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THE METABOLISM OF XENOBIOTICS BY CERTAIN EXTRAHEPATIC ORGANS AND ITS RELATION TO TOXICITY*

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INTRODUCTION

Considerable interest has developed in recent years in the bioactivation of xenobiotics in extrahepatic organs and in the accompanying toxic effects in these organs. However, the presence of covalently bound xenobiotic in, for example, kidney, coupled with selective pathologic changes in kidney, is not prima facie evidence for the generation of a toxic chemical species in that target organ. There is now abundant and further accumulating evidence that many reactive intermediates may be formed in liver and that they are sufficiently stable to diffuse back into the venous blood and be distributed to other organs where they may be covalently bound and produce tissue injury. Thus, one must exercise caution in equating high concentrations of covalently bound xenobiotics in specific organs and pathologic changes in those organs with in situ activation of the xenobiotic.

Williams (1) considered the metabolism of foreign chemical compounds, or xenobiotics, to occur via four chemical mechanisms: namely oxidation, reduction, and hydrolysis (Phase I reactions); and synthesis (conjugation) (Phase II reactions). Many excellent reviews (2–5) are available that discuss the specific types of reactions included with these categories, and they are not repeated here. It suffices to state that most of the reactions that fall within the scope of this review are oxidative reactions of a special type; in general they are catalyzed by enzyme systems known as monooxygenases or mixed function

oxidases. These enzymes present in the microsomal fraction of most mammalian organs and tissues, nonmammalian species, bacteria, molds, and plants usually require NADPH and an electron transport chain consisting of a flavoprotein—commonly referred to as NADPH cytochrome *c* (P-450) reductase—and a family of hemoprotein isozymes known collectively as cytochromes P-450. In most instances, interaction of this enzyme system with xenobiotics results in biological inactivation of the xenobiotic ("detoxication") and increased rate of clearance from the body. However, in other instances—those of specific interest to us here—xenobiotic substrates that are themselves biologically inert or of a low order of biological activity are converted by the monooxygenase system to highly reactive metabolic intermediates that form adducts with vital cellular macromolecules (DNA, RNA, proteins, lipids). These interactions may lead to a variety of detrimental biological consequences, including cellular necrosis, mutagenesis, and malignant transformations.

Because of its large mass and its richness in monooxygenases, much of the early work, relating bioactivation of xenobiotics to various toxic effects, was performed with, and concentrated upon, liver (6). More recently, however, an abundant literature has accumulated on the bioactivation of xenobiotics in extrahepatic organs with resulting toxicity either in situ or in distant sites that are the primary loci of toxicity.

Finally, we wish to emphasize that the primary purpose of this paper is not to review extrahepatic drug metabolism per se; many excellent reviews (7–9) and monographs (e.g. 10) perform that function quite adequately. The thrust of this paper instead determines if organ-specific toxicity has a metabolic basis, why it is selective (formation of a specific toxic metabolite, unusual sensitivity of that organ to a common metabolite, prolonged retention, etc), and, where possible, what biochemical mechanism(s) account for the selective toxicity.

The literature review for this manuscript was completed in February 1985.

RESPIRATORY TRACT

Level of the Respiratory Tree

NASAL EPITHELIUM Although the field of inhalation toxicology has proliferated immensely in the past ten years, systematic study of the metabolism of xenobiotics by the most proximal segments of the respiratory tree did not appear until 1982. Dahl and his co-workers (11) detected easily measurable levels of microsomal cytochrome P-450 and its related monooxygenases in nasal epithelium and ethmo- and maxilloturbinate membranes from dogs. The nasal carcinogen hexamethylphosphoramide was metabolized by nasal enzymes to another nasal carcinogen, formaldehyde. Later work from the same laboratory described the presence of cytochrome P-450, p-nitroansole O-demethylase,

and aniline hydroxylase in nasal membranes of six common laboratory species (12). Rabbit, guinea pig, and hamster were richest in these enzymes. Cytochrome P-450 levels in rat nasal epithelium were 1.6 times that of lung (per mg protein) (13). Brittebo and co-workers (14, 15) reported the metabolism of carcinogenic nitrosamines and of aminopyrine by nasal mucosa of mice and rats; they suggested a correlation between intranasal metabolism of a variety of substituted nitrosamines and their carcinogenicity in situ. Monooxygenases in nasal turbinates of dogs and rats catalyzed the activation of benzo[α]pyrene, 2-aminoanthracene (16), and 1-nitropyrene to bacterial mutagens in selected strains of *Salmonella typhimurium* (17).

