologic specimen substances (compounds) exist which are normal components of cells or cell membranes with which the assaulting chemicals are capable of reacting. Such normal components of cells may be referred to as "targets" or receptors for the assaulting chemical. The target may be very specific and vital to the function of the cell, and the chemical identity of the target is so altered by reacting with the assaulting chemical that it no longer carries on its function. The viability of the cell is thereby altered. For example, in the case of the mechanism of action of penicillin on susceptible bacteria the target for penicillin is a transpeptidase enzyme system in the bacteria that is involved in the synthesis of components of the cell wall necessary for its growth and stability. Penicillin reacts with and inactivates one or more of the transpeptidase enzymes, probably by an acylating reaction. The result is a weakening and eventual rupture of the cell wall of the bacteria, allowing extrusion of its contents into the surrounding medium with consequent death of the cell. Hence penicillin is most effective as a lethal agent to bacteria when they are in an actively growing phase. However, penicillin does not kill all forms of bacteria. This is probably because of structural differences between different strains of bacteria in their transpeptidase enzymes which alter their ability to act as targets for penicillin. Some bacteria can even destroy penicillin by producing penicillinase-type enzymes, thereby protecting themselves from the action of the drug. Thus penicillin is a selectively toxic chemical that affects only specific types of bacteria which have the transpeptidase system that is structurally specific to act as a target for the antibiotic drug.

In contrast to this, the target may be a protein or lipid which is not immediately vital to the function of the cell, and the reaction between the assaulting chemical and the target does not produce a direct alteration in cell function. In pharmacology, if the target with which the foreign chemical (or drug) reacts alters the function of the cell, such targets are given the general term of "specific receptors," signifying that a given drug intereacts or reacts with certain specific cell components. The same chemical may at the same time combine with, react with, or be adsorbed on extracellular proteins, but the function of the cells is not influenced by the product which is formed. Such combining sites on the proteins are referred to as "nonspecific receptors" for the drug.

In toxicology, a specific receptor is the cell component or components with which a foreign chemical interacts, thereby either directly or indirectly leading to the production of a harmful effect. When the receptor for a foreign chemical is known, it is not necessary to use such a noninformative term; rather, it is preferable to specifically identify the receptor. For example, the toxicologist may refer to the effect of mercury on the sulfhydryl groups of certain enzyme systems in which the sulfhydryl groups act as the specific receptors. The toxicologist recognizes that in the mammal one specific receptor for carbon monoxide is the hemoglobin molecule, that carbon monoxide has an affinity for hemoglobin, and that in reacting with hemoglobin it forms a hemoglobin–carbon monoxide complex which is not capable of carrying oxygen. Hemoglobin is therefore the receptor for carbon monoxide, and the kinetics of this reaction between carbon monoxide and hemoglobin have been well defined. However, in toxicology as in pharmacology, the exact receptor for many toxic chemicals remains to be defined.

The concept of specific receptors to chemicals is a useful concept in toxicology. Since the properties and structure of a chemical determine the affinity of that chemical for a biologic receptor, if the structure of one chemical entity is known and its receptor is known, then it is possible to predict the nature of the structure which is required to be more or less capable of reacting with the known receptor. Several useful drugs (for example, succinylcholine) and some of the most potent chemical agents known to man and of interest in toxicology were developed by prediction based on a knowledge of the chemical–receptor mechanism which was involved.

EFFECT OF IONIZATION AND LIPID SOLUBILITY ON TRANSLOCATION OF CHEMICALS

Many chemicals of interest in toxicology exist in solution in ionized and nonionized forms. Many drugs are weak organic acids or bases and only the nonionized forms are significantly soluble in fat. Since it has already been stated that the "pores" of the cell membrane occupy only a small part of the total area as compared to the lipid portion of the membrane, effective translocation of a chemical from extracellular fluid to the intracellular fluid should be facilitated by direct transfer of the agent through the lipid membrane. Current evidence strongly suggests that the nonionized, lipid-soluble form of an organic electrolyte is the predominant form capable of passing through the biologic cell membrane or the membranous barriers, which are composed of multiple cells.

The degree of ionization of an electrolyte in aqueous solution is dependent upon the pH of the solution. If the pH of an aqueous solution of an acid or base is adjusted so that the compound exists half in the ionized and half in the nonionized form, that pH is the acidic dissociation constant or pK_a of the compound. Conventionally, the dissociation constant for both acids and bases is expressed as the acidic dissociation constant or pK_a of the compound. An acid with a low pK_a is a strong acid, whereas a base with a low pK_a is a weak base. Conversely, a base with a high pK_a is a strong base and an acid with a high pK_a is a weak acid. At a pH above the pK_a of a compound, acids exist in aqueous solution mainly in the ionic form and bases in the nonionic form. Conversely, at a pH below the pK_a of a compound, acids exist in aqueous solution mainly in the nonionic form and bases in the ionic form. The pK_a of a compound may be derived from the Henderson–Hasselbalch equation as follows.

$$\text{for acids } pK_a = \text{pH} + \log \frac{\text{nonionized form}}{\text{ionized form}}$$

$$\text{for bases } pK_a = \text{pH} + \log \frac{\text{ionized form}}{\text{nonionized form}}$$

Therefore, if the pK_a of an acid or base is known and the pH of the aqueous solution of the compound is known, it is possible to calculate the ratio of the ionized to nonionized forms of the chemical in solution.

If two aqueous solutions of an electrolyte are separated by a biologic membrane that is permeable to only the uncharged molecules, in time a state of equilibrium occurs. At equilibrium the concentrations represented as the sum of the ionized and nonionized forms of the compound in each solution are identical if the pH values of the two solutions are identical, whereas the concentrations will be different if the pH values of the solutions are different. In the latter case, the concentrations of the electrolyte on the two sides of the membrane can be expressed as a ratio for any two pH values.

Since the pH on both sides of a cellular membrane in most organs of biologic specimens is essentially the same, if only the nonionized portion of the compound passes through the membrane, and if a compound is introduced to one side of the membrane, then it may be predicted that a compound that was highly ionized at that pH would fail to traverse the membrane as effectively as would a compound which was only poorly ionized at that pH. Compounds that exist at physiologic pH primarily in the nonionized state (provided the nonionized form is lipid soluble) would be expected to diffuse through membranes according to the direction of any existing concentration gradient until equilibrium is reached.

When a difference in pH exists on opposing sides of a membrane, a concentration gradient in regard to the nonionized moiety will be created so that when equilibrium is reached, the total quantity of electrolyte may be many times greater on one side of the membrane than on the other side. In the warm-blooded mammal, there are two sites at which the pH on the opposing sides of membranes may differ greatly. These are the mucosal surface of the gastrointestinal tract and the lumen of the tubules of the kidney. At these sites, the effect of pH on the ionization of organic electrolytes controls the transfer of the electrolytes across the membrane and therefore controls absorption of the electrolyte from the gastrointestinal tract and excretion of the compound by the kidney.

Shown in Fig. 4.1 is the proportion of nonionized to ionized forms of acetylsalicylic acid ($pK_a = 3.5$) in the stomach (at pH 1.0), in the intestine (pH 5.3), in the interstitial fluid or blood (pH 7.4), in acid urine (pH 6.8), and in alkaline urine (pH 7.8). At equilibrium, the concentration of the nonionized forms of acetylsalicylic acid in each fluid shown in the figure would be the same provided the membranes are permeable to the nonionized form of the drug. In order to reach equilibrium, the total quantity of drug present in each fluid will be different. Furthermore, that portion of the drug which is removed by nonspecific receptors such as protein would not contribute to the quantity of drug in each fluid as shown in the figure. The data in Fig. 4.1 are not corrected for protein binding. It is obvious that when a chemical is initially introduced into the stomach or the intestine, a concentration gradient for the chemical exists between the site of deposition and the other body fluids. If the chemical is capable of being absorbed,

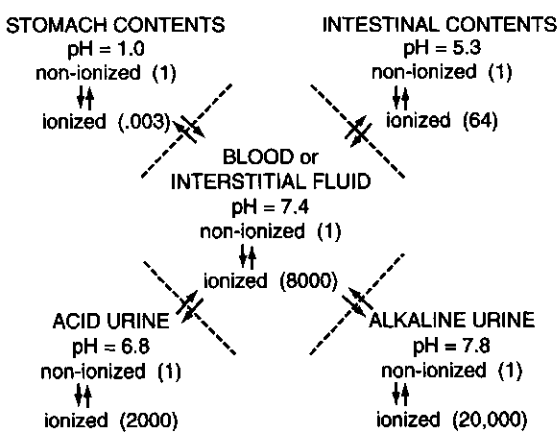


FIGURE 4.1 Proportions of nonionized and ionized forms of acetylsalicylic acid (pK_a = 3.5) in biologic fluids.

absorption progresses in the direction of the concentration gradient. If only the nonionized form is absorbed, the amount of the nonionized form determines the rate of absorption of the compound.

The direction of the concentration gradient for the ionized form of the drug across the mucosal membrane separating the stomach contents from the interstitial fluid would be highly favorable for rapid transfer of the drug from the stomach to the blood (Fig. 4.1). A similar, though less favorable, condition exists for the transfer of the drug from the intestine to the blood. Actual experimental results confirm the rapid absorption of aspirin from the stomach and intestine in humans.

When the kidney is forming acid urine, the concentration gradient of the nonionized form is in the direction of transfer of acetylsalicylic acid from the urine to the blood; under this condition the kidney would be expected to be a poor organ for excretion of the drug. However, if the kidney is forming alkaline urine, the concentration gradient for the nonionized form favors excretion of the drug from the blood into the urine. Actual experimental results confirm this concept (Fig. 4.2). The effect of alkalinization of urine on the excretion of acetylsalicylic acid from the dog, shown in Fig. 4.2, indicates that the excretion of acetylsalicylic acid is more than quadrupled by shifting the pH of the urine from 6.7 to 7.8. (Although

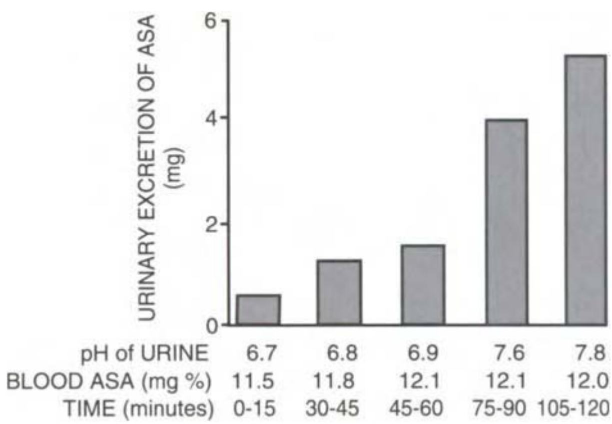


FIGURE 4.2 Effect of urinary pH on excretion of acetylsalicylic acid (ASA) by the kidney. Dog, male, 12 kg, hydrated with water (250 ml orally) 1 hr before the experiment. ASA (0.5 g orally) given 45 min before the experiment. ASA (50 mg) in 0.9% NaCl in water (150 ml) continuously infused during experiment. At 20 to 25 min $NaHCO_3$ (0.2 g) given intravenously. At 65 min $NaHCO_3$ (0.5 g) given intravenously. Urine collections made at 15-min intervals from an inlying urinary bladder catheter and each urine sample includes 50 ml water used to wash the bladder.

the above concept is valid for the purpose for which it is used, it involves an intentionally oversimplified version of the total number of mechanisms involved in urine formation by the kidney as well as the anatomic features of the kidney which permit formation of the blood filtrate and its ultimate appearance as urine.)

It is now well established that chemicals are readily absorbed from the gastrointestinal tract if the nonionized form is lipid soluble and if the pK_a of an acid is greater than 2 and for a base is less than 11. Chemicals are poorly absorbed from the intestine for several reasons if (1) the chemical is completely ionized in the intestine, (2) the nonionized form is not lipid soluble, (3) the chemical is destroyed by intestinal enzymes or microorganisms, or (4) the chemical is insoluble at the pH of the intestinal contents.

Provided they are organic electrolytes, chemicals that are strictly of toxicologic interest would be expected to follow these principles.

Chemical agents that do not undergo ionization or react with biologic fluids are absorbed and translocated according to the physical laws of diffusion and the solubility properties of the agent in water and lipids. In pharmacology, it is common practice to refer to the gaseous and vapor anesthetic agents (such as ether, chloroform, cyclopropane, nitrous oxide, ethylene, and divinyl ether) as substances that are nonreactive with tissue constituents simply because such agents generally are absorbed, translocated, and excreted from the body with little or no change in the chemical nature of at least 90% of the administered agent. In fact, except for minor losses of the compounds through the kidney and sweat, these agents are recovered primarily from the expired air of animals. Currently the mechanisms by which they produce anesthesia have defied precise description, but in general, anesthetic potency is directly related to lipid solubility.

Agents that are inspired through the pulmonary system diffuse across the pulmonary membrane according to Fick's law, which states that a gas will diffuse in the direction of a decreasing partial pressure gradient at a rate that is directly proportional to the diffusion coefficient and inversely proportional to the square root of the molecular weight or density of the compound. Thus the rate of diffusion of such agents into the blood of mammals is proportional to the partial pressure of the agent in the inspired air. This process of absorption of the drug from the air into the blood occurs at a sufficiently rapid rate so that equilibrium is reached by the time the blood makes one circuit through the lungs.

