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Environmental Aspects of Injury and Disease: Liver and Bile Ducts

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Evolutionary processes have not yet developed specific and safe ways to detoxify all chemical species new to our environment. Indeed, some are transformed and/or conjugated by the liver into more toxic species. Environmental factors can modulate hepatic enzyme systems. Particularly responsive are the mixed function oxidases, which initiate the transformation of many xenobiotics to excretable species via reactions which generate electrophilic intermediates such as free radicals, epoxides and aldehydes. Unless these reactive metabolites are rapidly removed by subsequent detoxification reactions or by endogenous defense systems, destructive cytotoxic reactions can be triggered or cell constitutents "attacked" thereby causing either acute injury and/or more latent molecular injury to long chain biopolymers resulting in chromatin damage, or tumors.

***In vitro* systems using purified, specialized cell fractions may be of considerable value in defining metabolic processes, but the results must be relevant to *in vivo* conditions. Although human liver is peculiarly resistant to tumorigenesis, liver microsomes (isolated endoplasmic reticulum) are extensively used as biological activators for *in vitro* mutagenicity test systems. The *in vivo* defense system of liver cells must be exceptionally efficient! Reactive metabolites generated in liver may be stable enough to migrate and cause injury to other tissues or organ systems. It is important to characterize metabolic pathways of toxic xenobiotics, subsequent molecular sites or modes of injury, and factors which depress or augment cellular defense systems including the biliary system responsible for the excretion of many xenobiotics. Only then can techniques or treatments be developed to screen individuals for risk to specific groups of xenobiotics, to protect those exposed, and to treat those injured.**

Introduction

The liver is a metabolically versatile organ responsible for the regulation of man's internal chemical environment. Exogenous and endogenous chemicals are absorbed, concentrated and then processed by the liver into more "useable," storable, or excretable forms. Many xenobiotics are converted at least initially to more excretable molecular species by components of the relatively nonspecific, mixed-function oxidases. These and other hepatic processing systems are responsive to multiple internal and external factors, such as sex, age, type of diet, hormone level, and prior or concurrent exposures to foreign chemicals (xenobiotics).

Environmental factors can sensitize individuals to potentially toxic chemicals by altering processes leading either to their "safe" removal from the body or their activation into molecular species capable of producing cellular injury. Mechanisms of alteration include induction or repression of enzymes involved in the metabolic processes, changes in levels of required cofactors and/or depletion of antioxidants or other hepatocellular defense systems. Chemicals activated by the liver may attack other organ systems. The metabolic capacity of the liver is the central factor in human environmental response.

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Pertinent Organization of Liver

Function

Mammalian liver is a "continuous mass of parenchymal cells tunneled by vessels through which venous blood flows on its way from the gut to the heart" (*1*). Foodstuffs absorbed from the external environment are processed in the liver for more specialized, less versatile tissues according to the body's needs. Dietary changes can be accommodated by shifts in metabolic pathways. Poly- and oligosaccharides, cleaved by the digestive system, reach the liver as a mixture of monosaccharides

where they are phosphorylated and either oxidized for energy or stored as glycogen. A vital function of the liver is the maintenance of normal blood glucose concentration through the hormone-controlled build up and breakdown of glycogen and synthesis of glucose (gluconeogenesis) from 3-carbon precursors. Dietary lipids are processed in the liver to provide sources of energy or raw material for synthesis. The liver is particularly important in the synthesis and regulation of circulating lipids, lipoproteins, triglycerides, cholesterol, cholesterol esters and in the degradation of cholesterol and steroids. Ingested proteins are cleaved to amino acids prior to absorption and transportation to the liver, where they are synthesized into structural and functional proteins for intrinsic and extrinsic use including plasma proteins, albumin and coagulation factors. Only the liver contains enzymes capable of converting nitrogen degradation products into urea for excretion.

The liver also functions as an endocrine gland in manufacturing, storing, concentrating and excreting cholic acid and other bile acids. The biliary system forms a major pathway for the excretion of conjugated xenobiotics. Iron reutilization and the breakdown and excretion of hemeporphyrins also require participation of the liver. Numerous nonspecific enzyme systems provide the liver with the flexibility to oxidize, reduce, dehalogenate and/or conjugate exogenous compounds in order to produce more excretable compounds, thus maintaining the hemostatis of the internal environment.

Structure

The massive bulk of the liver consists of myriads of hexagonal prismatic lobules approximately 3 mm in diameter which are the fundamental units of liver function. A branch of the hepatic vein is the center of each lobule. At the intersection of the interfaces between lobules are the portal triads which consist of small branches of the portal veins, hepatic artery and bile ducts, all three of which run concurrently.

Two major types of cells—parenchymal and reticuloendothelial Kupfer cells—populate the lobule. Parenchymal cells form irregular partitions one cell thick, lined on both sides by Kupfer cells, whose attenuated cytoplasm forms the tortuous labyrinth through which blood percolates on its way from the portal triad to a branch of the hepatic vein.

Kupfer cells form a discontinuous endothelium with phagocytic properties relatively efficient at removing particulate foreign matter from the blood. Their cytoplasm is rich in phagocytic lysosomes.

Parenchymal cells comprise the majority of the hepatocellular mass and their cytoplasm appears far more organized than that of Kupfer cells. Structurally, parenchymal cells are dodecahedrons. Parenchymal cell surfaces adjacent to vascular spaces and to the cytoplasmic extensions of Kupfer cells are microvillus in character, while the surfaces abutting adjacent parenchymal cells are relatively smooth. Bile capillaries which transport bile from the parenchymal cells to the bile ducts in the portal triads form a network of interfacial canals which run in the spaces between abutting liver cells.

Functionally the liver cells in the periportal regions (adjacent to a portal triad) differ from those in the centrolobular region (adjacent to a central vein). Succinic dehydrogenase, cytochrome oxidase, and glucose-6-phosphatase are more active in periportal parenchyma, while NAD- and/or NADP-dependent lactic, glutamic, and β-hydroxybutyrate dehydrogenase and 3,4-benzpyrene hydroxylase are predominately centrolobular (*2*). Xenobiotics have been found more potent inducers of smooth endoplasmic reticulum in centrolobular than periportal cells.

Cytoplasm of parenchymal cells contains abundant mitochondria, lysosomes, peroxisomes, Golgi apparatus, and rough and smooth endoplasmic reticulum, all of which are lined or delimited by phospholipid and protein-rich membranes of differing composition and function. These appear arrayed in a highly organized matrix. The plasma membrane with its many surface specializations forms the morphologic and functional interface between the cell and its immediate environment.

The endoplasmic reticulum (ER), a complicated network of membrane-lined cisternae permeating the cytoplasmic matrix, is particularly responsive to environmental changes. Many drugs, such as phenobarbital, and synthetic chemicals, such as polychlorinated biphenyls, cause marked proliferation and alteration of the metabolic properties of this organelle. The rough (RER) forms broad sheets, the outer surfaces of which are studded with myraids of ribosomes in rosettes and spirals. The other form, the smooth (SER), is a branching, interconnecting, sparsely granulated network of tubules 50 to 80 nm in diameter. Under normal conditions RER and SER are segregated, flat cisternae of the RER forming many-layered stacks of "ergastoplasm" and the SER forming loosely woven webworks of tubules which permeate the cytoplasmic matrix. Protein synthesis takes place on the ribosomes of the RER, glycogen storage is associated with the SER, and the entire membrane system of the ER appears to function in drug and steroid metabolism.