In view of the established nasal carcinogenicity of formaldehyde, Dahl & Hadley (18) examined 32 potential substrates that might be metabolized to formaldehyde by rat nasal mucosa. Substrates demethylated at similar rates by nasal and liver microsomes were methamphetamine, propylhexedrine, cocaine, nicotine, dimethylaniline, and hexamethylphosphoramide. Rat nasal epithelium was found to oxidize benzo[α]pyrene to dihydrodiols, quinones, and phenols 10 times more rapidly than lung microsomes (19). Autoradiographic studies with [ethyl-^{14}C] phenacetin in rats (20) revealed that 5 minutes after an i.v. injection, radioactivity was highly concentrated in nasal mucosa—specifically subepithelial glands—associated with olfactory epithelium and also trachea. Incubation of tissue slices with phenacetin revealed that the O-deethylase capacity of nasal mucosa of rats was 10 times that of liver and over twenty times that of lung. Nasal mucosa of rabbits was approximately 7 times as rich in O-deethylase activity as liver. These activities were markedly inhibited by the monooxygenase inhibitors metyrapone and SKF 525-A. Injection of mice with [^{14}C]-chlorobenzene followed by autoradiography demonstrated covalent binding of the material that was selectively concentrated in the nasal and tracheo-bronchial epithelium. In the nose, radioactivity was bound to the subepithelial glands (Bowman's glands) underneath areas of olfactory epithelium (21).

TRACHEA Like the nasal mucosa, the most common form of toxic insult to the tracheobronchial tree resulting from interaction with xenobiotics is malignant transformation. Recent reviews are available (22, 23), and because of the extensive size of the literature and the relative homogeneity of its toxic manifestations (carcinogenesis), we review the field briefly here.

Kaufman et al (24) incubated hamster tracheas with [^{3}H]-benzo[α]pyrene (BP), then isolated the tracheal epithelial cells, purified their DNA, and found it to contain covalently bound [^{3}H]-BP. Binding was enhanced if the tracheas were taken from hamsters previously exposed to BP plus Fe_2O_3. The authors concluded that tracheal epithelium is able to metabolize BP and that metabolism is required for the inducible binding of BP to tracheal epithelial DNA. Later

carcinogenesis studies clearly demonstrated that in golden hamsters, the tracheal epithelium is the target tissue for the development of tumors by polycyclic hydrocarbons such as BP (25). Tracheal organ cultures and isolated tracheal microsomes catalyzed the binding of [^{3}H]-BP to DNA in vitro. HPLC analysis revealed a variety of metabolic products such as phenols, quinones, tetrols, and diols (including the 7,8-dihydrodiol), subsequently shown to be the penultimate carcinogenic species.

When cloned hamster tracheal epithelial cells were incubated with [^{3}H]-BP, adducts with DNA were formed (26). The cells formed the ultimate carcinogen, 7,8-dihydro-7,8-dihydroxy BP-9,10-epoxide, which reacted with deoxyadenosine, deoxycytosine, and deoxyguanosine; the DNA-carcinogen adducts were partially repaired during the course of the incubation. In addition to these biochemical events (27, 28), it was reported that incubation of rat tracheal organ culture in media containing 7,12-dimethybenz-[α]anthracene (DMBA), benz[α]anthracene, BP, or 3-methylcholanthrene (3-MC) produced morphologic alterations consisting of hyperplasia, metaplasia, and other general cytotoxic features. Following intratracheal administration of BP-Fe_2O_3 to rats and hamsters, there was a striking species difference in the distribution of lesions (29). In hamsters, squamous metaplasia of the trachea and large bronchi were observed, while in rats squamous nodules of bronchioalveolar origin developed. These differences correlated with penetration and persistence of BP in hamster tracheal epithelium but not in rat. Interestingly, when BP was incubated with tracheal organ cultures (23), binding to DNA in hamster trachea was 17 times greater than in rat trachea; this appears to correlate nicely with the relative species specificity for BP carcinogensis.