The concentration of the anesthetic agent is distributed to the tissues in direct proportion to their blood supply and their water and fat content (which determines the solubility coefficients for the various tissues). Nervous tissue has no special affinity for these anesthetic agents. Thus, as long as the anesthetic agent is administered to the animal, a shift in distribution of the agent in the various animal tissues will continue to occur until equilibrium is obtained throughout the animal. Under the actual conditions of use of anesthetic agents, it is doubtful whether the animal ever reaches total equilibrium only because the administration of constant concentrations of such agents is not continued for long periods of time. When administration is discontinued, the body eliminates the agent because the diffusion pressure gradients are reversed. The rate of elimination of these agents then is proportional to the partial pressures of the agents in the tissues, blood, and inspired air.

In toxicology, exposures to various gases and vapors may be continued for long periods of time, such as under the conditions encountered in the course of living in a contaminated atmospheric environment. The same principles that determine the absorption and translocation of the gaseous and vapor anesthetic agents would apply to any gas or vapor. In toxicology, it is possible that gaseous or vapor types of atmospheric contaminants would reach equilibrium in the body. When such agents are encountered in concentrations that do not give rise to acute signs or symptoms in the biologic specimen, there is some question whether chronic exposure to such chemical atmospheric contaminants can induce harmful effects. Such a question is usually answerable only by acquisition of data through experience. The American Society of Industrial Hygienists recognizes this problem and has published estimates of maximal allowable concentrations that may be considered safe for 8-hr daily exposure for approximately 500 chemical agents encountered in the atmosphere. A discussion of the basis for these estimates is given in Chapter 5.

Compounds that are inhaled and undergo ionization in biologic fluids would be expected to be absorbed, translocated, and excreted by the organism according to the conditions described for electrolytes.

BIOTRANSFORMATION MECHANISMS

Many foreign (xenobiotic) chemicals that are introduced into the body undergo chemical transformation, and this process is generally referred to as "metabolic transformation" or "biotransformation." The transformation processes are enzymatically induced and result in either the alteration of the parent molecule or the formation of products involving combinations of normally occurring substances and the parent molecule. Two categories of enzyme systems are known to exist in mammals. One category consists of enzymes that normally occur in the tissues and are responsible for transformation of normal endogenous chemicals in the tissues. The second category consists of enzymes that alter the structure of many foreign chemicals but have no established normal endogenous substrates.

A number of the enzyme systems that induce the tranformation of normal chemical substrates in the body are also active in catalyzing alterations of foreign chemicals that structurally are sufficiently similar to the normal substrate. For example, the nonspecific esterase-hydrolyzing enzyme cholinesterase not only hydrolyzes acetylcholine (a normally occurring neurohormone), but also will hydrolyze the local anesthetic agent procaine, as well as the muscle-paralyzing drug succinylcholine. Another example is the enzyme, monoamine oxidase, which is important in the metabolism of normally occurring biologic amines such as epinephrine and tyramine. This enzyme also oxidizes foreign short-chain amines such as benzylamine.

An enzyme system that is important in toxicology is that which has been extensively investigated in regard to the metabolism of drugs. These enzymes have become classed as "drug-metabolizing enzymes" and are frequently referred to as the "drug detoxication enzymes." These terms are misleading and should be discontinued not only because the enzymes catalyze transformation of many compounds that are not drugs, but also because the reactions do not always result in detoxication of the foreign compound. Rather, the toxicity of the product for many foreign compounds that are transformed by these enzymes is shown to be greater than that of the parent compound, by way of a process that may be termed "toxication" or "activation." These enzymes are referred to as drug-metabolizing enzymes only because of the common use of this term for identification of this group of enzymatic substances.

The drug-metabolizing enzymes consist of a group of enzymes that are present in many tissues but are particularly abundant in liver cells. Of the various components of the liver cells, the endoplasmic reticulum can be visualized with the electron microscope as filamentous-like structures of two types, smooth- and rough-surface filaments. It is the smooth-surface endoplasmic reticulum that contains the large proportion of the drug-metabolizing enzymes, whereas the rough-surface reticulum is concerned with enzymes involved in protein synthesis. When the liver cells are ruptured by homogenization of the cells, the endoplasmic reticulum undergoes fragmentation; these fragments can be separated by ultracentrifugation from the other parts of the liver cell. The fragments of the smooth reticular endothelium are then commonly called "microsomes."

Much of the information that has been obtained regrading the drug-metabolizing enzymes is based on *in vitro* studies utilizing the microsomal fraction of liver cells as the source of the enzymes. These microsomal enzymes are capable of catalyzing a variety of biotransformation reactions, among which are hydroxylation, dealkylation, deamination, alkyl side chain oxidation, hydrolysis, and reduction. The microsomal enzymes generally do not act on lipid-insoluble materials. In fact, they generally convert lipid-soluble compounds to less lipid-soluble compounds, thereby forming more polar substances that can be easily excreted by the kidney and by the biliary tract.

Shown in Fig. 4.3 are the types of metabolic transformation mechanisms important in toxicology. The figure is composed of mechanisms that have been shown to exist in several conventional laboratory animals. However, there are distinct variations in the pathways of metabolism for individual compounds, not only between species, but also within species. The figure includes an example of each type of metabolic transformation pathway. Several of the examples are of drugs and represent data obtained from studies on drug metabolism, which initially led to the demonstration of the existence of the enzyme system listed in the figure.

The microsomal material contains a membrane-bound, mixed-function oxidase system. It consists of a system enabling electron transport between compounds through the action of a variety of reductases plus a group of heme proteins that possess oxidase properties. The oxidase system is capable of attacking molecular oxygen (O_2) by reducing one atom of oxygen with the formation of water and incorporating the other atom of oxygen into a substitute xenobiotic chemical. The microsomal system more specifically operates as follows. It requires the presence of reduced nicotinamide–adenine–dinucleotide (NADPH) and molecular oxygen. NADPH reduces a component of the microsomes which reacts with molecular oxygen to form an active oxygen intermediate which oxidizes the drug. This may be viewed as a stepwise process involving initially the oxidation of NADPH by the action of a flavin enzyme (cytochrome *c* reductase); subsequently, in the presence of a reduced heme protein called P450, active oxygen is formed from molecular oxygen. P450 is so named because after complexing with carbon monoxide it shows maximal spectral absorption at 450 millimicrons. It is now recognized that there are several similar heme proteins (for example, P448) that have similar functions and which are identified by their maximal spectral absorption when complexed with carbon monoxide. The active oxygen oxidizes the drug. The reactions are listed as follows.

$$\text{NADPH} \xrightarrow[\text{reductase}]{\text{cytochrome } c} \text{NADP} + \text{H} \tag{1}$$

$$(\text{Fe}^{3+})\text{P-450} + \text{H} \xrightarrow[\text{P-450 reductase}]{\text{cytochrome}} (\text{Fe}^{2+})\text{P-450} \tag{2}$$

$$(\text{Fe}^{2+})\text{P-450} + \text{O}_2 \longrightarrow \text{“active oxygen”} \tag{3}$$

$$\text{“active oxygen”} + \text{drug} \longrightarrow \text{oxidized drug} \tag{4}$$

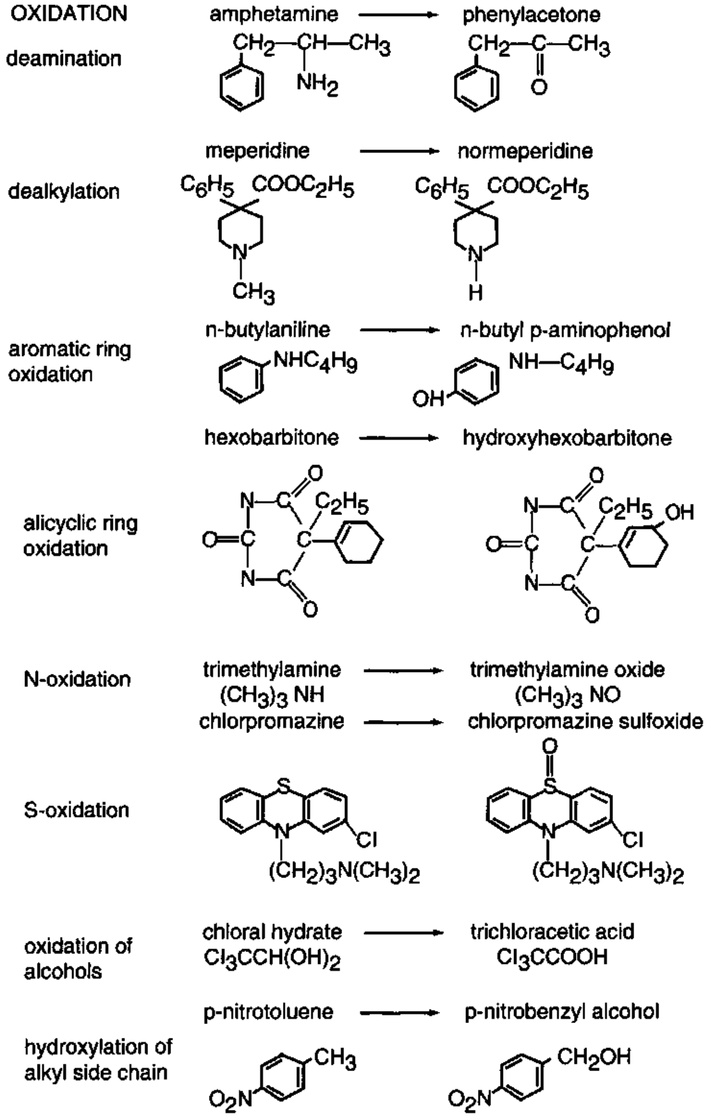


FIGURE 4.3 Some types and examples of biotransformation mechanisms in animals. The oxidation and reduction reactions are catalyzed by liver microsomal enzyme systems. The hydrolysis, acetylation, and conjugation reactions may involve enzyme systems from other tissues.

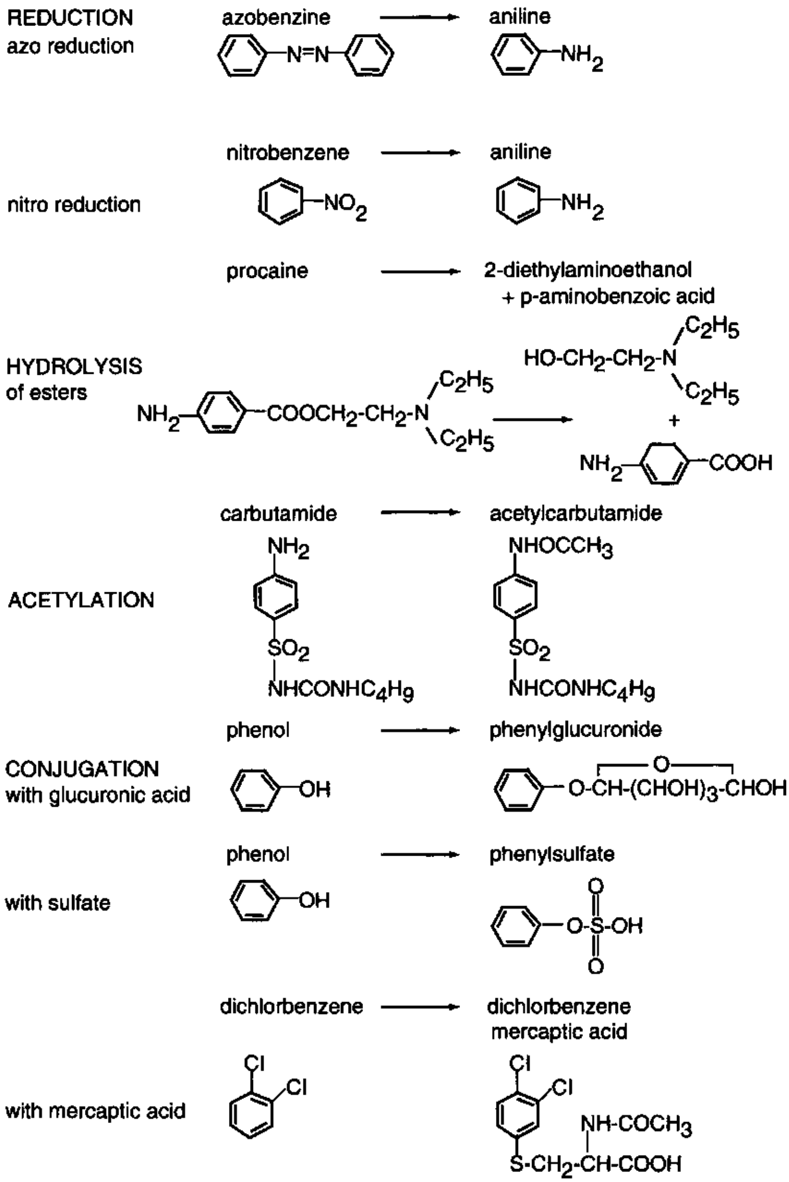


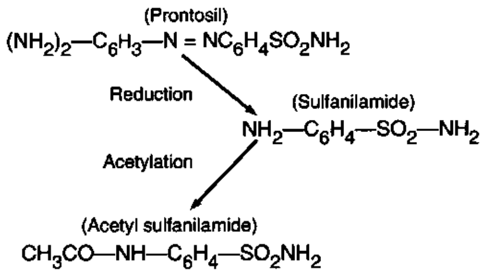
FIGURE 4.3 (*continued*)

The family of microsomal enzymes known as the cytochromes P450 also metabolize chemicals other than through oxidative and reductive mechanisms. In recent years much has been learned about the function of these enzymes and their action on xenobiotics through the discovery of selective inhibitors of members of this family of cytochromes P450.