Fragmented liver endoplasmic reticulum is isolated as a relatively heterogeneous membrane frac-

tion, called microsomes, which is used widely in experimental molecular biology, pharmacology and toxicology. Liver microsomes are currently extensively used as biologic activators of chemicals in mutagenicity *in vitro* test systems. Oddly, liver is perculiarly resistant to tumorigenesis, hepatomas developing in adult human liver only after long-standing prior cirrhogenic chronic injury. Thus, although components of liver endoplasmic reticulum are capable of activating chemicals to mutagens *in vitro*, the *in vivo* mutagen defense system of the liver cells seems exceptionally efficient!

It should be pointed out that liver size (relative to animal size) is carefully regulated. If portions are removed surgically or chemically, the liver quickly returns to normal size through hyperplasia of remaining hepatocytes. Causes of increase in size include conditions which favor storage of glycogen, fat, or water, and agents which cause proliferation of endoplasmic reticulum and induction of drug (xenobiotic) metabolizing enzymes (*3*).

Cellular Organelles

Different morphologic structures of the liver parenchymal cell carry out specialized functions. The nucleus, nucleolus, and polyribosomes are responsible for the replication of DNA and the translation of genetic information into RNA, proteins and other forms of molecular species necessary for cellular function and replication. Xenobiotics or their electrophilic metabolites may interact—either directly or indirectly —at any point in this system. Mutagens are considered to bind covalently to the electron-rich centers of DNA bases.

Membranes of the cytoplasmic constituents of the liver cell, endoplasmic reticulum, mitochondria, peroxisomes, and lysosomes, carry out distinctly different forms of oxidative metabolism. Substrate oxidations (and reductions) are also carried out by the enzymes of the cell sap.

The membranes of the endoplasmic reticulum hydroxylate endogenous, such as fatty acids and steroids, and numerous exogenous compounds by a multimolecular system known as the mixed function oxidase because one atom of molecular oxygen is reduced to water while the other is incorporated into substrate (*4–6*).

$$RH + 2H + 2e^- + O_2 \rightarrow ROH + H_2O \qquad (1)$$

In Eq. (1), H^+ and e^- are derived from either NADPH or NADH, or both. The versatile mixed function oxidase systems of liver endoplasmic reticulum carry out numerous reactions including: nitro reduction, *N*-dealkylations, *O*-dealkylations, *S*-dealkylations, epoxidations, dechlorinations and alcohol oxidations. Multiple species of the terminal oxidase of this system, cytochrome P-450, have been characterized and demonstrated not only to have different metabolic capacities but also to be preferentially inducible (*7*).

It should be noted that completion of many biotransformation processes initiated by components of mixed function oxidases of liver endoplasmic reticulum requires participation of other enzyme systems. These processes are thus affected by the availability of cofactors such as NADPH, NADH, NAD, O_2, reduced glutathione, glucuronate, UTP, UDP-G, and ATP. Interference with or depletion of one or more components could impede electron transport and complex formation or decomposition causing uncontrolled release of "activated" free radical or epoxide intermediates to the immediate intracellular environment. The complexity of mixed function oxidase sytems and related conjugation systems are schematically depicted in Figure 1. "Safe" transformation of xenobiotics to water-soluble (oxidized or conjugated) species requires adequate energy sources, oxygen, and above all ample endogenous antioxidants to protect cellular components from the ongoing reactions.

In contrast to the reactions of mixed function oxidases [Eq. (1), Fig. 1], electron transport in mitochondria is tightly coupled to the generation of high-energy phosphate bonds and terminates in cytochrome oxidase, which carries out the reduction of molecular oxygen to water [Eq. (2)].

$$4e^- + 4H^+ + O_2 \rightarrow 2H_2O \qquad (2)$$

Michaelis (*8*) postulated that this takes place by a series of one electron transfers involving free radical intermediates. Parts of the mitochondrial electron transport chain are embedded within hydrophobic membrane interiors. Mitochrondial membrane integrity appears to be disrupted at an early stage in the toxic course of some hepatotoxins such as 1,1-dichloroethylene ($Cl_2C{=}CH_2$) (*9*).

Peroxisomes carry out the stepwise reduction of molecular oxygen with the formation of H_2O_2 as an intermediate [Eq. (3)]. H_2O_2 may either be broken down by catalase or peroxidase [Eq. (4)] (*10*).

$$2e^- + 2H^+ + O_2 \xrightarrow[\text{(}l\text{-}\alpha\text{-HO acid oxidases)}]{\text{oxidases}} H_2O_2 \qquad (3)$$