A vast literature exists on the organotropy of nitrosamines, specifically diethylnitrosamine (DEN) for the respiratory tract of the hamster (30). Although the bronchiolar nonciliated epithelial (Clara) cell is clearly the cellular site of covalent binding of [^{3}H]-DEN and both metaplastic and neoplastic transformations, it should be noted that the mucous cells of the trachea and the ciliated and Clara cells of the bronchi are also cellular targets (31, 32).

BRONCHI The capacity of human bronchus to metabolize and covalently bind carcinogenic aromatic hydrocarbons was reported by Harris et al (33). Specimens of bronchi (1 cm^2) were cultured for one week and then incubated with [^{3}H]-BP. Total binding of BP depended on its concentration, incubation time, and temperature. [^{3}H]-BP and its metabolites were bound to cellular macromolecules and to DNA isolated from bronchial mucosa. The cell types involved were predominantly ciliated and mucous cells. Earlier work by this group had shown that cultured human bronchial epithelium could also metabolize 7,12-dibenz[α,h]anthracene to molecular species that bound tightly to macro-

molecules including DNA. The same group demonstrated that cultured human bronchial epithelium catalyzed the demethylation of dimethylnitrosamine and 1,2-dimethylhydrazine to products that bound to both cellular DNA and protein (34). Bronchial DNA was methylated in both the O-6 and N-7 positions of guanine. Autrup et al (35) reported that cultured human bronchial epithelium in vitro converted [^{3}H]-BP to 7,8-dihydroxy-7,8-dihydro BP (the "dihydrodiol"), the penultimate carcinogenic species of BP. Aryl hydrocarbon hydroxylase (AHH) is a microsomal enzyme that catalyzes the oxidation of BP to metabolic products (phenols, quinones, epoxides; for review see Ref. 4) that ultimately bind covalently to DNA and initiate carcinogensis. Kahng et al (36) found that exposure of bronchial epithelium during culture to benz[α]anthracene induced AHH activity and the binding of [^{3}H]-BP to the epithelial DNA by as much as 29-fold.

BRONCHIOLES, ALVEOLI, AND VASCULAR ENDOTHELIUM A number of excellent reviews have appeared on the role of metabolic activation of xenobiotics in lung injury (37, 39). Most of these involve attack of a reactive electrophilic intermediate on bronchiolar nonciliated (Clara) epithelial cells, alveolar type I or type II cells, or vascular endothelium. There are at least three general mechanisms by which xenobiotics elicit lung injury; others and/or combinations undoubtedly exist of which we are currently unaware. The first involves activation of the xenobiotic to a chemically reactive species that may covalently bind to vital macromolecules, frequently in a specific cell type, and cause malignant transformation or necrosis. This reactive intermediate may be formed in situ and act directly in the lung or be formed elsewhere (e.g. liver) and be transported to lung, where it exerts its toxic action. The second mechanism involves xenobiotics with appropriate redox characteristics that accept an electron from, for example, NADPH, with the formation of a free radical, such as $R\text{-}\dot{N}O_2^-$. These radicals can transfer the electron to dioxygen (O_2), resulting in formation of another free radical called superoxide anion radical, $O_2^{\cdot-}$; through a series of redox steps, $O_2^{\cdot-}$ may give rise to $^1O_2$ (singlet oxygen), H_2O_2, or $\cdot OH^-$, all four of which are toxic in varying degrees. Finally a few xenobiotics (bleomycin, O_2) attack the tight junctions between capillary endothelial cells causing leakage of serous, serofibrinous, or even hemorrhagic exudate into the alveolar space.