Synthetic and Nonsynthetic Mechanisms

It is useful to divide the biotransformation mechanisms into two major types: (1) the nonsynthetic reactions involving oxidation, reduction, or hydrolysis and (2) the synthetic reactions involving generation of a product that is biosynthesized from the xenobiotic agent or its metabolite plus an endogenous agent or radical such as glucuronide or sulfate. One or both types of transformation may occur in the case of a single xenobiotic compound, and in various species the pathways for transformation may vary, depending on the availability in the species of the enzymatic system or endogenous products necessary for the reactions. Examples are seen in the fact that ethereal sulfate synthesis from phenols is a universal mechanism found in all species. The sulfation process involves activation of inorganic sulfate to an active sulfate which is 3 phosphoadenosine-5-phosphosulfate (PAPS) and serves as the sulfate donor. PAPS appears to sulfate aryl amines but not aliphatic amines. Glucuronide conjugation involves uridine diphosphoglucuronide, which probably represents a metabolically active form of glucuronic acid and has a normal role in conjugation of compounds such as the steroids, but which will also conjugate the salicylates, cinchophen, morphine, or codeine. Glycine conjugation occurs in most laboratory animals as well as in man, but glycine conjugation is replaced in hens by ornithine conjugation and in spiders by arginine conjugation.

An example of a foreign chemical that gives both the nonsynthetic and the synthetic types of reaction during metabolic transformation is one of the first compounds investigated, the diazo compound Prontosil. Prontosil, which was used initially as a dye material, showed antibacterial activity when administered to animals infected with hemolytic streptococci. It was soon discovered that the antibacterial activity was predominantly due to a metabolic product of Prontosil which was identified as sulfanilamide. It was then subsequently found that most animals were not only capable of reducing Prontosil to sulfanilamide, but also were capable of acetylating the sulfanilamide. Also, the acetylated compound was ineffective as an antibacterial agent. This series of transformation steps follows.



The metabolism of Prontosil serves as an example of several basic concepts of toxicology:

1. In regard to antibacterial activity, the first transformation reaction leads to activation of the compound, but the second reaction leads to inactivation of the compound. In the intact animal the first reaction takes place at a rate which exceeds that of the second reaction, thereby leading to accumulation of concentrations of sulfanilamide so that antibacterial activity is present in the animal. Had this not been the case, the mechanism of antibacterial action of Prontosil in all probability would have been overlooked. Many sequential transformation mechanisms lead to the formation of intermediate products, which exist as transient or only hypothetical products and would have to be extremely potent to have any significant toxicologic effect on the organism.

2. In regard to toxicologic effect on the host and in regard to acute lethal toxicity, the first step in the metabolism of Prontosil may be said to lead to the formation of a more toxic compound than the parent compound; the second step in the metabolism of Prontosil may be said to lead to the formation of a less toxic compound. However, in actual practice, Prontosil (or its metabolic products, sulfanilamide, and acetylsulfanilamide) produce several toxicologic effects which are not necessarily lethal. For example, in man sulfanilamide induces the formation of methemoglobin; it inhibits carbonic anhydrase, and it may produce fever, skin rashes, or blood cell dyscrasias. Since sulfanilamide is a metabolic product of Prontosil, some of these toxicities are induced by administration of the parent compound. In animals (the dog is an exception since it does not acetylate sulfanilamide) acetyl sulfanilamide is prone to precipitate in the kidney tubules, owing to its relative insolubility in urine as compared to sulfanilamide or Prontosil, and can obstruct the flow of urine. Therefore, as far as the host animal is concerned, both the first and second steps in metabolic transformation of Prontosil lead to the formation of products with some form of toxicity greater than that of the original compound.

In another example, understanding the pathways of metabolism of the analgesic drug acetaminophen helps to explain the mechanisms responsible for the hepatic toxicity of overdoses of that drug. Figure 4.4 shows these pathways. Following ordinary doses of acetaminophen in humans a portion of the drug undergoes conjugation with glucuronide and sulfate and the conjugates are then excreted in the urine. Simultaneously, a major portion of the drug is biotransformed via cytochrome P450 oxidative metabolism to form reactive metabolites which combine with hepatic glutathione and are converted to mercapturic acid derivatives. These are also excreted in the urine. Large doses of acetaminophen deplete the liver of its stores of glutathione, thereby allowing the reactive metabolites of acetaminophen to covalently bind to various liver cell proteins. This leads to death of the liver cells. Although the above sequence of reactions does not identify the ultimate "toxicant," clearly the depletion of glutathione stores is the critical problem leading to the hepatic cell death. Also it is now recognized that replacement of the thiol glutathione with a similar thiol, *n*-acetylcysteine, effectively reduces acetaminophen hepatic toxicity. (See Chapt. 11 on antidotal therapy.)

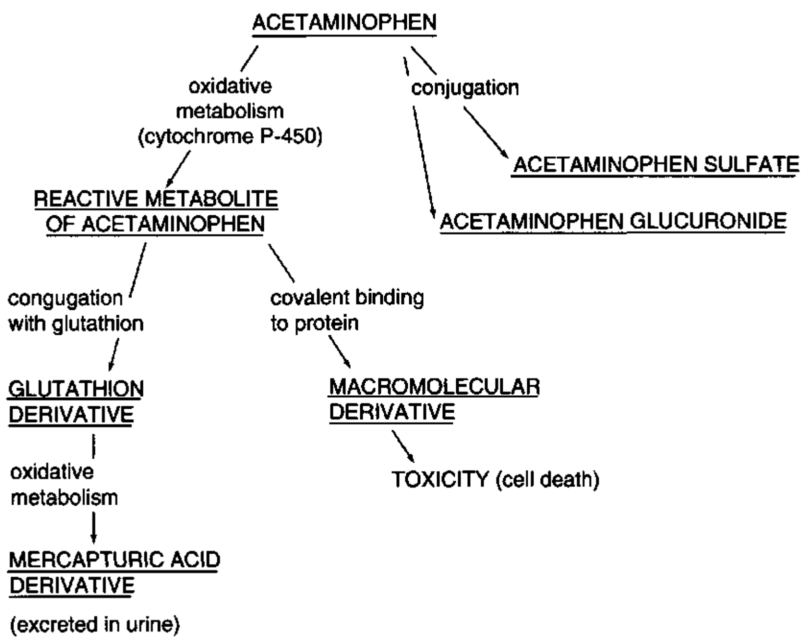


FIGURE 4.4 Acetaminophen metabolism and probable mechanism of hepatic toxicity.

It is now evident that determination of the toxicity of any compound which is metabolically transformed is in essence the determination of the toxicity of the parent compound and its metabolites. In fact, some of the most widely used pesticides are not effective pesticides and have low orders of toxicity to a host animal until they are metabolically transformed in the host to an active substance. A good example is the conversion of Parathion to Paraoxon. It is possible that parent compounds may exist for such a short period of time in the biologic organism that their usefulness as therapeutic agents is extremely limited. Such mechanisms help us to understand the difficulties encountered in transposing data that are acquired from *in vitro* observations of effects of chemicals to the intact animal.

There are many instances in which metabolic conversion of a chemical results in the formation of products that are more toxic than the original compound (Table 4.1). Such a process may be referred to as metabolic toxication. It should be recognized that whether or not metabolic toxication has practical significance depends on the quantity and potency of the metabolic product that is made available to the animal or biologic specimen

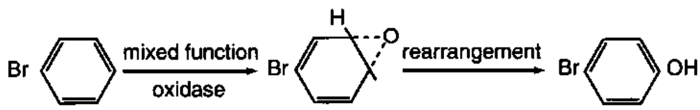
TABLE 4.1 Increased Toxicity Resulting from Metabolic Conversion

Compound	Product
Sulfanilamide	Acetylsulfanilamide
Ethylene glycol	Oxalic acid
Methanol	Formate
Fluoroacetate	Fluorocitrate
Parathion	Paraoxon
Tremorine	Oxytremorine
Tri-o-cresyl phosphate	Cyclic phosphate
Dimethyl nitroso amine	Diazomethane
Schradan	Schradan-N-oxide
Heptachlor	Heptachlor epoxide
Pyridine	n-Methyl pyridinium chloride
Chloral hydrate	Trichlorethanol chloride
Nitrobenzene	Nitrosobenzene, phenylhydroxylamine
Acetanilid	Analine
Pentavalent arsenicals	Trivalent arsenicals
Selenate	Selenite
2-Naphthylamine	2 Amino-l-naphthol
Codeine	Morphine
Phenylthiourea	Hydrogen sulfide

Note. Data from Schuster, L.: Metabolism of drugs and toxic substances. *Ann. Rev. Biochem.* **33**:590, 1964.

under consideration. It is apparent that metabolic toxication resulting from the conversion of Parathion to Paraoxon in an insect is significant; otherwise Parathion would be a most ineffective insecticide. In regard to conversion of sulfanilamide to acetyl sulfanilamide, this is a critical conjugation reaction in regard to lethal effect on bacteria, but is of little practical importance (except for changes in solubility leading to crystalluria) in regard to lethal toxicity in the average clinical case which is treated with sulfanilamide or similar therapeutic agents.

The effect of biotransformation of a compound may be of great significance in some examples but may be of no significance in other examples as far as the overall toxicity to the animals is concerned. An example in which the biotransformation process is of great significance is shown when a chemically unreactive compound is converted to highly reactive derivatives, where the derivatives are alkylating agents that are incorporated into macromolecules in the tissue cells. The enzymes that perform this conversion are in the mixed function oxidase system of the microsomes. The chemically reactive compound that is created may be only an intermediate in the total biotransformation reaction. For example, para-bromobenzene forms several derivatives in the liver. It is first converted to the active alkylating agent, bromobenzene epoxide, which undergoes rearrangement to form parabromophenol. These reactions are shown below.



In this example the epoxide is probably the principal compound that covalently binds with the macromolecules in the liver resulting in liver toxicity. An important concept resulting from the above example is that if the biotransformation process results in the formation of highly reactive derivatives such agents are capable of modifying vital macromolecules.

The microsomal oxidizing system has acquired added significance in toxicology because it is the basis of some popular theories about the mechanism of chemical-induced necrosis (i.e., death) of cells. One theory is that compounds that produce cell death selectively in specific species or organs and tissues within a given species do so because of the abundance in those tissues of the microsomal oxidizing system, and because intermediate reactive products capable of binding with essential proteins in the cell are formed by the oxidation of the parent compound. In fact, it is often possible to show a direct relationship between the severity of the tissue lesion and the amount of metabolite that is covalently bound in the damaged tissue. Also, by inhibiting the microsomal system, the tissue lesion is favorably influenced. Metabolic activation of a chemical resulting in tissue injury is the most probable mechanism responsible for local tissue damage occurring in only selected species as well as in only selected organs.

An example in which biotransformation of a compound to a more toxic agent has little significance in normal humans is the case of the metabolism of ethyl alcohol. The principal metabolism of ethyl alcohol does not involve the microsomal system. It involves conversion of ethanol to acetaldehyde by alcohol dehydrogenase (ADH) in the presence of nicotinamide adenine dinucleotide (NAD). The aldehyde is then converted to acetate. The acetate forms acetyl CoA which then enters the citric acid cycle. The end products are then carbon dioxide and water as follows:

$$\text{Ethanol} + \text{NAD} + \text{ADH} \rightarrow \text{acetaldehyde} + \text{NADH}$$
$$\text{Acetaldehyde} + \text{aldehyde dehydrogenase} \rightarrow \text{acetate}$$
$$\text{Acetate} + \text{CoA} \rightarrow \text{acetyl CoA} \rightarrow CO_2 + H_2O$$

In the human small amounts of acetaldehyde produce nausea, vomiting, headache, palpitation, and a fall in blood pressure. In the normal human, acetaldehyde never accumulates to any significant amount following ingestion of alcohol since it is rapidly converted to acetate. However, the enzyme system responsible for conversion of acetaldehyde to acetate can be blocked by administration of several compounds, among which is tetraethylthiouram disulfide; only when the aldehyde dehydrogenase enzyme is blocked does the ingestion of alcohol produce the characteristic symptoms of accumulation of acetaldehyde.

Whether or not toxicity occurs as a result of biotransformation to a more toxic substance is dependent on the affinity of the products of the reaction for receptors, the concentration of the product at the receptor sites, and the duration of its presence in the biologic system.