$$H_2O_2 + 2e^- + 2H^+ \xrightarrow{\text{catalase}} 2H_2O \qquad (4)$$

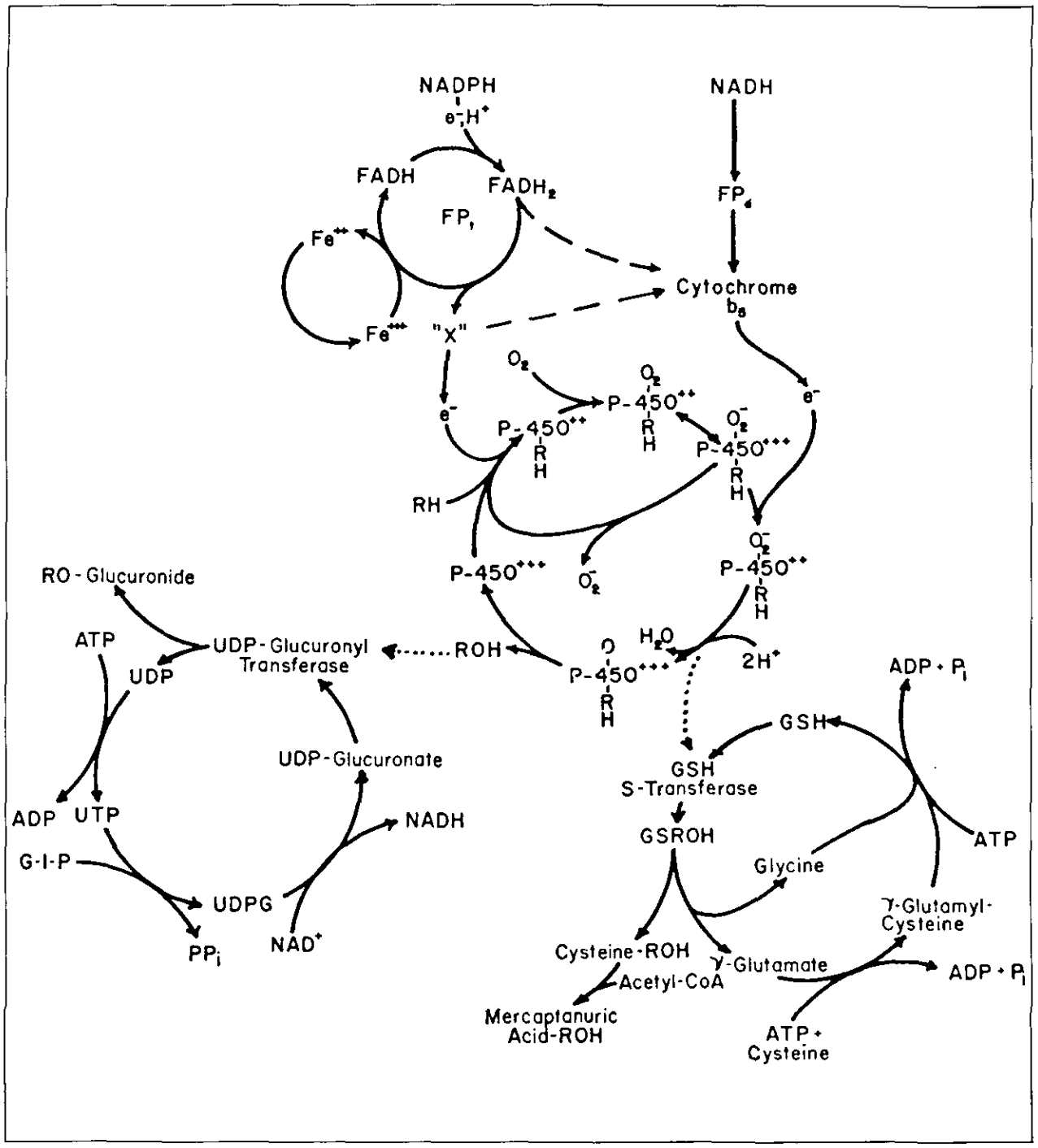


FIGURE 1. A schematization of the nonspecific mixed function oxidase system of the liver endoplasmic reticulum and related conjugation systems. In the basis reaction (center wheel) substrate (RH) binds to the central hemoprotein known as cytochrome P-450, is reduced by an electron transferred via flavoprotein (FP) from NADPH and complexes with molecular oxygen. The complex is then reduced by a second electron derived from NADH (or NADPH). The triplex of substrate, cytochrome P-450 and O_2 decomposes with one atom of oxygen reduced to water and the other incorporated into the substrate. Oxidized substrates (alcohols, aldehydes, phenols, epoxides) may then undergo conjugation reactions with glutathione (lower right), glucuronate (lower left), water, phosphoadenosine phosphosulfate (PAPS) etc. Modified after Gillette and Jollow (5).

Aliphatic alcohols, including ethanol, have also been postulated by some to be metabolized by this pathway. Certain hypocholesterolemic compounds (e.g., ethyl α-*p*-chlorophenoxyisobutyrate) increase cellular populations of peroxisomes (*11*).

Lysosomes, which are more closely associated with Kupfer cells than parenchymal cells, have phagocytosis-related NADH oxidase which generates H_2O_2 or other forms of active oxygen within phagolysosomes. In the presence of peroxidases, H_2O_2 generates active halides, superoxide, singlet oxygen, hydroxyl radical and/or aldehydes, all or some of which may participate in bacterial killing reactions and the chemical modification of phagocytized cellular or extracellular constituents (*12*). Contents of lysosomes appear to evolve through a series of oxidative and polymerization reactions to relatively inert residual bodies. Pathologic processes which increase the amounts of materials being phagocytized causing more rapid turnover of cellular constitutents in autophagosomes (e.g., vitamin E deficiency states, copper toxicity, iron overload) should increase residual body contents of lysosomes.

Biliary System

Functions of the biliary system (components of liver parenchymal cells, bile canaliculi, bile ducts, and gall bladder) include the synthesis of cholic acids, other bile salts, cholesterol, bilirubin glucuronides, and their secretion by hepatic parenchymal cells. Bile is collected and concentrated in the gall bladder. Excretion of this concentrated bile is mediated on demand by cholecystokinin. The biliary system also forms a major pathway for the excretion of detoxified xenobiotics. Drugs and chemicals and xenobiotics all may affect the synthesis and/or rates of excretion of components of bile normally present and affect the stability of this easily supersaturatable system. Many compounds excreted in bile are reabsorbed by the intestinal epithelium only to be re-excreted in the bile (enterohepatic circulation).

Klaassen (*13*), in a recent review (1975), has summarized research into the biliary excretion of xenobiotics as follows: 'The biliary route is very important for the elimination of some foreign compounds from the body. For many of these compounds, an increase in the rate at which they are excreted into the bile will decrease their toxicity and vice versa. A number of factors which are known to alter the biliary excretion of xenobiotics, as well as the current concepts of the physiological mechanisms responsible for the excretion of foreign compounds, have been enumerated. However, much remains still to be understood; essentially nothing is known at the subcellular level about the biliary excretion of foreign compounds. It has recently been concluded that our knowledge of biliary excretion of compounds is about 40 years behind that of the renal excretion mechanism."

Mechanisms of Hepatic Injury by Environmental Chemicals

One of the liver's activities is the processing of chemicals derived from the environment. Modern man is literally deluged with molecular species unknown even fifty years ago. Evolutionary processes have not yet developed specific and safe pathways to process and excrete all species of this deluge from our synthetic era. Appendix I lists the most extensively produced compounds which are demonstrated or potential hepatotoxins. Appendix II lists types of occupational and environmental exposure to these hepatotoxins. Entry of certain of these foreign species into our internal environment can cause interference with hepatic processes by depletion of cofactors or through "accidental" insertion into complex molecules during synthesis. For example, ethionine interferes acutely with cellular function, protein synthesis and nuclear and polyribosomal structure by sequestering adenine thereby producing a conditioned deficiency of the ATP necessary for DNA, RNA, and protein synthesis (*14*). Mercury and arsenic combine with sulfhydryl enzymes and thus impair hepatic metabolic functions.

Another perhaps more serious danger is that xenobiotics will be activated by the relatively indiscriminate" enzyme system into reactive electrophilic molecular species capable of "attacking" cellular constituents, or initiating destructive processes such as lipid peroxidation. The liver mixed-function oxidase system is thought to activate many xenobiotics including haloalkanes and haloalkenes (carbon tetrachloride, halothane, vinyl chloride, trichloroethylene) (*15*), haloaromatics (hexachlorobenzene, bromobenzene, lindane, DDT) and carcinogens (3,4 benzpyrene, 2-acetylaminofluorene, aflatoxin B) (*16*), and dimethyl or diethylnitrosomine (*17*). Appendix III provides examples of mechanisms by which these toxins are activated.