Pulmonary Toxicity of Oxygen

If adult rats are placed in 100% O_2 at 1 atmosphere pressure, virtually 100% of the animals are dead within 60–72 hours. The cause of death is pulmonary insufficiency resulting from massive fibrinous pulmonary edema (40). Microscopically one finds that the initial lesion is loosening and then necrosis of capillary endothelial cells followed by loss of type I alveolar epithelial cells. In-

terestingly, if rats are initially exposed to 85% O_2 (1 atm) for five or more days, they acquire tolerance and are able to survive for prolonged periods in 100% O_2. The chemical and biochemical mechanisms of acute O_2 toxicity and the origins of tolerance are reviewed extensively elsewhere (41, 42). Dioxygen (O_2) is thought to be toxic to tissues including lung because of its ability to generate free radicals (43). Dioxygen readily undergoes one electron reduction in biological systems with the formation of $O_2^{-\cdot}$, superoxide radical anion (41). This reaction may occur nonenzymatically or may be catalyzed by numerous enzymes such as NADPH cytochrome P-450 reductase, xanthine oxidase, and several mitochondrial enzymes. Once formed, superoxide may produce a number of further reduction products known collectively as reactive oxygen or oxyradicals. Superoxide may produce damage to mammalian cells and tissues either directly (i.e. by inactivating enzymes) or indirectly by stimulating lipid peroxidation. The sequential four-electron reduction of dioxygen may be depicted as

$$O_2 \xrightarrow{e^-} O_2^{-\cdot} \xrightarrow{e^-} H_2O_2 \xrightarrow{e^-} {\cdot}OH \xrightarrow{e^-} H_2O$$

Superoxide, hydrogen peroxide, singlet oxygen, and especially the highly reactive hydroxy radical (·OH) are individually and collectively toxic to tissues and cells. Normally, tissues have defense mechanisms that protect them against the toxic effects of oxyradicals (44). Among these are superoxide dismutase (SOD), catalase, and glutathione peroxidase, together with tissue stores of α-tocopherol, reduced glutathione (GSH), and ascorbate. However, when these defenses are overwhelmed as in oxygen toxicity or paraquat poisoning (see below), toxic levels of various oxyradicals accumulate in tissues.

That oxyradicals are directly capable of evoking tissue damage was cleverly demonstrated by Johnson et al (45). Xanthine and xanthine oxidase (which together generate $O_2^{-\cdot}$) when instilled into the trachea of rats produced acute lung injury that was not produced by either of the two components singly or by saline. The lung injury was markedly reduced by intratracheal SOD (which degrades $O_2^{-\cdot}$) but not by catalase. Similar administration of glucose and glucose oxidase (which generate H_2O_2) produced lung injury of lesser magnitude; this was prevented by catalase that degrades H_2O_2. Finally, intratracheal administration of glucose and glucose oxidase plus lactoperoxidase, which presumably generate $^1O_2$ or HOCl, resulted in massive pulmonary edema and a fibrinous exudate in the aleveolar space. Fourteen days later, the lungs were markedly hypercellular and exhibited extensive interstitial fibrosis. The progression to pulmonary fibrosis suggests that oxygen metabolites may be an important vector in the pathogenesis of interstitial pulmonary fibrosis.

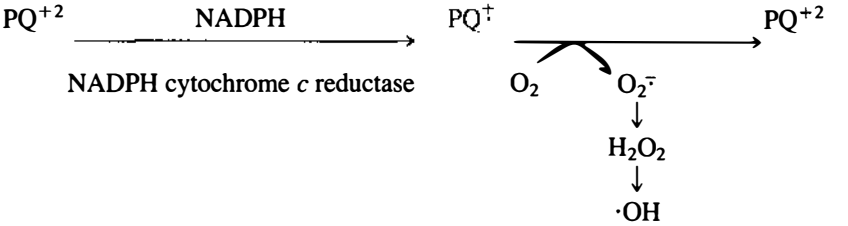
Toxicity of Paraquat and Other Agents that Generate Reactive Oxygen Radicals

Paraquat, also known as methyl viologen, has been used as a redox reagent in chemistry for some years; it can accept an electron and be converted to a free radical; in the process it is transformed from colorless to blue. Its tremendous economic utility as a broad spectrum herbicide led to its widespread use and to the discovery that it is a lung toxin in animals and man.

Parenteral administration of paraquat to most mammalian species results in its selective accumulation in lung relative to other organs; 30 hours after dosing, there was six to seven times more paraquat in lung than in plasma of rats (46). Similarly, incubation of lung slices with paraquat reveals the remarkable capacity of this organ to concentrate the xenobiotic (46); slice/medium ratios ranging from 5:1 to 15:1 can be obtained. Accumulation does not occur by covalent binding. Paraquat uptake by lung is an active, energy-dependent uptake process that occurs against a concentration gradient and can be blocked in the usual ways; anaerobiosis, cold, iodoacetate, etc, and also by a variety of other amines that are themselves actively accumulated by lung slices, such as 5-hydroxytryptamine, norepinephrine, spermine, propanolol, imipramine, chlorpromazine, diphenhydramine, and chlorphentermine (37).