Inhibition of Biotransformation Mechanisms

Various circumstances can influence the level of the microsomal P450 enzyme system and thereby influence the toxicity of compounds that are dependent on this system for conversion to metabolites that are more or less toxic than the parent compound. It has been shown that even the diet can influence the level of the P450 system in experimental animals; that is, low-protein diets suppress P450 levels and associated enzyme activity.

The microsomal enzyme systems can be inhibited by several compounds in concentrations which by themselves have little definite pharmacologic activity. One of the first examples of a nonspecific microsomal enzyme inhibitor was SKF-525A (diethyl aminoethanol ester of diphenylpropyl acetic acid). This compound was initially investigated in 1954 and 1955 and was shown to increase the duration of a barbiturate-induced anesthesia, probably by directly combining with and inactivating the microsomal drug-metabolizing enzymes that are largely responsible for termination of the action of the barbiturate. The action of SKF-525A is not limited to an effect on barbiturate metabolism; rather, it has a general inhibitory action on many of the microsomal drug-metabolizing enzymes, and therefore may affect the metabolism of many xenobiotics normally metabolized by these enzymes.

When the product of metabolic transformation is of greater toxicity (metabolic toxication) than that of the parent compound, the inhibition of the metabolizing enzymes would be expected to protect the animal from toxicity resulting from metabolism of the parent compound. A good example is in regard to the liver lesions produced by large doses of the commonly used analgesic drug acetaminophen, which has been discussed previously in this chapter and is shown in Fig. 4.4. When animals are pretreated with inhibitors of drug-metabolizing enzymes, the liver lesions are prevented from occurring. Such experiments initially suggested that the acetaminophen-induced hepatic necrosis is caused by a toxic metabolite rather than acetaminophen itself. However, this is not always the case; actual experiments with the phosphorothionates which are biotransformed to the more toxic O-analogs show that their toxicity is not affected by SKF-525A. This is because both the formation (metabolic toxication) and the hydrolysis (metabolic detoxication) of the O-analog are only partially blocked by SKF-525A; thus the two effects cancel each other. In contrast to this, in the case of procaine which is in part detoxified by microsomal esterases (metabolic detoxication), pretreatment of mice with SKF-525A decreases the LD_{50} of procaine from 188 to 79 mg/kg. In this case, the toxicity of procaine may be said to be increased by the pretreatment of the mice with SKF-525A. Since this effect of SKF-525A is the result of prolonging the metabolic conversion of procaine, it is a practical demonstration that the toxicity of a compound may be related to its duration of action in the organism, which is in turn related to the efficiency of the mechanisms involved in termination of action of the chemical in the organism. Other compounds that have the same action as SKF-525A are piperonyl butoxide and cobalt chloride.

Other factors also can be the cause of decreased microsomal enzyme activity. At least in rats, starvation for as little as 16 to 36 hr leads to the formation of an endogenous inhibitor of microsomal N-demethylation. There is also ample evidence that the livers of newborn as well as immature animals are deficient in the microsomal drug metabolizing enzymes. Therefore, these as well as various as yet undetermined factors may alter the activity of the microsomal enzymes and may account in part for variations in toxicity within members of a species.

Inhibition of microsomal as well as nonmicrosomal enzyme systems is occasionally involved in drug interactions that result in toxicity. Many drugs produce their therapeutic effects by inhibition of specific enzyme systems; when a second drug is given in several doses to the patient, if that second drug is dependent on the affected enzyme system for termination of its presence in the body, the second drug will accumulate in the patient with each subsequent dose. For example, allopurinol is a xanthine oxidase inhibitor that reduces the synthesis of uric acid in some species of animals and in man. It is used in the treatment of gout and other clinical states associated with increased levels of uric acid. Other drugs such as 6-mercaptopurine (an antileukemic drug) and azathioprine (an immunosuppressant drug) are normally inactivated by xanthine oxidase. Concomitant repeated administration of allopurinol with 6-mercaptopurine or azathioprine will increase the plasma levels of the latter drugs, and because of the profound suppressant effect of these drugs on the tissue responsible for the formation of blood cells, such interactions can be fatal.

It is practically impossible to determine the effects of all possible drug or chemical combinations. If the mechanism of termination of biologic action of any agent is via an enzyme system, then it is highly probable that other agents which influence the enzyme will alter the toxicity of the first compound. Therefore whenever a drug is a known enzyme inhibitor, toxicity tests should be conducted in combination with other drugs that are dependent on that enzyme system for their inactivation.

Induction of Biotransformation Mechanisms

The total quantity of microsomal drug-metabolizing enzymes can be increased in humans and in higher animals by prior administration of a large variety of chemical substances. Such substances include the anesthetics, such as nitrous oxide, ether, and chloroform; the sedatives, such as barbiturates and urethane; analgesics, such as glutethimide and phenylbutazone; the hypoglycemic agents, such as tolbutamide and carbutamide; and the insecticides, the particularly effective ones being chlordane, DDT, hexachlorocyclohexane, dieldrin, aldrin, and heptachlor.

Induction of enzyme activity usually involves repeated exposure to the inducing chemical. It is usually temporary and lasts from 2 to 4 weeks following the administration of the inducing chemical. When phenobarbital is injected daily for 5 days in anesthetic doses in the dog, the duration and half-life of the drug in the blood are shortened. Four weeks later the half-life of the drug in the blood as well as the duration of anesthesia has returned to normal. Currently it is not clear what the duration of enhanced microsomal enzyme activity may be following repeated daily exposure to organochlorine pesticides which may exist as residues on food consumed by humans or animals.

The phenomenon of chemical-induced enhancement of the activity of enzyme systems that are responsible for their own degradation apparently results in an increased rate of metabolic transformation of the compound. Since metabolic transformation has been shown to result in the formation of more or less toxic products as compared to the parent compound, enzyme induction may be protective to the animal (when detoxication is involved) or detrimental to the animal (when toxication is involved). The induction of enzymes by the administration of chemicals basically represents a mechanism of adaptation by the animal to repeated assault by foreign chemicals. This type of adaptation does not represent a true tolerance, which has been defined as a modified response of the receptor.

It is now recognized that the microsomal mixed-function oxidases of the endoplasmic reticulum are initially inhibited by high concentrations of those compounds which are normally used as substrates for the system. Good examples of such substrates are the barbiturate drugs and the halogenated hydrocarbon pesticides. The initial inhibition of the enzymes by these compounds is believed to be an important step in the subsequent induction of the same enzymes, that is, substrates that are slowly metabolized act as inhibitors and initiate a feedback mechanism that calls for additional *de novo* synthesis of the oxidase proteins. Furthermore, the nature of the induced enzyme is influenced by the type of inducer involved. For example the carcinogenic polycyclic aromatic hydrocarbons such as benzo(a)pyrene and 3-methyl-cholanthrene are slow inducers which initially produce a conformational change in cytochrome P450, converting it to P448; the new enzyme is synthesized *de novo* as cytochrome P448. Currently cytochrome P450 has become recognized as the terminal oxidase that is induced by many drugs and pesticides, whereas cytochrome P448 is induced by recognized carcinogenic agents. Furthermore, cytochrome P450 may actually be destroyed by reacting irreversibly with some highly reactive intermediate radicals that it is responsible for producing. For example, many organic sulfur-containing compounds are desulfated and converted to their oxygen analogs by the microsomal mixed function oxidases; the sulfur which is released forms a stable complex with cytochrome P450, thereby inactivating it.

An important consequence of enzyme induction is that when the microsomal enzymes are induced by one compound and a second compound is then introduced into the animal, if the second compound is metabolized by the same enzyme system its metabolism will also be altered. In this manner the toxicity of the second compound will be either increased or decreased depending on the products of the biotransformation reaction. In chronic drug therapy indirect drug interactions due to this mechanism can seriously alter the therapeutic efficiency and toxicity of other drugs that may be concomitantly administered to the patient.

The foregoing discussion of metabolic transformation mechanisms suggests at least two important possibilities by which chemical-induced toxicity can be altered. The first is that toxicity of a given compound can be distinctly different within members of a species or between species if the suitable enzymatic systems between the test organisms are not identical. The second is that prior exposure to a chemical can alter the toxicity of the same chemical and other chemicals to which the biologic specimen may be exposed on a subsequent occasion.

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CHAPTER 5
Influence of Route of Administration on Systemic Toxicity

Under normal day-to-day conditions humans, as well as essentially all mammals, are exposed to chemicals in the air, in food, and in drinking water. In addition, humans are exposed to a wide variety of agents which are applied to the skin for cleansing or cosmetic reasons or ingested for therapeutic or recreational purposes. The chemical and physical properties of each compound largely determine the route by which exposure can occur. For example, although solids can be suspended in the air as "dusts," vapors and gases would be the most common, readily available agents for inhalation via the respiratory route. Xenobiotic materials which are dissolved or suspended in water would be ingested by the oral route, in which case absorption could take place through the gastrointestinal tract. Thus the percutaneous (or dermal), the oral (or gastrointestinal) route, and the inhalation (or respiratory route) are the common routes by which xenobiotic agents gain access to biologic systems in animals. However, under experimental conditions in the laboratory in which the toxicologist wishes to produce and study harmful effects of chemicals, additional routes of exposure are commonly used. These routes involve a group of techniques by which agents are injected into various body compartments. In this case the common routes of exposure involve injection directly into the blood (intravenous route), into the abdominal fluid (intraperitoneal route), beneath the skin (subcutaneous route), into the spinal subarachnoid fluid (intrathecal route), or into a muscle (intramuscular route). The route of administration of an agent determines the barriers that the agent will encounter in regard to absorption, distribution, and biotransformation. Although the route of administration has little to do with the qualitative nature of the toxicity of a compound, it can greatly influence the quantitative toxicologic response to an agent; that is, it can alter the slope and position of the dose–response curve.

PERCUTANEOUS ROUTE

The skin of humans is fundamentally a modified membrane comparable to the mucous membranes of the mouth and gastrointestinal and respiratory tracts. It acts as a barrier to the transfer of xenobiotics in a manner similar to the other mucous membranes. It consists of two layers, an outer epithelial layer known as the epidermis and an underlying connective tissue layer known as the dermis (or corium). The epidermis consists of continuous multilayers of cells pierced only by the orifices of the hair follicles and sweat gland ducts. The sweat glands and hair follicles are embedded in the dermis. The sebaceous glands generally open into the hair follicles. The effectiveness of the skin as a barrier to the transfer of xenobiotics varies considerably at different sites on the body and for different xenobiotics.

When chemicals are applied to the skin toxicity may be manifested at the site of application and the agent may be translocated through the skin, resulting in adverse systemic effects. In general it is clear that the amount of any compound that passes through the skin is dependent on the applied dose, the time over which the agent is in contact with the skin, the concentration involved, and the location as well as the surface area involved. In addition a compound may be exposed to multiple enzymes in the skin that may transform the initial compound into products having different chemical properties and toxicities. When these factors are determined for a given agent dermal absorption rates can be predicted. This has been very successfully utilized in the drug industry to administer such drugs as nitroglycerin and scopolamine which are incorporated in patches that are applied to the skin. A single patch is designed to supply the drug in therapeutic amounts slowly and uniformly over a 24-hr period.

The barrier properties of whole skin vary with the site of application and with the properties of the chemical which is applied, both in the same species and in different species. Pig skin appears to have a higher diffusion rate for water than does rat or guinea pig skin. As an example Table 5.1 indicates the species variation in percutaneous toxicities of two organic phosphates. Furthermore, the integrity of the skin barrier can be altered by application of chemicals which specifically produce a breakdown in the surface layer, an example being formic acid. Methyl and ethyl alcohol, hexane, and acetone applied to the skin and washed off may be used as solvents for the normal lipids in the skin resulting in a moderate change in permeability. A marked change in skin permeability can be produced by application of chloroform–methanol (2:1) mixture. The normal rat skin can be penetrated by a variety of chemical agents. Simple organic amines such as propyl, butyl, and pentyl amines have been shown to penetrate rat skin at a rate that increases linearly with concentration. These amines penetrate the skin only in an unchanged state, so that below the isoelectric point where the amines exist as cations, penetration through the skin is poor.

The physicochemical properties of the substance under consideration are the principal determining factors with respect to percutaneous absorption of the compound. In general, it may be thought that gases penetrate quite freely through the epidermal tissues, liquids less freely, and solids which are insoluble in water or lipids probably are incapable of penetrating to a significant degree. Solids that are soluble in the secretions of the skin may dissolve in the secretions to a variable extent and thereby be put into solution. Penetration of materials through the skin is time-dependent, and this can be demonstrated by the application of occlusive bandages to prevent loss of the material from the site of application.

TABLE 5.1 Relative Percutaneous Toxicities of Two Organophosphorus Compounds Tested in Eight Animal Species[a]

Species	Compound A[b]	Compound B[b]	B/A
Rabbit	1.0	5.0	5.0
Pig	10.0	80.0	8.0
Dog	1.9	10.8	5.7
Monkey	4.4	~13.0	~3.0
Goat	~3.0	~4.0	~1.3
Cat	0.9	2.4	2.7
Mouse	6.0	~9.2	~1.5
Rat	17.0	20.0	1.2

[a] Data from McCreesh, A. H.: Percutaneous toxicity. *Toxicol. Appl. Pharmacol.* 7:20, 1965.