Recent biologic studies of the industrially important compound vinyl chloride ($ClHC{=}CH_2$) illustrate how many factors can be involved in xenobiotic activation. Exposure to vinyl chloride under occupational conditions (long-term, low-dose) has been associated with nonmalignant liver injury and

angiosarcoma of the liver in both man and experimental animals (*18, 19*). A single 6-hr exposure to 5% vinyl chloride causes acute injury to liver endoplasmic reticulum in animals pretreated with potent inducers of cytochrome P-450, the terminal oxidase of the liver mixed function oxidase system (*9*). Diminution of liver cytochrome P-450 contents and some functional activities of the mixed function oxidase system of animals by one day after vinyl chloride exposure implies that the activated vinyl chloride species either directly "attacks" membrane constituents or initiates reactions leading to their destruction (*20*). *In vitro* studies of vinyl chloride have shown that the vinyl chloride molecule can covalently bind to proteins and nucleic acids or cause mutagenesis of test bacteria systems when activated by a microsomal enzyme system containing the required cofactors (*21*). Inhibition of cytochrome P-450 markedly reduces the binding of vinyl chloride to proteins (*21*).

The complexity of pathways by which vinyl chloride and other heptotoxins such as halogenated aromatics can be activated to electrophilic species and subsequently deactivated is suggested by Figure 2. Such schemes may provide insight into the complexity of the multienzyme interactions and how easily they may be thrown out of balance. For example, bromobenzene is activated by the mixed-function oxidase system to an epoxide which then rearranges to a phenol or is converted enzymatically either to a dihydrodiol or to a glutathione conjugate. When the rate of glutathione depletion exceeds the rate of glutathione synthesis, bromobenzene epoxide covalently binds to proteins of the intracellular environment—presumably directly causing injury. Impaired synthesis of reduced glutathione could enhance bromobenzene's cytotoxic effect, while induction of the dihydrodiol pathway or alternate pathways of bromobenzene metabolism could minimize its toxic effect (*4*). Actions of other factors which perturb the membrane system in which the xenobiotic-metabolizing enzyme systems (see Fig. 1) are embedded or attached could disturb the intermolecular relationships between the various components causing profound imbalances in the detoxification pathways.

Which metabolite is carcinogenic? Which acutely hepatotoxic? What are the structural parameters of xenobiotics which allow prediction of cytotoxicity and/or of carcinogenicity? What parameters of xenobiotic metabolites determine stability and selective chemical reactivity, i.e., which electrophilic metabolites are sufficiently stable to migrate from endoplasmic reticulum to nuclei where they selectively and covalently bind to electron-rich groups of DNA bases? Indeed, how are these carcinogenic electrophiles which may be generated in liver exported to nuclei of other cellular species of liver, to nuclei of cells of other organs, or across placentas to developing fetuses? All these parameters are but poorly understood, indeed, are poorly substantiated *in vivo*!

Definition of a Person at Risk in the Environment with Regard to Liver Injury

Species, sex, age, diet, habits, genetic differences, prior environmental or occupational exposure, neonatal exposure to compounds with hormonal activity, and other factors yet unknown modulate the responses of individuals to chemical agents in their environments.

Some differences are obvious. Rats, whales, and deer do not have gall bladders. Thus they do not have gall stones. Male rats metabolize drugs more rapidly than females. Other differences are more subtle, such as exposure to aromatic wood products, polycyclic hydrocarbons in tobacco smoke, and consumption of charcoal broiled meats, rancid (peroxidized) fats, or food products containing xenobiotics with estrogenic activities.

Since many potentially injurious environmental agents produce their effects through interaction with the mixed function oxidases of liver endoplasmic reticulum, the ability of this enzyme system to activate them to proximate toxins, or to subsequently deactivate them is significant. Fetal and newborn rats have very low contents of cytochrome P-450 and other drug metabolizing components, as well as the enzymes of the glucuronide and glutathione conjugating systems (*6*). These enzymes rapidly develop about the time of birth. Fetal and neonatal primates including man appear to have mixed function oxidase activities that are higher than rats, approaching 20 to 40% of the levels in adults. Since components of the mixed function oxidase system not only activate (toxify) xenobiotics but also detoxify the activated metabolites, imbalances may occur in such a system, particularly when specific components are present in different amounts at different developmental stages, or are selectively induced or destroyed by the xenobiotics themselves. Depending upon the developmental stage, production of electrophilic xenobiotic metabolites may greatly exceed or be balanced by the ability to safely detoxify them. Since mixed function oxidases are responsive to multiple internal and external factors, conditions at certain times may favor pathways which produce relatively stable xenobiotic electrophiles. If these electrophilic

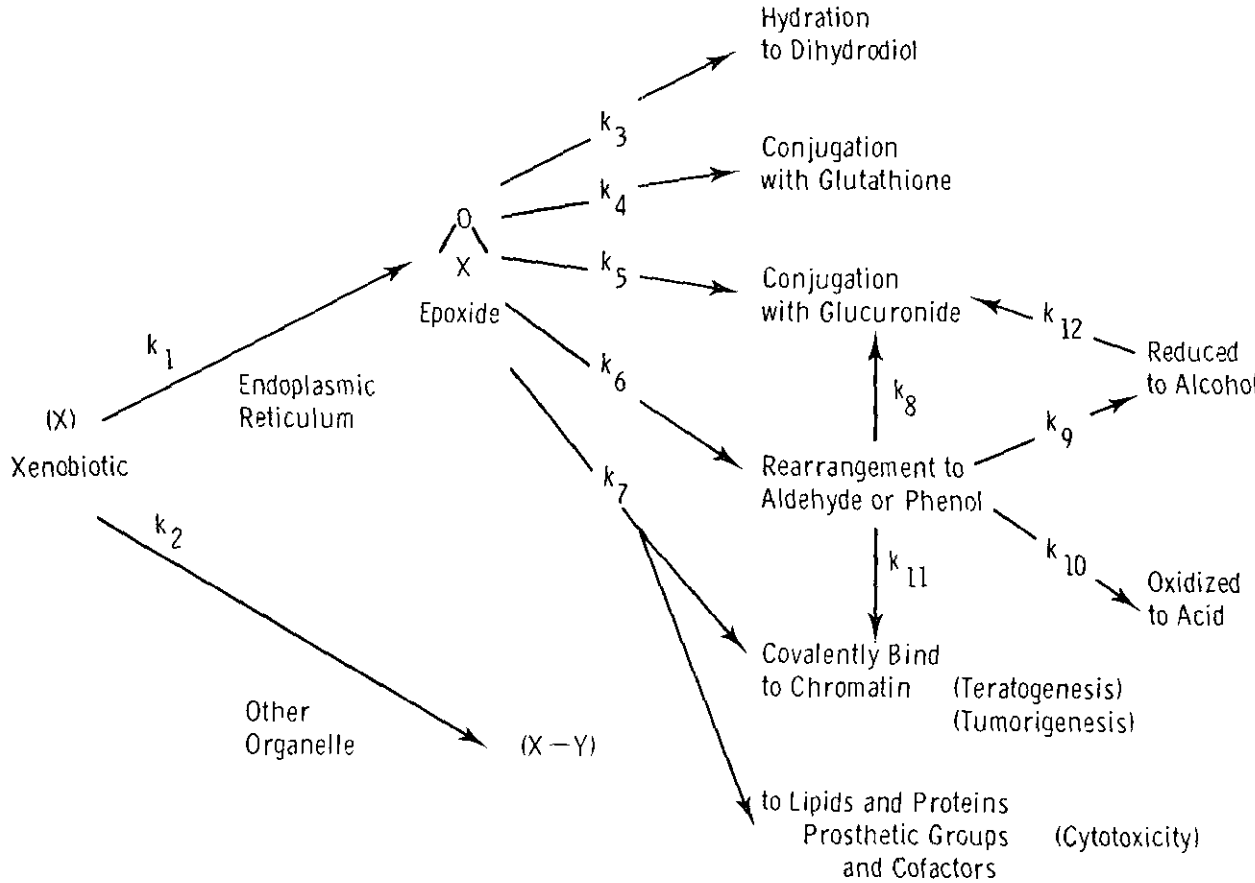


FIGURE 2. Simplified kinetic flow diagram of xenobiotic toxification-detoxification. Xenobiotics may be metabolized by more than one pathway (k_1 or k_2) which may be differentially inducible. The assumption is made that the epoxide product of k_1 is a cytotoxic or mutagenic electrophile capable of interaction with vital cellular components (k_7). Hydration (k_3), conjugation (k_4, k_5), and rearrangement (k_6) transform the epoxide intermediate, presumably to less toxic compounds. However, compounds formed subsequently, such aldehydes may also be toxic (k_{11}). Induction of alternate pathways of initial metabolism (k_2) may diminish epoxide formation and induction of k_3, k_4, or k_5 may shorten its existence. Depletion of conjugation cofactors (glutathione, glucuronide) would minimize detoxification pathways (k_4, k_5) and allow increased covalent binding (k_7, k_{11}) to occur. Modified after Gillette and Jollow (*5*) and Robinson et al. (*6*).

metabolites migrate to the cell nuclei, to nuclei of other liver cells, or indeed are exported to nuclei of cells of other organs, their covalent binding to DNA may result in tumors. If such events occur during the early weeks of gestation, developmental defects or teratogenic effects may ensue or the molecular seeds may be sown in the fetus for the subsequent development of a tumor. Cytotoxic tissue injury due to metabolism of xenobiotics by the mixed function oxidase system usually require relatively extensive or intensive exposure, whereas mutagenesis may occur with low dose exposure, if stable electrophiles ensue.

What is the evidence that such events do occur? Covalent binding in lipids and proteins has been correlated with cytotoxic tissue injury (*22, 23*). Oesch (*24*) found that induction of arylhydrocarbon monooxygenase in fetal rat liver is not accompanied by induction of an epoxide hydrase, one of the detoxifying enzyme systems, and that microsomes of rat fetuses transplacentally exposed to benzopyrene provided a more potent activation system in mutagenic assays of benzpyrene to S Typhimurium TA 1573 than microsomes from fetuses of control rats not exposed to benzpyrene transplacentally. Robinson et al. (*6*) demonstrated genetic differences in the inducibility of arylhydrocarbon monooxygenase in liver (determined by the Ah locus) in mice and in man and the placental induction of arylhydrocarbon hydroxylase in smoking

mothers. Furthermore, in mice, responsiveness at the Ah allele is a predictor of protection against hepatocellular injury following lindane or bromobenzene. Deficiency in a potential detoxification pathway, such as the glucuronide conjugating enzyme system, i.e., Gilbert's Disease, may represent but one of many of the types of deficiencies—genetic or otherwise—that may develop. Slow acetylators of isoniazid may be another group. People such as these may be among those who have untoward reactions towards xenobiotics in the environment. Ways to detect these persons potentially at risk must be developed.

Types of Injury Related to Environmental Exposure

Developmental defects, hepatomas, acute hepatocellular necrosis, chronic persistent hepatitis, post-necrotic scarring, micronodular cirrhosis associated with alcoholism, biliary cirrhosis, gall stones all may result from environmental exposure to xenobiotics. Unfortunately we do not yet know how, in these instances, to make a definitive diagnosis of chemical etiology. Only when the time between exposure to a specific compound and injury is brief or chronic (such as occupational) are we able to make a presumptive diagnosis of chemical etiology. Development of jaundice following exposure to a compound is usually taken as presumptive evidence for cause and effect, particularly if the compound is also an acute (or chronic) hepatotoxin in experimental animals. In the case of carbon tetrachloride, perhaps the most intensively studied hepatoxin, we still do not know what distinctive morphologic or chemical changes to seek to make a definitive diagnosis of carbon tetrachloride poisoning, i.e., those changes which differentiate it from injury following exposure to other acute hepatotoxins.

Halothane has been suspected of being an acute hepatotoxin in man since its introduction as an anesthetic. Also, continued exposure to low concentrations has been associated with increased incidence of spontaneous abortions and congenital defects in operating room personnel (*25*). Yet a nation-wide inquiry into the possible relationships between halothane anesthesia and postoperative jaundice (National Halothane Study, 1969) did not find morphologic patterns of acute liver injury that were distinctive for injury caused by halothane. Halothane has recently been shown to be an acute hepatotoxin in polychlorinated biphenyl-pretreated rats (*26, 27*), and under similar circumstances of mixed-function oxidase induction it may be directly hepatotoxic in man. Definitive diagnosis awaits chemical definition of the lesion, including detection and determination of injury-associated halothane metabolites in breath, blood, and urine.

Chronic liver injury in vinyl chloride workers is manifest by a rather nonspecific periportal fibrosis which in the most advanced instances may resemble primary biliary cirrhosis (*18*). Vinyl chloride-induced angiosarcoma develops in those chronically injured livers. It is assumed that metabolites of vinyl chloride cause both the chronic injury and the angiosarcoma.

Ethanol is a classic case. It is still not known how this ubiquitous compound consumed in billions of pounds yearly produces its devastating effect on the liver.

Clearly, more effort needs to be expended in these areas. Injury producing pathways of xenobiotic metabolites need to be delineated, the metabolites sought and the products of their interaction with cellular constitutents determined. Chemical, functional, and morphologic correlates should be sought to provide better basis for the recognition of the causes of similar lesions in the future. Unfortunately for the diagnostician, the early hepatic injury caused by CCl_4, halothane, vinyl chloride, or trichloroethylene exposure all resemble one another morphologically with formation of tubular tangles of smooth endoplasmic reticulum and functionally with deactivation of cytochrome P-450 and mixed function oxidase components. Yet the first two haloalkanes are presumed to act through free radical mechanisms and the second two haloalkenes through epoxide intermediates (*15*). Characterization of molecular changes in the membrane may provide a definitive answer. Fortunately, once you know what to look for, it is easy to identify.

Detection of Injury-Producing Potential

Quantitative predictors of the toxin-generating reactivity of chemicals in biological systems are needed. Physical chemical parameters of molecular structure related to the ability to produce overt injury or more subtle changes in hepatic function need to be defined and structure-activity guidelines developed. However the development of such guidelines requires the development of methods to isolate and quantify metabolites of xenobiotics, and to detect, define and quantitate the magnitude of injury. Noninvasive methods to determine xenobiotic body burden, and rates and routes of xenobiotic metabolism should be developed utilizing breath, blood, urine, and bile, particularly in instances where there is the possibility of a relationship between xenobiotic exposure leading to overt

or covert injury.