[b] All values expressed as ratio of the LD_{50} of that compound to the rabbit LD_{50} of Compound A.

Although it is not clear to what extent lipid solubility of the compound is important, it is apparent that both water and lipid solubility influence percutaneous penetration of a compound. The insecticide DDT is considerably more soluble in lipids than it is in water. It is also more poorly absorbed from the skin than it is from the gastrointestinal tract. The comparative LD_{50}'s for DDT in rats for the oral and dermal routes of administration are 118 and 2510 mg/kg, respectively. In contrast to this the insecticide Isolan is quite soluble in water, is well absorbed from the skin, and is more toxic by dermal than by oral administration to rats. Prominent among the lipid-soluble compounds readily absorbed into the skin are phenol and phenolic derivatives; hormones such as estrogen, progesterone, testosterone, and desoxycorticosterone; vitamins D and K; and organic bases such as strychnine and nicotine. As far as polarity is concerned, it appears that nonpolar compounds pass through the skin more readily than ionic materials, but not exclusively so. Salts of some alkaloids may pass freely through the skin.

A variety of factors such as pH, extent of ionization, molecular size, and water and lipid solubility are all involved with the transfer of chemicals through the skin. Local factors such as temperature and blood flow to the site will influence the rate of absorption and therefore the percutaneous toxicity of potent chemicals.

INHALATION ROUTE

Exposure to chemicals in the atmosphere is accomplished by unavoidable inhalation of such agents unless devices are used to remove the atmospheric contaminants before they enter the respiratory tract. However, in order for any particular chemical contaminant to reach the alveoli of the lungs, it must be a gas, a vapor, or of sufficiently proper particulate size so that it is not removed in the airway to the lungs. Although some atmospheric contaminants present little more than a nuisance, others are capable of inducing local as well as systemic toxicity. The actual and potential hazards associated with exposure to chemicals via the respiratory tract are particularly evident in regard to industrial working environments and in regard to pollution of atmospheres in urban areas of high-density human populations.

Because of the widespread use of a large number of chemicals in industrial working environments, it is not surprising that the atmosphere in which people work is more or less contaminated with a variety of such chemicals. It has therefore become necessary to establish some standards regarding the limits of contamination of the atmosphere which would be considered safe. The data necessary to establish a maximum safe concentration of a chemical in the atmosphere for humans who are exposed over an 8-hr working day are only rarely obtainable. Those values that are available for specific chemicals represent estimations based on information obtained by experience in industry and by experiments on humans and animals.

The American National Standards Institutes (ANSI) initially recognized and developed some guidelines for use in industrial toxicology regarding safe exposure concentrations to some chemicals in the work environment. These were soon adopted by the American Conference of Governmental Industrial Hygienists (ACGIH) who initially published what are now recognized as Threshold Limit Values (TLVs). Initially a TLV was a maximum concentration of an agent in air that was believed to be "safe" for exposure in the working environment for a lifetime. In the United States in 1970 the National Institute of Occupational Safety and Health (NIOSH) Act emphasized the need for some regulatory standard consensus of opinion regarding safe levels for inhalation exposure to chemical contaminants in the workplace. NIOSH adapted TLVs as the legal permissible levels (PELs) and issued criterion documents for many of the common industrial air contaminants. Actually, TLVs are reviewed annually by the ACGIH committee but have no legal status, whereas PELs have legal status in the United States and can be changed only by legislative action. More recently the degree of sophistication of TLVs has greatly improved so that ACGIH now lists TLVs as time-weighted averages (TWAs) and may include short-term exposure limits (STELs) and a ceiling concentration limit (TLV-C). STELs represent a maximum concentration limit for a period of not more than 15 min. As the term indicates, TWAs are the average allowable values over an 8-hr period of time and take into account periodic exposures above and below the average. Although PELs have legal status they have no more scientific validity than TLVs. For details on this subject the reader is referred to the National Research Council publication in 1983 on "Risk Assessment in the Federal Government: Managing the Process" and in 1994 on "Science and Judgment in the Risk Assessment."

Inhalation of toxicants is an unintentional means or route of exposure to xenobiotics whether it involves an industrial or urban environment. Consequently it has become a subject of considerable public interest and federal regulatory action. The current extremely conservative policies of the federal regulation agencies in the United States have resulted in questions about the ability of both science and the regulatory agencies to accurately evaluate the threat of harmful effects from inhalation of toxicants. This book has stressed that from a scientific perspective toxicity is a graded effect, and that there is no sharp line that distinguishes between harmful and safe doses for any xenobiotic agent. Further consideration of this subject is beyond the scope of this book, which acknowledges the respiratory route as a principal route of exposure to gases, vapors, and even particulate materials. This has resulted in a vast body of literature with its own terminology, as well as controversial regulatory legislation.

TLVs serve a useful purpose in that they represent a gross classification of the relative harmfulness or safeness of a large variety of compounds that become atmospheric pollutants in industry. Their use for any other purpose is grossly erroneous, if one accepts the concept that all harmful effects of chemicals are graded responses that are dose-dependent and that there is no exact concentration of a chemical above which that chemical is harmful or below which it is safe. The only way in which any value which represents a safe value for human exposure can be established is through extensive experience; even then, such a value would not represent a "limit," but would only represent an estimated safe level for exposure. The 6th edition (1991) of *TLV Documentation for Chemical Substances in the Work Environment* lists data for approximately 700 compounds. Some examples of the TLVs listed are given in Table 5.2.

ORAL ROUTE

The oral route is probably the third most common means by which a chemical enters the body. The gastrointestinal tract in the experimental

TABLE 5.2 A Selected List of Threshold Limit Values

Compound	TLV–TWA (ppm)[a]	TLV–STEL (ppm)[a]
Bis (chloromethyl) ether	0.001	—
Toluene-2,4-diisocyanate	0.005	0.02
Methyl isocyanate	0.02	—
Nickel carbonyl (as Ni)	0.05	—
Acrolein	0.1	0.3
Chloropicrin	10	—
Hexane	50	—
Turpentine	100	—
Methyl alcohol	200	250
Gasoline	300	500
Acetone	750	1000
Butane	800	—
Ethyl alcohol	1000	—
Carbon dioxide	5000	30,000

Note. From *Documentation of TLVs and Biological Exposure Indices*, 6th ed., American Association of Governmental Industrial Hygienists, 1991.

[a] Parts of vapor or gas per million parts of air volume at 25°C and 760 mm Hg.

animal may be viewed as a tube going through the body, starting at the mouth and ending at the anus. Although it is within the body, its contents are essentially exterior to the body fluids. Therefore, chemicals in the gastrointestinal tract can produce an effect only on the surface of the mucosal cells that line the tract, unless absorption from the gastrointestinal tract takes place. Caustic or primary irritant agents, such as strong alkalis and acids or the phenols, in adequate concentration can result in a direct necrotizing effect on the mucosa of the tract. Most orally administered chemicals can otherwise have a systemic effect on the organism only after absorption has occurred from the mouth or the gastrointestinal tract.

Although alcohol, nitroglycerin, and even several of the steroid drugs can be absorbed directly through oral mucosa, they must be retained in the mouth for a suitable time interval if any significant absorption is to take place. Under ordinary conditions, chemicals or even foods remain in the mouth and esophagus for too short a time to permit any significant degree of absorption. Rather, the first site from which orally administered chemicals can be effectively translocated is the stomach (or the rumen in those species that have such organs).

The effect of the special condition of pH in the stomach and the influence of pH on the ionization of the weak organic acids and bases have been described in the previous chapter. In the stomach the chemical comes in contact with preexisting stomach contents (such as food particles and gastric mucin) and secretions (such as pepsin, renin, and gastric lipase) in addition to hydrochloric acid. If the chemical was to be absorbed, react with, or act as a substrate for any of these components of the gastric contents, the amount of free chemical would be altered, thereby leading to an altered absorption rate of the agent. Products of reactions that take place in the stomach may be more or less readily translocated or more or less toxic than the parent compound. As the orally ingested compound is carried from the stomach into the intestine, the pH is again shifted and the chemical is mixed further with additional agents such as the food residues, bile, and the additional enzymes in the pancreatic juice.

The toxicity of orally administered chemicals may vary with the frequency with which they are given, and with the conditions under which they are given (that is, whether they are mixed with food or given on an empty stomach). Studies regarding two examples show that the toxicity of a drug given by oral gavage (introduction via stomach tube) may be considerably different from the same drug administered by admixture in the diet. The drugs used were Dimethline (a respiratory stimulant) and Dixyrazine (a phenothiazine type drug used as a tranquilizing agent). Dimethline possessed much greater lethal toxicity when administered by gavage than when given in the diet, whereas Dixyrazine showed the opposite behavior. When Dimethline was administered to fasted rats by gavage, the LD_{50} was found to be about 12 mg/kg. When the rats remained unfasted, this value was 30 mg/kg. With repeated daily gavage, 5 mg/kg was tolerated, whereas 10 mg/kg was fatal. The symptomology was the same in all cases. When the same drug was administered in the diet, the rats tolerated 100 mg/kg, which is 10 times the lethal gavage level. Further studies indicated that the drug remained unchanged chemically in the diet, that acute toxicity was less in unfasted than in fasted rats, that by employing divided dose procedures the normally acute lethal dose could be tolerated for several weeks. Similar studies performed with Dixyrazine indicated that it was appreciably more toxic when administered in the diet than when given by gavage. In this case, analysis of the test diet showed that 60% of the chemical underwent degradation when mixed with the diet, and it appeared that the products of degradation were more toxic than the original material.

An example in which oral toxicity is greater when the substance is administered in the feed than when given by stomach tube or gavage is the case of griseofulvin; administered by the oral route, this is normally a substance of low toxicity. When this substance is added to the feed of mice, it leads to pathologic changes in the liver, although such changes are not encountered in rats, guinea pigs, or rabbits. In mice, the addition of griseofulvin to the diet resulted in changes in the biliary tract in 10 to 12 days and tumors of the liver at 140 days. When an equivalent dose of griseofulvin was given as a single dose by stomach tube, only slight liver damage was observed even after a period of 122 days. When the single daily dose was divided into nine fractional doses given at 1-hr intervals during each day, microscopic changes in the liver did occur on the third and fourth day. Therefore, it would appear that if the objective of the test is to obtain toxicity, it would be preferable to administer the griseofulvin in the feed rather than by gavage.

Following oral administration of a compound to animals, absorption of the agent from the gut necessarily involves translocation of the agent either to the lymphatic system or to the portal circulation. Those agents that appear in the portal circulation are carried directly to the liver. A large number of foreign compounds that appear in the blood following their absorption from the gut are known to be excreted by the liver into the bile. Thus, a cycle involving translocation of the chemical from the intestine to the liver and to the bile and back to the intestine is established. This cycle is referred to as the *enterohepatic circulation.* Some compounds simply diffuse from the blood into the bile, whereas others are actively excreted into the bile. For example, the bile salts and Bromsulphalein appear in the bile in concentrations from 10 to 1000 times greater than the concentrations of the compound in the blood, whereas compounds such as glucose appear in the bile in a concentration less than that which is present in blood.

Furthermore, the liver may biotransform or conjugate a chemical, for example with glucuronide or sulfate, and excrete the conjugate into the bile where the metabolite is then carried to the intestine, and reabsorbed back into the portal circulation. The drugs madribon and chloramphenicol appear to be actively excreted in the bile as the glucuronides and the conjugates are then hydrolyzed in the gut to yield the initial form of the drugs, which are in turn absorbed again into the portal circulation and thereby enter the cycle of the enterohepatic system. Studies of the enterohepatic circulation of a series of nitro- and hydroxybenzoic acids in rats and have shown that both molecular size and degree of conjugation influence biliary excretion of the compounds. Several of the chlorinated hydrocarbon insecticides are also known to undergo enterohepatic circulation in various laboratory animals. Prominent among such insecticides are DDT (2,2-bis [parachlorphenyl] 1,1,1-trichlorethane), aldrin, dieldrin, and methoxychlor. The liver appears to be an important site for biotransformation of DDT to DDE (2,2-bis [parachlorphenyl] 1,1-dichlorethane) and other metabolites, a process which leads to the excretion of DDE in the bile. This mechanism is the principal source for the appearance of DDT metabolites in the feces. Surgical obstruction of the biliary duct in rats that are given isotope-tagged DDT leads to increased excretion of the isotope in the urine, indicating that the enterohepatic circulation also consitutes a mechanism of termination of action of this compound.

Oral administration of chemicals that are rapidly absorbed from the gastrointestinal tract would theoretically expose the liver to concentrations of the agent that would not be obtained by other routes of administration. Furthermore, if a compound entered the enterohepatic cycle, at least a portion of the compound would be localized in the organs involved in the cycle. Compounds that are known to be toxic to the liver would be expected to be more toxic following oral administration on repeated occasions, whereas their administration by other routes may be less hazardous. An example of this is in the use of thiopental. This drug is a short-acting thiobarbiturate which is commonly administered intravenously to produce anesthesia. Intravenous use of this drug has not been noted for its hepatotoxicity. The compound is readily absorbed from the stomach and intestine, but repeated use of the compound by the oral route of administration in experimental animals is likely to produce degenerative changes in the liver; therefore, its use by the oral route is not recommended in humans.