In order to know what metabolite to seek, relationships between xenobiotic metabolism and injury must be sought in experimental animals —preferably whole animals. Studies with isolated organs, cell suspensions, parts of cells and soluble extracts may not relate readily to conditions *in vivo*. Only *in vitro* systems which resemble defined *in vivo* systems are valid. Choice of animal is important for results must ultimately relate to disease in man and screening for injury-related metabolites should be applicable to man.

Clearly, there is a need for more definitive and more sensitive detection of hepatic injury in man exposed to the modern world of chemicals. Current methods are acceptable for symptomatic acute or chronic liver disease but usually fail to indicate ongoing subclinical or occult liver disease. Changes in liver-derived serum enzyme activities should continue to be explored in a search for more sensitive indicators. Rates of liver synthesis or removal of serum lipids, proteins, lipoproteins, corticosteroids and sex hormones might provide some insight into functional defects brought about by xenobiotic exposure. Serum determinants of the status of the antioxidant defense of the liver (reduced/oxidized glutathione, vitamin E oxidation products) could relate to increased intracellular peroxide generation. Increased mercapturic acid excretion in urine could relate to increased xenobiotic detoxification. To determine the status of mixed function oxidases in people who might be at high risk of liver injury because of exposure to certain xenobiotics, noninvasive tests of the metabolic capabilities of the mixed function oxidases should be developed.

Similarly, better morphologic and cytochemical parameters of the types and extents of injury to the liver need to be developed. Because the liver is such a heterogeneous organ with segregation of function and toxic response by zone and cell type, histologic, histochemical, and physical techniques are particularly valid. Large changes in the function or composition of small numbers of cells may become indiscernible when averaged out over the total population as would occur following homogenization of the liver.

Certain hepatotoxins produce striking ultrastructural changes in liver parenchymal cells. Although several different general classes of injury can be recognized by electron microscopy—mitochondrial swelling and/or calcification, dilitation of rough endoplasmic reticulum, "denaturation" of the smooth, production of "finger print" whorls of the smooth, segregation of nucleolar and nuclear components, increased cytoplasmic contents of peroxisomes or autophagosomes—for the most part, the molecular causes of these changes are not known. Ultrastructural studies could localize the initial site of hepatocellular change, however it should be recognized that structural alterations results from rather complex molecular changes, the basis of which is poorly understood. If the chemical causes were known, it would render the ultrastructural studies more definitive and thus, more useful as screening procedures.

New and Novel Approaches

Increasing awareness that xenobiotic-induced cellular injury results from finite chemical changes in the constituents of the cell, and that elucidation of the injury-producing mechanism depends upon the detection of these molecular changes emphasizes the need for new and better techniques to analyze and to reproduce the injury in more uniform populations of cells. One must always keep in mind, however, that the lesion produced *in vitro* must always replicate that produced *in vivo*.

Suspensions of parenchymal cells, or reticuloendothelial cells, either from dissociated livers or cell cultures might be used to test injury producing potential(s) of chemical agents. Kinetics would be simpler and different parameters of injury might be selectively enhanced or suppressed. Cellular suspension of dissociated hepatocytes can be further separated into large and small hepatocytes (assumed centrolobular and periportal, respectively) with differing metabolic functions. Fractionated organelles would then also be more homogeneous. Clearly, the screening potential of such superspecialized systems, for example in mutagenesis assays, need to be examined.

Only by understanding the molecular basis of injury can we learn what to look for to detect early injury and to provide methods for its prevention or reversal. Prediction of injury producing potential depends upon information which still needs to be gathered.

Recommendations

RECOMMENDATION 1: Since most xenobiotics present in the environment interact with constituents of liver and cause alterations and/or injury to that organ and possibly others through their metabolites, metabolic pathways of xenobiotics need to be better understood and the types of metabolites associated with injury classified.

RECOMMENDATION 2: Although the association between xenobiotic toxic action and metabolism must be worked out in the experimental animal, metabolite production and associated liver injury

must be sought in persons exposed to such compounds in their environment. Detection of injury-associated compounds in serum, breath, or urine of persons could be used to estimate extent of exposure and risk.

RECOMMENDATION 3: Simple test systems using perfused livers, suspended hepatocytes obtained from enzymatically disassociated livers, hepatocytes in culture, or organelle fractions should be developed for screening of metabolic pathways of xenobiotics metabolism and detection of the production of potentially injurious metabolites.

RECOMMENDATION 4: Since the actual molecular mechanisms responsible for hepatocellular injury by chemical agents are poorly understood, ways to better define the molecular events involved in hepatocellular injury by specific chemical agents and to characterize and quantify injury should be developed.

RECOMMENDATION 5: Better noninvasive or minimally invasive methods to detect alterations of liver structure and function should be developed and applied to people who may be at risk because of their environment but who are not overtly ill.

RECOMMENDATION 6: More rational definitions of risk and of conditions which enhance or protect against injury must be made on the basis of direct and not inferred evidence.

Appendix I

Compounds of Significance in the Environment by Quantity of Production (Top 100 in 1970) Which are Potential or Demonstrated Hepatotoxins, Cohepatotoxins, or Inducers of Hepatotoxicity

A. Hepatotoxins, demonstrated:
- Vinyl chloride
- Carbon tetrachloride
- Carbon disulfide
- Perchloroethylene
- Trichloroethylene
- 1,1,1-Trichloroethane
- Chloroform
- Chlorobenzene
- Methylene chloride
- Dichlorobenzene

B. Hepatotoxins, potential:
- 1,2-Dichloroethane
- 1,2-Dibromoethane
- Styrene
- Ethylene oxide
- Acetaldehyde

C. Cohepatotoxins (compounds without demonstrated primary hepatotoxin potential which potentiate hepatotoxicity of other chemical hepatotoxins) (Term reserved for compounds which do not or have not been demonstrated to modulate the mixed function oxidases).
- Isopropyl alcohol
- Acetone
- *n*-Butyl alcohol
- Ethyl alcohol

D. MFOS inducers, not necessarily cytotoxic in themselves but which distort the physiologic responsiveness of the animal
- DDT
- Benzene
- Xylene
- Toluene
- Polychlorinated biphenyls (chlordane and other halogenated or chlorinated pesticides, herbicides)

E. Other industrial, agricultural, pharmaceutical and natural chemicals of demonstrated or potential hepatotoxic occupational or environmental risk
- Vinyl bromide
- Vinylidine chloride
- Vinylidine bromide
- Iodoform
- Methylmercury
- Halothane
- Manganese
- Arsenic
- Thorium
- Chromium
- Cadmium
- Antimony
- Mercury
- Yellow phosphorus
- Selenium, tellurium
- Flame retardants
- Ethylene-chlorohydrin

Appendix II

Examples of Types of Occupational and Environmental Exposure to Potential Hepatotoxins

1. Food production
   - Pesticides
   - Herbicides
   - Fumigants

2. Food processing
   - Preservatives
   - Dyes
   - Solvents
   - Packaging
3. Manufacturing
   - Plastic monomers
   - Petrochemicals
   - Synthetic fabrics
   - Industrial degreasers
   - Metal and electrical equipment
   - Fabrics
   - Forest products
4. Service
   - Vehicle supply and maintenance: (degreasers, refrigerants, solvents)
   - Dry cleaning solvents for degreasing
   - Janitorial solvents for dewaxing, carpet cleaning, window washing
   - Medical:
     - Anesthetic gases
     - Solvents for tissue processing and chemical analysis
   - Personal care
     - Beauticians
     - Cosmetologists
     - Barbers
5. Housekeeping
   - Cleaning aids, solvents, and waxes
   - Grooming aids
     - Propellants
     - Solvents
     - Emulsifiers
   - Paints, varnishes
   - Foodstuffs, including packaging

Appendix III

Example of Known or Postulated Chemical Mechanism of Toxin Activation and Detection of Toxin-Related Injury (Toxigenic Reactions)

Free-Radical Reactions: Free radical reactions are postulated to take place as a consequence of single-electron capture reactions by xenobiotic molecules and their subsequent dissociation into xenobiotic free radicals and anions. The most likely source of such electrons is considered to be the electron transport chain of the mixed function oxidases of liver endoplasmic reticulum, although similar reactions may be initiated by the mitochondrial electron transport chain, and through interaction with peroxides in peroxisomes and phagolysosomes.

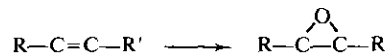
Carbon tetrachloride is thought to be the best example of a "free-radical" toxin. Its initial homolytic cleavage is considered to occur as shown in Eq. (A-1).

$$CCl_4 + e^- \rightarrow CCl_3 + Cl^- \qquad \text{(A-1)}$$

The CCl_3 radical then either abstracts labile hydrogens with the formation of $CHCl_3$ or covalently binds to electron-rich areas of proteins and lipids. In both instances, protein and lipid free radicals are produced which react subsequently in free-radical propagation or diradical annihilation reactions, resulting in lipid peroxidation or molecular polymerization.

Direct evidence for the free radical metabolism of CCl_4 includes the formation of $CHCl_3$ (the result of abstracting labile hydrogens), C_2Cl_6 (the diradical annihilation product), and the presence of increased conjugated diene content in lipids (a consequence of scouring of labile hydrogens from methylene bridges of polyunsaturated fatty acids). Preferential covalent binding to lipids is suggestive, but the chemical sites of covalent binding have not been characterized.

Epoxide Formation: Xenobiotics are converted into epoxides by components of mixed function oxidase as shown in Eqs. (A-2) and (A-3).



(A-2)

(A-3)

Both alkenes and arenes may be metabolized. Their intermediates may either rearrange spontaneously to less toxic compounds, conjugate with H_2O, reduced glutathione, or bind covalently to electrophilic groups in tissue macromolecules. Cytotoxicity is considered to be related to the magnitude of covalent binding. Carcinogenic intermediates of benzanthracenes are also considered to be epoxides (specifically the 9-10 epoxide).

Evidence of epoxide formation of alkenes is the formation of trichloroethanol from trichloroethylene, a process involving chloride shift under the influence of an active oxygen at cytochrome P-450. The shift of hydrogen between the 3 and 4 position in the formation of *p*-bromophenol from bromobenzene (the NIH shift also supports initial metabolism via formation of an epoxide in-

termediate). Epoxide intermediates of benzanthracenes have also been isolated. However, for the most part, unless the epoxide is remarkably stable, the existence of the epoxide is deduced from the analysis of its more stable products. Specific cytotoxicity-related covalent binding targets in tissue macromolecules have not been elucidated.

Aldehydes: Aldehydes are generated by alcohol dehydrogenases in the cytoplasmic matrix, through metabolism of xenobiotics by mixed function oxidases, and through the activities of catalase or peroxidase in peroxisomes. Allyl alcohol, which produces a severe coagulative necrosis of periportal hepatocytes, is considered to be converted to the presumptive noxious agent allyl aldehyde (acrolein) by alcohol dehydrogenases [Eq. (A-4)].

$$H_2C{=}CH\text{-}CH_2OH \xrightarrow{NAD} H_2C{=}CH{-}\overset{\overset{\displaystyle O}{\diagup}}{\underset{\underset{\displaystyle H}{\diagdown}}{C}} \qquad \text{(A-4)}$$

Aldehydes are then presumed to form Schiff's bases with primary amines, redox condensation products with peptide bonds, alcohols, thiols, etc.

Epoxides of unsaturated haloaliphatics such as those formed from trichloroethylene by the mixed function oxidases may rearrange non enzymatically to form aldehydes which then react with their chemical environment. Products such as these which might be related to injury have not been verified.

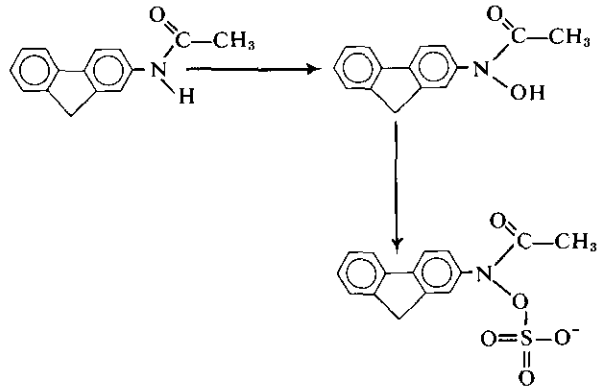
Oxidative X Dealkylation (X = N,S,O): Compounds such as the diallyl nitrosamines are *N*-demethylated by cytochrome P-450 associated enzymes to monoalkylnitrosamines, which spontaneously rearrange to unknown active metabolites which may include electrophilic alkyl carbonium ions.

$$\begin{matrix} CH_3 \\ CH_3 \end{matrix}\!\!>N{-}N{=}O \longrightarrow CH_3{-}NHN{=}O + CH_2O \qquad \text{(A-5)}$$

$$CH_3{-}NHN{=}O \longrightarrow CH_3^+ \qquad \text{(A-6)}$$

Dimethyl nitrosamine is acutely cytotoxic at high doses and a carcinogen at low doses. Not only is it an hepatocarcinogen, but it will also induce tumors in other organs—the electrophilic alkyl radicals which have been preferentially recovered covalently bound to amino groups of purine and pyrimidine bases of DNA are considered by most to be the carcinogenic vector. The molecular basis of its cytotoxic action is not understood, but it should be noted that the leaving methyl group in reaction (A-5) is oxidized to formaldehyde.

N (S) Oxidation: The liver carcinogen 2-acetylaminofluorene is *N*-hydroxylated by either cytochrome P-450 related enzymes or amine-*N*-oxidase [Eq. (A-7)]. The hydroxyl amine group may then form a sulfate ester.



(A-7)

Both the *N*-hydroxyl and the *N*-hydroxyl sulfate ester bind to nucleophilic centers in bases of liver DNA *in vivo*.