PARENTERAL ROUTES

Introduction of chemicals into the organism by means of injection of the chemical from a syringe through a hollow needle at specific sites in the animal is a common procedure used in the administration of drugs. By this means, the natural body orifices are bypassed and specific amounts, or doses, of chemicals may be introduced into the animal. These routes of administration are collectively called the parenteral routes of administration of chemicals. They consist of administration of chemicals by injection into the skin (intrademal), beneath the skin (subcutaneous), in the muscle (intramuscular), into the blood of the veins (intravenous), or into the spinal fluid (intrathecal). Specific agents may on infrequent occasions be administered into the blood in the arteries (intraarterial), into tumors, or into the chest fluid (intrapleural). In laboratory animals, the injection of chemicals into the abdominal fluid (intraperitoneal) is a very common procedure, whereas this is only done in humans on extremely rare occasions. In the laboratory, it is even possible to inject solutions into single cells (intracellular) by use of micropipettes.

It is apparent that the most rapid means of achieving a high concentration of a chemical within a given tissue is to introduce the chemical directly into that tissue. Whereas intravenous administration of a chemical bypasses the biologic barriers presented by the normal body surface or orifices, other parenteral routes may impose additional barriers to translocation of the chemical. In the latter case, the chemical remains at its site of deposition until absorption or diffusion carries it to the sites in the animal where it can be chemically modified or excreted. Therefore, except for a local action at the site of injection, parenteral administration of chemicals still necessitates translocation of the agent in the organism if the chemical is to reach distant specific receptor sites.

Lethal toxicity of a chemical may be dependent or variously independent of the route of parenteral administration. Examples of compounds for which the LD_{50} is dependent and independent of the route of administration are given in Table 5.3. In general, it may be assumed that the intensity of the toxicity of a compound will be different following different parenteral routes of administration if the rate at which translocation of the compound takes place is influenced by the injection route. For example, if the rate of absorption from the site of administration is less than the rate of excretion (or termination of action of the compound), there will be little opportunity for accumulation of a biologically effective systemic concentration of the compound. In contrast to this, if the rate of termination of action of the compound is less than the rate of absorption from the site of administration, it would be reasonable to expect the compound to achieve systemically effective concentrations.

These facts are utilized in the development of drug formulations when it is desired to achieve a constant systemic concentration of a drug over a period of time. This condition is practically accomplished by developing a

TABLE 5.3 Effects of Administration of Compounds in Which Lethal Toxicity Is Independent (Isoniazid), Partially Dependent (DFP and Pentobarbital), and Completely Dependent (Procaine) on Route of Administration

Route of administration	Procaine[a] (mouse)		Isoniazid[a] (mouse)		DFP[b] (rabbit)		Pentobarbital[a] (mouse)	
	LD_{50} (mg/kg)	Ratio (X/IV)	LD_{50} (mg/kg)	Ratio (X/IV)	LD_{50} (mg/kg)	Ratio (X/IV)	LD_{50} (mg/kg)	Ratio (X/IV)
Intravenous	45	1	153	1.0	0.34	1.0	80	1.0
Intraperitoneal	230	5	132	0.9	1.00	2.9	130	1.6
Intramuscular	630	14	140	0.9	0.85	2.5	124	1.5
Subcutaneous	800	18	160	1.0	1.00	2.9	130[c]	1.6
Oral	500	11	142	0.9	4 to 9	11.7 to 26.5	280	3.5

[a] Data from Barnes, C. D., and Eltherington, L. G.: *Drug Dosage in Laboratory Animals.* University of California Press, Berkeley, CA, 1964.
[b] DFP, diisopropylfluorophosphate. Data from Spector, W. S. (Ed.): *Handbook of Toxicology,* Vol. I, Acute Toxicities. W. B. Saunders, Philadelphia, 1956.
[c] Personal data from author's laboratory.

formulation of a drug which only permits slow liberation and absorption of the drug following intramuscular or subcutaneous administration of the preparation. A good example of such a preparation is procaine penicillin as compared to penicillin. The former preparation permits slow absorption of the penicillin from intramuscular sites as compared to the latter preparation which is rapidly absorbed. The natural counterpart of this mechanism is the buffering effect involved in adsorption of drugs to plasma protein, thereby limiting the quantity of free active drug in the circulation regardless of the route of administration of the drug.

Intraperitoneal injection of chemicals represents a selective site of administration in which an absorbable chemical will first be translocated to the liver via the portal circulation. This is possible because the major venous blood circulation from the abdominal contents of mammals is effected via the portal circulation. Therefore, an intraperitoneally administered compound is subjected to the special metabolic transformation mechanisms existent in the liver, as well as to the possibility of excretion of the compound in the bile before it gains access to the remainder of the animal. A hypothetical compound which is selectively toxic to any system in the animal other than the liver, and which is detoxified in the liver, would be expected to have a greater toxicity following subcutaneous or intravenous administration than following intraperitoneal administration. An example of such a compound is the organic phosphate Soman (methyl pinacolyl phosphonofluoridate) for which the LD_{50}'s in the mouse are, respectively, 0.165 and 0.425 mg/kg by the subcutaneous and intraperitoneal routes of administration. The LD_{50} values of compounds that were not biotransformed or excreted into the bile would not be expected to be different by the intraperitoneal as compared to the subcutaneous or intravenous routes of administration unless other factors, such as differences in absorption from the three sites, were involved. Therefore, it is possible to predict some information regarding translocation, deposition, inactivation, or site of excretion from comparative evaluation of LD_{50}'s of a given compound which are determined by various routes of administration.

The specific biologic barriers that are effective in blocking translocation of compounds in an animal effectively protect certain tissues from exposure to a large number of foreign chemicals, although the chemicals may be present in the blood of the animal. An excellent example of this is the blood–brain barrier in mammals, which inhibits translocation of quaternized nitrogen-containing compounds from the blood to the central nervous system. Intrathecally administered chemicals bypass the blood–brain barrier, thereby permitting the brain to receive concentrations of the agent that could not be obtained by any other route of administration. Certain antibiotic drugs are therefore administered by direct intrathecal injection for treatment of infections of the brain and spinal cord. Administration of proper volumes as well as amounts of local anesthetic agents, such as procaine or pontocaine solutions, by intrathecal administration produce spinal anesthesia, whereas the same compounds are ineffective as spinal anesthetic agents when given by other routes. Thus, toxicity following intrathecal administration varies as compared to other routes of administration depending upon the site of action of the agent under consideration and on the barriers to translocation of the compound.

The intravenous route of administration of liquids and the inhalation route for gases and vapors achieve rapid systemic distribution of the compound throughout the animal. The compound reaches all organs of the animal in periods of time limited only by the time required for the blood to circulate and the time necessary for translocation of the compound from the capillaries to the extracellular fluid. Compounds with rapid biologic action therefore generally show greater toxicity following intravenous administration than when they are given by other parenteral routes.

The foregoing discussion has been oriented toward the influence of the various routes of administration of chemicals on toxicity in the species of animals commonly utilized in the toxicology laboratory or in man. Comparable variations in routes of administration are adapted to other species, such as fish, birds, or insects, but are uncommon except for specific laboratory investigational work. The usual route of administration of chemicals to these latter species involves exposure via the environment of the species. Comparisons of toxicities between species of animals by similar routes of administration for various foreign chemicals frequently shed light on mechanisms of action, mechanisms of biotransformation, and mechanisms of excretion of chemicals of interest in toxicology.

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CHAPTER 6
Genetic Factors That Influence Toxicity

In most modern toxicology texts the subject of "Genetic Toxicology" deals entirely with the interaction of chemical agents with the hereditary mechanism. In this context Genetic Toxicology is a new specialty that has received recognition only during the past 30 years, and it is concerned with the demonstration of chemical-induced genetic damage. The discipline has developed both animal and clinical laboratory test methods to detect mutants as well as the mutations that they produce. These methods are described in Chapter 13. Chemical-induced mutagenicity is unique in toxicology because it can lead to acute toxicity in contemporary generations as well as in the subsequent offspring of affected subjects.

In this chapter the subject of Genetic Toxicology will be presented in a broader context; that is, it will consider not only chemicals acting as mutagens that damage the hereditary system alone, but also effects of variations in the "normal" genetic code as a cause of toxicity when man is exposed to other nonmutagenic xenobiotic chemicals.

THE GENETIC MECHANISM

The morphologic and biochemical makeup of biologic systems is determined by the heredity of the individual members. The basic units of inheritance are the genes, which are submicroscopic entities located at various areas on the chromosomes. Various species of animals possess various numbers of such gene-containing chromosomes which always exist in pairs (Fig. 6.1). Humans, for example, have 23 pairs of chromosomes. Each member of a pair is an autosome; therefore the human has 44 autosomes plus two sex chromosomes. In the female, each of the two sex chromosomes possess the "X" or sex-determining gene. In the male, one of the sex chromosomes contains the "X" gene and the other chromosome contains the "Y" gene. Thus, females possess the "XX" chromosomes and males

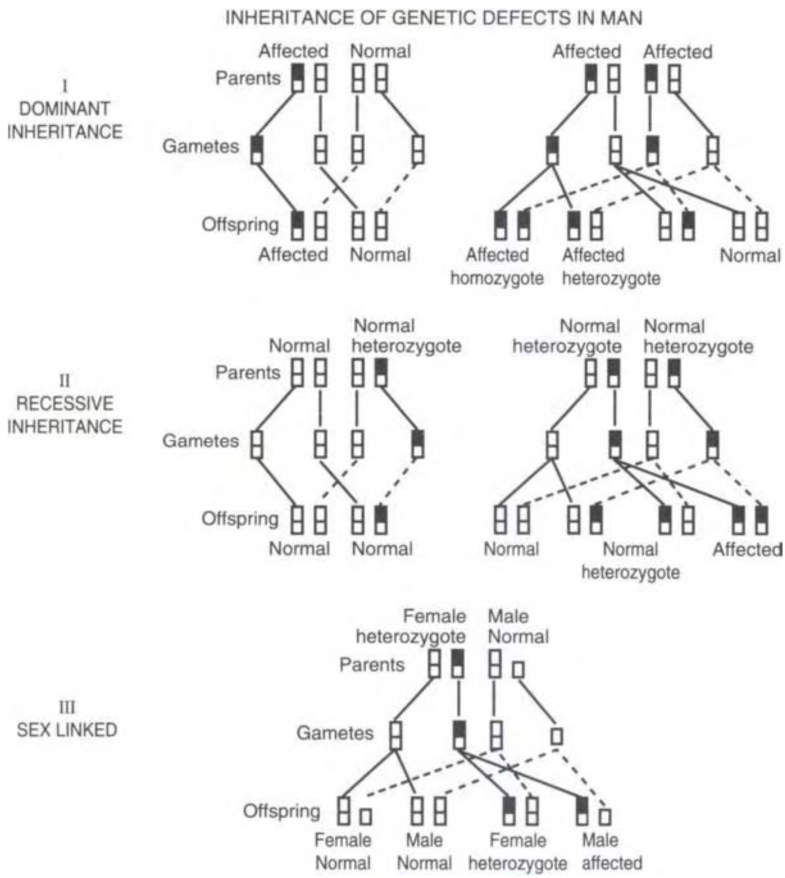

FIGURE 6.1 According to Hsia, D. Y., *Inborn Errors of Metabolism,* Year Book of Medical Publishers, Chicago, 1959, pp. 19–23. Examples consist of heterozygous state of the abnormality in which the paired chromosomes contain one normal gene (⊟) and one abnormal gene (▆).

possess the "XY" chromosomes. Those genes that are located at the same points on paired chromosomes are known as alleles.

A specific single gene or many genes may be responsible for a specific trait of the individual. If many genes are involved, the condition of polygenic inheritance for the specific trait is said to exist. Abnormalities that manifest themselves as altered morphology or altered ability to direct the synthesis of proteins originate as mutations. Since the normal is only apparent when a mutation occurs, a mutation (altered gene) may involve the ability of the progeny to carry on a vital function and such progeny would not survive. However, a mutation that affects the genetic template for the formation of a nonvital enzyme system may permit the progeny to survive, and may be manifested by the ability of the organism to produce either a deficient enzyme or a completely inactive enzyme. Such a genetically induced deficiency may go undetected until the deficient system is suitably challenged.

Drugs and chemicals represent possible challenging agents for the determination of the existence of certain enzymes within the body if these enzymes are involved in metabolic alteration of the drug or chemical. The origin of such mutations is not understood, although there is some evidence that a mutation can be induced by exposure of the reproductive cells to high-energy radiation or to formaldehyde or nitrogen mustard. The mutated gene may be dominant or recessive with respect to its ability to result in a manifested abnormality in the biologic organism. Figure 6.1 depicts the possible conditions that can exist when a single abnormal gene is considered in regard to inheritance of disease in man. Such concepts may be applicable to deficient enzyme systems that may be involved in metabolic transformation of chemicals in the intact biologic specimen. It is estimated that humans have at least 50,000 expressed genes; currently, some information is available for about 5000, and about 1900 have been mapped in terms of their chromosomal location. For information on the current status of Mendelian inheritance in man the reader is referred to McCusick, V. A., *Mendelian Inheritance in Man: Catalogs of Autosomal Dominant, Autosomal Recessive, and X-Linked Phenotypes,* Johns Hopkins Press, 1992.