Cofactor Depletion: The complexity of structure and of metabolic processes in liver cells emphasizes the importance of the distribution and concentration of cofactors and their precursors in the regulation of normal cell function and responses of cells to injury. Indeed, certain xenobiotics have profound effects on cofactor levels. Ethanol increases liver contents of NADH and NADPH. Ethionine through the formation of *S*-adenosyl ethionine depletes the liver of adenine nucleotides, thus bringing nucleic acid and protein synthesis to a halt. Component of nucleoli segregate but cells do not die. This affect is apparently unrelated to its ability to produce tumors.

Similarly, galactosamine through the formation of uridine diphosphogalactosamine depletes the liver cell of uridine phosphates and uridine sugars and brings RNA and protein synthesis and glycosylation reactions to a halt. Large doses produce necrosis, not because they interfere with protein synthesis, but because increased plasma membrane permeability to calcium develops. Thus xenobiotic impairment of cell coat renewal processes may have lethal consequences.

Diethyl maleate depletes liver of reduced glutathione but does not produce necrosis. Bromobenzene depletes cells of glutathione and produces necrosis. Bromobenzene is metabolized to potent electrophiles, which in the absence of glutathione covalently bind to liver macromolecules; yet bromobenzene-induced hepatic injury is not manifest until hours after the depletion of glutathione.

Hepatic Porphyria: Certain xenobiotics grossly disturb the synthesis of porphyrins in livers of experimental animals. Similar mechanisms may be operative in man. Since the rate of synthesis is controlled by intracellular free (?) heme levels, decreases in heme brought about through the destruction of heme [2 allyl-2-isopropylacetamide (AIA)], inhibition of ferrochelatase which inserts iron into the porphyrins β,5-diethyoxy carbonyl-1,4-dihydrocolkdene and increased synthesis of hemoproteins (perhaps coupled with increased destruction of hemoproteins) (hexachlorobenzene?), greatly stimulate δ-aminolevulinic acid synthetase and porphyrin production, presulting in porphyria.

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REFERENCES

1. Elias, H. The anatomy of the liver. In: The Liver, Vol. I. C. Rouiller, Ed., Academic Press, New York, 1963, p. 41.
2. Novikoff, A. B. Cell heterogeneity within the hepatic lobule of the rat. J. Histochem. Cytochem. 7: 240 (1959).
3. Hermann, R. S. Induction of growth by xenobiotic compounds and other stimuli. Crit. Revs. Toxicol. 3: 97 (1975).
4. Gillette, J. R., Mitchell, J. R., and Brodie, B. B. Biochemical mechanisms of drug toxicity. Ann. Revs. Pharmacol. 14: 271 (1974).
5. Gillette, J. R., and Jollow, D. J. Drug metabolism in liver. In: The Liver: Normal and Abnormal Functions. F. Becker, Ed., 1974.
6. Robinson, J. R., et al. Pharmacogenetic aspects of drug toxicity and teratogenesis in the newborn. In: Basic and Therapeutic Aspects of Perinatal Pharmacology. T. L. Morselli et al., Eds., Raven Press, New York, 1975.
7. Lu, A. Y. H., et al. Induction of different types of cytochrome P-450 in liver microsomes by drugs and carcinogens. Anticonvulsant Drugs and Enzyme Induction, Study Group 9 of the IRMMH, March 1976.
8. Michaelis, L. Free radicals as intermediate steps of oxidation reduction. Cold Spring Harbor Symp. Quant. Biol. 7: 33 (1939).
9. Reynolds, E. S., et al Hepatotoxicity of vinyl chloride and 1,1-dichloroethylene: role of mixed function oxidase system. Amer. J. Pathol. 81: 291 (1975).
10. De Duve, C., and Baudhuin, P. Peroxisomes (microbodies and related particles). Physiol. Rev. 46: 323 (1966).
11. Reddy, J., et al. Microbodies in experimentally altered cells. J. Cell Biol. 44: 226 (1970).
12. Klebanoff, S. J. Antimicrobial mechanisms in neutrophilic polymorphonuclear leucocytes. Seminars Hematol. 12: 117 (1975).
13. Klaassen, C. D. Biliary excretion of xenobiotics. Crit. Revs. Toxicol. 4: 1 (1975).
14. Farber, E., et al. Biochemical pathology of acute hepatic adenosinetriphosphate deficiency. Nature 203: 34 (1964).
15. Reynolds, E. S. Liver endoplasmic reticulum: target site of halocarbon metabolites. In: Membrane Toxicity. M. W. Miller and . E. Shampoo, Eds., Plenum Press, New York, 1977, p. 117.
16. Felton, T. S., and Nebert, D. W. Mutagenesis of certain activated carcinogens associated with genetically mediated increases in mono-oxygenase activity and cytochrome P-450. J. Biol. Chem. 250: 6769 (1975).
17. Frantz, C. N., and Maling, H. V. Factors affecting metabolism and mutagenicity of dimethyl- and diethylnitrosamine. Cancer Res. 35: 2307 (1975).
18. Thomas, L. B., et al. Vinyl chloride induced liver disease from idiopathic portal hypertension (Banti's Syndrome) to angiosarcomas. New Engl. J. Med. 292: 17 (1975).
19. Maltoni, C., and Lefemine, G. Carcinogenicity bioassays of vinyl chloride. I. Research plans and early results. Environ. Res. 7: 387 (1974).
20. Reynolds, E. S., et al. Vinyl chloride-induced deactivation of cytochrome P-450 and other components of the liver mixed function oxidase system. An *in vivo* study. Res. Comm. Chem. Pathol. Pharmacol. 12: 685 (1975).
21. Kappus, H., et al. Rat liver microsomes catalyse covalent binding of ^{14}C-vinyl chloride to macromolecules. Nature 257: 134 (1975).
22. Reid, W. D., and Krishna, G. Centrolobular hepatic necrosis related to covalent binding of metabolites of halogenated aromatic hydrocarbons, Exptl. Mol. Pathol. 18: 80 (1973).
23. Ilett, K. F., et al. Chloroform toxicity in mice: Correlation of renal and hepatic necrosis with covalent binding of metabolites to tissue macromolecules. Exptl. Mol. Pathol. 19: 215 (1973).
24. Oesch, F. Transplacental control of epoxide forming and inactivating enzymes of rat fetal liver by clinically used drugs and environmental chemicals. In: Basic and Therapeutics Aspects of Perinatal Pharmacology. T. L. Morselli, et al., Eds., Raven Press, New York, 1975.
25. Cohen, E. N., et al. Occupational disease among operating room personnel: a national study. Anesthesiology 41: 321 (1974).
26. Reynolds, E. S., et al. Modulation of halothane and vinyl chloride induced acute injury to liver endoplasmic reticulum. In: Recent Advances in Biochemical Pathology, Toxic Liver Injury. M. U. Dianzani, Ed., Minerva Medica, Turin, Italy, 1976.
27. Sipes, I. G., and Brown, B. R. An animal model of hepatotoxicity associated with halothane anesthesia. Anesthesiology 45: 622 (1976).