CHEMICALS AS MUTAGENS

The evaluation of thousands of chemicals in the laboratory for their ability to produce damage to DNA has demonstrated that many agents possess this capability. Furthermore, it is generally recognized that it is highly probable that many carcinogens are mutagens and that many but not all mutagens are carcinogens. In addition, both of these classes of toxicity can be induced in cells which subsequently can undergo repair to normal. The recent human genome project to define the amino acid sequence of the genetic code has helped to increase the total body of knowledge about the genetic basis of human disease.

Currently it is believed that mutations of any cell type (somatic or germ) can be produced by some xenobiotic chemicals. Such mutations are subject to repair (removal of the damage) and may not result in permanent changes in the genetic code. Mutations in somatic cells that are lethal to the cell (such as those that are produced by cancer chemotherapeutic agents) are very useful as a therapeutic approach to the treatment of cancer. Nonlethal mutations in germ cells which do not undergo repair present the possibility of transfer to subsequent generations. Only in the past few years have the techniques in molecular biology become available to supply data for study by epidemiologic methods in humans. Consequently, when the simple, commonly encountered xenobiotic agent ethylene oxide was demonstrated in 1990 to produce genetic changes in male mouse germ cells, it supplied an impetus to evaluate the germinal changes (in terms of risk to humans) associated with occupational and environmental exposure to the compound.

Basically, current knowledge from animal and cellular experiments demonstrates that DNA of somatic and germ cells can be damaged by a variety of xenobiotic agents and that chromosomal and point mutations result from replication by the damaged template. To date, there are no conclusive examples of inheritable xenobiotic-induced disease in humans. Considerable effort is directed toward a better understanding of the risk involved when humans are exposed to "mutagenic" agents. In constrast all chemical-induced carcinogenic agents that have been demonstrated in man have also been demonstrated in animals.

PRINCIPLES OF GENETIC-INDUCED CHEMICAL TOXICITY

Termination of biologic action of chemicals in an organism is accomplished by excretion, by metabolic transformation processes, or by deposition mechanisms. Of the three processes, only excretion permanently removes the chemical from the body so that it can no longer produce a biologic effect. In contrast to this, metabolic transformation of a compound may lead to the formation of a more or less potent toxicant.

In cases in which the metabolic transformation process would convert the chemical to a *more* toxic compound, theoretically it would be better for the organism to have a deficiency of the enzyme involved, to ensure that *less* of the more toxic compound would be produced. In cases in which the metabolic transformation process would convert the chemical to a *less* toxic compound, a deficiency of the enzyme would be detrimental, because the organism then would not be able to remove the chemical by metabolic transformation, but would have to rely on other processes.

Since the enzymes involved in metabolic transformation of chemicals exist according to the genetic templates characteristic of each member of a population of organisms, genetic defects in members of a species may result in a deficiency or complete lack of certain enzymes. Such genetic defects within members of a species have been shown to be responsible for some specific types of toxicities from chemical agents. These forms of toxicity appear in the affected genotypes, and can be shown to occur at the frequencies stipulated by the laws of genetics. Genetically controlled deviations in individuals within a population therefore may be the reason certain "idiosyncratic" toxicities occur in a few members of a supposedly homogeneous population. Genetic deviations also represent one mechanism involved in biologic variation as it is manifested in relation to chemicals. The study of the genetically controlled factors that influence the pharmacologic actions of drugs has been termed "pharmacogenetics," but the usual example in pharmacogenetics involves examples of toxic effects of drugs as they are seen in relatively small numbers of a total population.

DISCOVERY AND CLASSIFICATION OF GENETIC-INDUCED CHEMICAL TOXICITY

The use of statistical procedures for the evaluation of data obtained on the effects of drugs and other chemicals tends to obscure the recognition of deviations in response of individual contributors to the data. The investigator may observe an occasional animal which deviates markedly from the majority of his animals in response to a chemical. Such deviants may be disregarded and the data may be discarded because of a variety of unscientific reasons, or the data may be included in the final data. The experimental investigator conventionally applies statistical tests to determine whether his data are sufficiently homogeneous so that statistical methods of analysis of the data are applicable. Ordinarily, unless the deviant data are sizable, such statistical tests for homogeneity of the data may not detect the deviants. For example, the crude data that are acquired for the determination of the LD_{50} of a chemical compound almost never form a true, uniform Gaussian curve when plotted as a frequency–response relationship (Fig. 6.2). Rather, a skewed curve is obtained, and by statistical manipulation the curve is subsequently normalized to give the normal Gaussian form.

The occurrence of a few mutant animals within the group under study may be statistically unimportant because of the small number of animals involved in the study. The discovery of the existence of such mutants

Doses tested (arbitrary units)	1	2	3	4	5	6	7	8	
Animals responding (number)	0	2	1	6	15	14	8	4	50
Animals responding (percent)	0	4	2	12	30	28	16	8	100

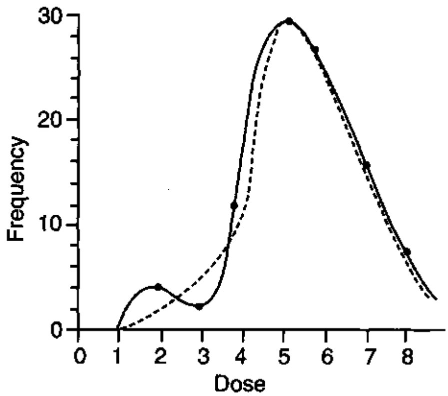

FIGURE 6.2 Graphic representation of data obtained with a hypothetical anesthetic agent in which the dose of the agent required to produce respiratory paralysis was determined in mice. The solid line represents the true curve. The broken line represents the best fitting skewed Gaussian distribution curve.

(abnormal responders) has resulted from recognition that the deviant animals may represent a population separate from the normals. The deviant animals represent mutants and the mutation may be the cause of toxic effects resulting from the administration of agents that are relatively innocuous when given in the same dose to normal members of the population. When such mutants occur and the data are plotted as a frequency–response curve, multiphasic curves are obtained. When such diphasic or triphasic curves are obtained, they are highly suggestive of the existence of mutant animals within the group involved in the study.

Although a mutant that results in the formation of a defective enzyme system may be responsible for the failure of the biologic organism to detoxify a chemical agent, it is entirely possible that the mutant could result in the development of a new, or at least a "more efficient," detoxifying enzyme. In the latter case, if the more efficient enzyme was responsible for detoxication of the chemical agent, then the mutant would protect the organism from the toxicity. If the chemical involved was a drug, then the overall consequence would be not only resistance to any toxic effect of the drug, but also resistance to the therapeutic effect of the drug. However, if the mutant produced a more efficient enzyme and the function of the enzyme was activation (conversion to a more active form) of the agent, then the biologic organism would be liable to exhibit a greater therapeutic, and possibly a greater toxic susceptibility to any given dose of the drug involved. It is also not necessary that the mutant be limited to an enzyme or an enzyme system; rather the mutant can be in the form of an abnormal protein. Such a protein would be one in which the normal amino acid sequence, which identifies the protein, is disrupted by the presence of an incorrect amino acid somewhere along the sequence. If the mutant protein constitutes a target (or receptor) for the foreign chemical, then the protein may exhibit a greater or lower, or even an absence of, affinity for the foreign chemical. Some examples of altered responses to chemical agents due to genetic-based mechanisms are listed in Table 6.1.

For the purpose of categorizing the various toxicologic responses that are the result of mutant enzymes or proteins, it is convenient to identify the toxicity as being (1) the result of a *prolongation* of the action of the agent to the point that it becomes a distinctly detrimental response and there is no accumulation of the agent in the biologic system; (2) the result of repeated exposure to the drug whereby the agent *accumulates* in the biologic system so that concentrations of the agent are reached that will produce toxicity; or (3) the result of a change in the *sensitivity* of the receptor system so that the response represents an altered susceptibility to the chemical agent. This classification of genetic-based toxicity is described more fully below and is followed by some examples.

1. *Prolongation* of the action of a chemical as the result of a deficient biotransformation mechanism, in which case the administered chemical is the primary toxic agent. This condition is exemplified by the prolonged succinylcholine-induced apnea as observed in humans who have a genetically deficient cholinesterase enzyme.

2. *Accumulation* of the chemical as a result of a genetically deficient or absent metabolic transformation mechanism (enzyme system), in which case the administered chemical is the primary toxic agent. This condition would readily occur with drugs that are given in multiple doses at specified intervals. Examples of this condition are the variations between individuals with respect to the acetylation of isoniazid and variations with respect to metabolism of Dicumarol in various members of a given species.

3. *Hypersensitivity*, involving a defective enzyme which causes a borderline level of activity with borderline symptoms of enzyme deficiency when the administered chemical is the primary toxic agent. Examples of this

TABLE 6.1 Altered Responses to Chemicals Due to Genetic-Based Mechanisms

Agent involved [type of drug(s) or chemical(s)]	Mutant involved		Reaction involved [detoxication (D) or activation (A)]	Consequence	Mechanism [prolongation (P), accumulation (A), sensitivity change (S)]
	Type of enzyme or protein	Deficient (D) or more efficient (ME)			
Succinylcholine	Cholinesterase	D	D	Prolonged apnea	P
Succinylcholine	Cholinesterase	ME	D	Drug resistance	
Isoniazid	Acetyl transferase	D	D	Neuropathy	A
Isoniazid	Acetyl transferase	ME	D	Drug resistance	
Hydralazine	Acetyl transferase	D	D	—	
Phenelzine	Acetyl transferase	D	D	—	
Sulfamethazine	Acetyl transferase	D	D	—	
Nitrites	Methemoglobin reduction	—	—	Methemoglobinemia	S
Nitrates	or abnormal	—	—	Methemoglobinemia	
Chlorates	hemoglobin	—	—	Methemoglobinemia	
Quinones		—	—	Methemoglobinemia	
Methylene blue		—	—	Methemoglobinemia	

Primaquine	Glucose-6	D	—	Hemolytic anemia	S
Antipyrine	phosphodehydrogenase,				
Acetanilid	stability of reduced	D	—	Hemolytic anemia	S
(fava beans)	glutathione				
Diphenyl hydantoin	Hydroxylation enzyme (vitamin K dependent system?)	D	D	Ataxia, dysarthria	A
Warfarin		ME	—	Drug resistance	
Coumarin	Coumarin hydroxylase	D	—	Hemorrhage	A
Barbiturates	(d-amino levulinic acid	—	—	Porphyria	
Sulfonamides	synthetase)				
Ethanol	Alcohol dehydrogenase	D	—	Altered metabolism conversion rate	
Ethanol	Alcohol dehydrogenase	ME	—	Drug resistance	
Benzo(a)pyrene	Aryl hydrocarbon hydroxylase	—	A	Resistance to induction of the enzyme	

condition involve the primaquine-induced hemolytic anemia in which there is a genetically altered stability of reduced glutathione and an altered glucose-6-phosphodehydrogenase activity. Additional examples are the abnormal hemoglobins in which there is an altered ability of the hemoglobin to remain in the reduced state, and the sulfonamide and barbiturate-induced porphyrias which are involved with the deficiency of the inhibitor system which normally controls the level of α-amino levulinic acid synthetase.

GENETIC FACTORS IN ACCUMULATION OF CHEMICALS

In the normal human, the antitubercular drug isoniazid undergoes acetylation as one mechanism of metabolic termination of the action of the drug. Formation of the acetylating enzyme is under the influence of a single major gene. Following conventional repeated doses of the compound to a mutant individual who lacks the acetylation gene, the compound can accumulate in the blood. High blood levels of isoniazid are prone to induce the toxicity of polyneuropathy.

Studies of variation between human individuals in regard to metabolism of isoniazid demonstrate the existence of three classes of subjects which may be described as slow, intermediate, and rapid inactivators of the drug. When the blood levels of isoniazid are determined 6 hr after a standard dose of the drug (4 mg/kg) and when these data are plotted as a frequency distribution graph (Fig. 6.3), a trimodal curve is obtained, thereby indicating the three types of subjects. This information, together with observations which indicate that the differences between subjects are not related to

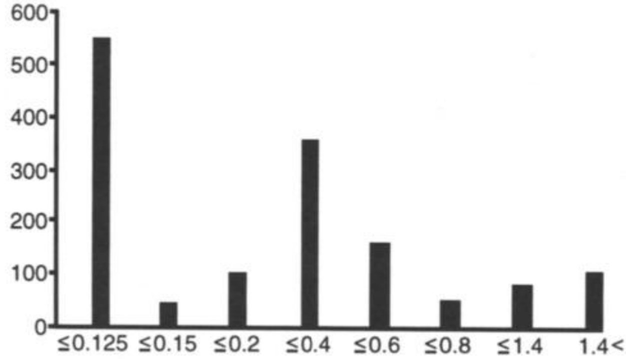

FIGURE 6.3 Frequency distribution curve of 6-hr blood levels of INH (isoniazid) in μg/ml after single dose of 4 mg/kg of INH in 1386 Japanese (from Sunahara, 1961). Data from Motulsky, A. G. (In Steinberg, A. G., and Bearn, A. G., Eds.) *Progress in Medical Genetics*, Grune & Stratton, New York, 1964, p. 52.

differences in intestinal absorption, protein binding, or urinary excretion of the drug, further suggests the existence of a genetically induced deviation from normal. Further investigation disclosed that more of the drug in acetylated form is excreted in the urine in the rapid inactivators. When liver slices of the rapid and slow inactivators were tested for ability to metabolize isoniazid, the compound disappeared at a faster rate in the liver from rapid inactivators. This indicated that the slow inactivators lacked the acetylating enzyme. Genetic studies of families also indicated that the slow inactivators were homozygous. Siblings from slow inactivators always were slow inactivators. Further genetic studies on many populations indicate that the isoniazid-acetylating gene deficiency has the lowest frequency in Eskimos, is common in Negroes and Europeans in general, and is of intermediate frequency in Japanese and Chinese people.

The anticoagulant drug dicumarol undergoes biotransformation in the liver. Failure of such metabolic alteration of the drug would be expected to permit accumulation of the drug following repeated doses, thereby leading to overdose toxicity in the form of hemorrhage. Dicumarol metabolism has been known to vary between members of a species since 1943. The fact that there is also variation in intestinal absorption between animals following oral intake of the drug, together with the demonstration that high doses of the drug would retard the biotransformation rate of the drug initially, cast some doubt on the significance of metabolic variation. Data obtained in subsequent studies of the half-life of the drug in humans still failed to establish that a genetically controlled system was operative in the variation of metabolism of dicumerol. However, studies of families strongly suggest that there are, besides the normal rapid metabolizers, a second group of possible mutants which are slow metabolizers of the drug. Biphasic frequency response curves for dicumarol have been demonstrated.

GENETIC FACTORS IN PROLONGATION OF ACTION OF CHEMICALS

The drug succinylcholine is capable of producing profound, generalized neuromuscular blockade. The intensity of the blockade is dose-dependent, and the action of the drug is terminated by hydrolytic biotransformation induced by the enzyme cholinesterase, which exists in the extracellular fluid of the body. This is a rapid biotransformation process. The average dose of succinylcholine (0.5 to 1 mg/kg) results in a duration of action in the human of not more than 8 to 10 min.

The use of succinylcholine in electroshock therapy to prevent peripheral skeletal muscle convulsive effects disclosed the existence of persons who developed prolonged respiratory paralysis following the administration of doses of the drug. Initial studies of such subjects indicated that they possessed a deficient cholinesterase enzyme system. Subsequent, more intensive investigations have shown that there are persons who are completely lacking in cholinesterase (pseudocholinesterase) enzymes. As a result of many investigations, the conclusion reached is that the prolonged succinylcholine-induced apnea is due to a genetic deficiency in the enzyme involving termination of the action of the drug.

The enzyme pseudocholinesterase is capable of hydrolizing many organic esters and many of these esters are also capable of inhibiting the enzyme by simply increasing the concentration of the ester. When succinylcholine is used as an inhibitor of the enzyme, it can be readily shown that higher concentrations of succinylcholine are required to inhibit the mutant enzyme than are required for inhibition of the normal enzyme. Thus, the enzyme from a mutant subject possesses cholinesterase which is less susceptible to inhibition by succinylcholine, and the mutant enzyme is less capable of hydrolizing succinylcholine. Using the anesthetic dibucaine as the enzyme inhibitor and benzoylcholine as the substrate, the trimodal (normal, deficient, and absent) distribution and the three distinct genotypes of cholinesterase in serum from different persons was demonstrated.

GENETIC FACTORS IN INCREASED SENSITIVITY TO CHEMICALS

Several relatively harmless drugs are known to induce hemolytic anemia in a few members of the population. The antimalarial drug primaquine, an 8-aminoquinoline derivative, is one example of such a drug. Extensive investigations of the primaquine-induced hemolytic anemia have led to the conclusion that this toxicity is due to a genetically controlled red blood cell abnormality which results from an enzyme deficiency. This enzyme deficiency, like the pseudocholinesterase deficiency which is demonstrated only after administering succinylcholine, is a harmless defect unless the system is challenged by certain drugs.

The mechanism of primaquine-induced hemolytic anemia was confusing for 15 years after the introduction of these types of drugs because the drugs also produced methemoglobinemia, a toxicity which was confused with, but not related to, the ultimate mechanism involved with the hemolytic anemia toxicity. The actual demonstration that the defect in the primaquine-sensitive person was localized in the blood cells involved experiments in which the erythrocytes from sensitive individuals were labeled with ^{51}Cr and transfused into nonsensitive recipients who were subsequently given primaquine. Rapid destruction of the primaquine-sensitive cells occurred.

Also, erythrocytes from nonsensitive subjects were given to sensitive subjects who were subsequently given primaquine. The primaquine-sensitive recipients in this latter case hemolyzed their own cells without destroying the transfused (normal) cells. These experiments established that sensitivity to primaquine was not due to abnormal degradation of the drug or to abnormal immune mechanisms in sensitive individuals, and that the defect was in the erythrocyte.

The discovery that reduced glutathione of primaquine-sensitive cells was uniquely sensitive to destruction enabled the development of the "glutathione stability test." This test involved incubation of the red blood cells with acetylphenylhydrazine. This test, when applied to primaquine-sensitive and nonsensitive individuals, readily demonstrated the bimodal distribution of the red-cell-reduced glutathione in the population. Subsequently several groups of investigators established that the sensitive cells also possessed a deficiency in the enzyme glucose-6-phosphate dehydrogenase. It is currently believed that the reduced glutathione instability is the result of a deficiency in glucose-6-phosphate dehydrogenase, although other metabolic lesions may be involved.

The familial and racial nature of the incidence of primaquine sensitivity suggests that the red-cell abnormality is genetically transmitted. Many investigators have shown that the red-cell defect is greater in families of its carriers than in the population at large, that there are less reactor females than males, and that intermediate degrees of deviant glutathione are present in females but not in males. The current concept is that the defect is due to either a sex-linked autosomal gene or a sex-linked gene.

Although several drugs are known to induce hemolytic anemia, the above mechanism has been limited to the primaquine-induced condition. Sulfanilamide- and acetanilid-induced hemolytic anemia appears to be involved with the same mechanism. The chemical, phenylhydrazine, if given in sufficiently high doses, produces hemolytic anemia in all subjects, but primaquine-sensitive subjects are more sensitive to phenylhydrazine. The exact mechanism of action of these drugs on the glucose-6-phosphate dehydrogenase or on the reduced glutathione is not clear, but the evidence indicates that the genetic mutant produces a deficient enzyme which is incapable of maintaining the red blood cell in its normal state when challenged by certain drugs. This system appears to be hypersensitive to the drugs.

DRUG-SENSITIVE HEMOGLOBINS

The drug-sensitive hemoglobins represent additional examples of genetically controlled factors which influence the occurrence of harmful effects from drugs. In the normal animal, the iron in hemoglobin is maintained in the reduced state (as ferrous iron) and remains in the reduced state when combined with oxygen. Oxidation of the iron to the ferric state converts the hemoglobin to methemoglobin. Methemoglobin is not capable of carrying oxygen and therefore fails to carry on one of its main functions, that of oxygen transport in the animal. If sufficient methemoglobin is present (10% in humans), a visible cyanosis is evident. The maintenance of hemoglobin iron in the reduced state in the normal animal is in part accomplished by the presence of the enzyme diaphorase (or methemoglobin reductase).

A variety of chemicals are capable of inducing the formation of methemoglobin, either by a direct stoichiometric action in which 1 mole of the chemical (as exemplified by nitrites) reacts with 1 mole of the hemoglobin to form 1 mole of methemoglobin, or by metabolic transformation to derivatives (as exemplified by conversion of acetanilid to phenylhydroxylamine) which acts directly on the hemoglobin. In the case of phenylhydroxylamine, it reacts with oxyhemoglobin to form nitrosobenzene complexed with hemoglobin and hydrogen peroxide, the latter of which is unstable and yields methemoglobin. The nitrosobenzene is in turn reduced by the enzyme diaphorase, resulting in the reformation of phenylhydroxylamine. Thus, one molecule of phenylhydroxylamine can result in the formation of several molecules of methemoglobin.

The rare clinical condition of hereditary methemoglobinemia has been recognized for over 40 years. The two forms of the disease are: (1) the molecular form in which the molecular structure of hemoglobin differs from the normal hemoglobin, and (2) the enzymatic form in which an enzyme or coenzyme (diaphorase 1) is deficient or absent, and in its absence the normal equilibrium state between hemoglobin and methemoglobin is shifted toward the formation of methemoglobin. Whereas the molecular form of the disease is transmitted as a dominant trait, the enzymatic form is transmitted as a recessive trait by an autosomal recessive gene.

The presence of a genetically deficient mechanism for maintaining the hemoglobin in the reduced state, as exemplified by that condition present in the enzymatic form of hereditary methemoglobinemia, predisposes such mutants to the development of clinical signs of cyanosis when they are administered methemoglobin-forming drugs. Such subjects in the absence of drugs may have from 6 to as much as 50% of their hemoglobin as methemoglobin, and the addition of hemoglobin-oxidizing drugs would be expected to show clinical effects, even though the contribution to the total methemoglobin supplied by the drugs was no greater than that achieved in the normal person. In the case of the mutant exhibiting absence of diaphorase, it is quite unlikely that this mutant would survive doses of acetanilid which would be innocuous in the normal person.

Unlike the enzymatic form of hereditary methemoglobinemia, the mutants exhibiting a molecular deviation involve the type of hemoglobin which does not show increased amounts of methemoglobin in the absence of drugs. The latter mutants are known to be predisposed to severe hemolytic anemia and methemoglobinemia only when challenged by such drugs as the sulfonamides. Two such mutant hemoglobins have been described and have been named after the cities in which the subjects resided (hemoglobin Zurich and hemoglobin Seattle). The alteration of these mutants involves the presence of a histidine residue on the 63rd position of the beta chain on the globin. It is apparent that such mutations in the hemoglobin molecule result in a hemoglobin that is unable to maintain its iron in the reduced state and this condition may lead to methemoglobinemia and possibly hemolytic anemia on administration of oxidizing drugs. Thus, this type of mutant is sensitive to the amounts of drugs which may induce only minor effects on the normal population.

Acute porphyria is an excellent example of a clinical condition that is inherited from a dominant gene according to Mendelian concepts. The condition is characterized by intermittent excretion of porphobilinogen and aminolevulinic acid in the urine and porphyrins in the feces. In humans who have the inherited defect, drugs such as barbiturates and sulfonal will precipitate the condition. Furthermore, several chemicals such as hexachlorobenzene, allyl isopropylacetamide, and certain collidines can induce the condition in experimental animals.

Studies on experimentally induced porphyria using one of the collidines have shown that excretion of the porphyrin precursors results primarily from enhancement of the enzyme activity of aminolevulinic acid (ALA) synthetase in the mitochondria of liver. Normally the liver cells control the porphyrin-synthesizing mechanism by controlling the production of the enzyme ALA-synthetase, which is the first enzyme in the porphyrin biosynthetic chain. When a drug, such as collidine, produces porphyria, it is postulated that the drug will activate the gene for ALA-synthetase by combining with, or inactivating, a repressor control. In the Mendelian disease, it is postulated that an operator gene may be defective and the repression of formation of the ALA-synthetase is held in balance. Therefore, the potential porphyria individual will be highly sensitive to small doses of specific drugs that affect the repressor control system.

GENETIC FACTORS IN SPECIES AND STRAIN RESISTANCE TO TOXICITY

The foregoing discussion pertains to some genetic deviations within a species which may account for increased toxicity from specific drugs or chemicals in relatively few members of the entire population. Genetically induced alterations in metabolic processes in the organism may result in protection of the organism from a harmful effect of a chemical. Such genetic deviations in the levels of "atropinase" in rabbits result in marked protection of the "high atropinase level" animals from the biologic effects of atropine.

Certain members of a species of bacteria may show marked resistance to the biologic effects of specific antibacterial agents which are bacteriocidal or bacteriostatic to the normal majority of that species of bacteria. A similar condition exists in the common house fly, which may be resistant to the lethal effect of the chlorinated hydrocarbon insecticides. Such resistant members of a species probably represent mutants. In either case, it is not well established whether the mutant resistant members of the species existed prior to widespread use of the chemicals, or whether exposure to the chemicals resulted in the development of the mutation, although the latter is strongly suggested in some examples. It is apparent that the resistant mutants could become the comon form of the organism solely because of effective eradication of the susceptible organisms by widespread use of antibacterial or insecticidal chemicals. The occurrence of mutant resistant forms of bacteria and insects is a continuing economic problem, which limits effective control of these organisms by the use of existing antibacterial and insecticidal chemicals. These conditions are considered in greater detail in Chapter 10, The Basis of Selective Toxicity.

It is very probable that there are many as yet undiscovered genetically induced deviants which would account for the rather rare individual who responds untowardly to chemical agents. By systematic investigation of the relatively rare intoxications occurring on the basis of hereditary disposition we may gradually learn of the mechanisms responsible for some of the factors that predispose to chemical intoxication.