Liver or lung microsomes incubated aerobically with NADPH slowly oxidize NADPH; addition of paraquat results in massive increases in the rates of NADPH oxidation and oxygen uptake (47). Associated with this oxidative burst, one discovers a marked increase in $O_2^{\cdot-}$ formation. These reactions are not blocked by carbon monoxide (CO) and are therefore not cytochrome P-450 dependent (48).

It is currently held that the mechanism by which paraquat damages tissues (lung, kidney, liver) is as follows:



In the presence of microsomes and NADPH, paraquat undergoes one-electron reduction to its free radical. This reaction is catalyzed by NADPH cytochrome *c* reductase (49). Under aerobic conditions, the paraquat free radical immediately transfers its electron to dioxygen with the formation of $O_2^{\cdot-}$ and the regeneration of the paraquat cation. The net result of these reactions is that a reduction-

oxidation cycle is set up in which paraquat functions catalytically, NADPH is consumed (NADPH/NADP falls) (47), and most importantly, enormous quantities of $O_2^{\cdot-}$ are generated. Since oxyradicals (see the section on O_2 toxicity) are (*a*) themselves toxic and (*b*) able to stimulate lipid peroxidation that is destructive to biomembranes and also yields products (hydroxynonenals) that are cytotoxic (50), it is not possible at this time to identify the more toxic of these coincidental events.

As noted above, mammalian organisms have a variety of endogenous mechanisms to protect themselves from oxidant stress, the generation of large amounts of oxyradicals, and it is not until these defenses are overwhelmed that oxidant toxicity occurs. For example, superoxide dismutase (SOD) degrades $O_2^{\cdot-}$, catalase degrades H_2O_2, and glutathione peroxidase converts fatty acid hydroperoxides to alcohols (41, 42). In addition, tissue stores of α-tocopherol, reduced glutathione (GSH), and ascorbate scavenge oxyradicals. The toxicity of paraquat, defined either in terms of LD_{50} or LT_{50}, can be manipulated with the knowledge of these oxidant protective mechanisms. For example, hyperoxia (e.g. 100% O_2) markedly increases paraquat toxicity while hypoxia (10% O_2) reduces it (51). Depletion of tissue GSH stores by pretreatment with diethylmaleate or vitamin E deficiency dramatically increases paraquat toxicity. Similarly, selenium deficiency produced by dietary means also increases paraquat toxicity. Selenium is a component of glutathione peroxidase. There are two final points regarding paraquat worthy of note. All mammalian species studied to date except the rabbit, respond to paraquat with pulmonary lesions (52, 53). It is not clear whether the rabbit lung is absolutely refractory to the effects of paraquat or whether a higher dose is required in it than in other species. Finally, there is no convincing evidence to date that, other than the cyclic reduction and oxidation undergone by paraquat, there is any net biotransformation of the compound in vivo or in vitro.

The pathologic changes induced by paraquat in mammalian lung have been described in great detail (54). Animals receiving lethal doses of paraquat die in three phases; the most acute deaths (4–18 hours) result from massive pulmonary edema and hemorrhage into the alveolar space. A second group of animals die in 3–6 days from renal failure resulting from widespread necrosis of proximal renal tubular epithelium. Finally, depending on the dose and the species (14–20 days or longer in humans), death results from chronic respiratory failure coincident with massive pulmonary fibrosis. The latter stage, referred to as the proliferative phase (54), begins with the influx into lung of large numbers of fibroblasts with abundant rough endoplasmic reticulum. Fibroblastic activity results in the synthesis and deposition of large amounts of collagen into the extracellular space. The increased septal cellularity and increases in interstitial and intra-alveolar collagen eventually obliterate the alveolar and bronchiolar spaces, and the lung becomes a solid glandular-like organ incapable of either

inflation or gas exchange. When the thorax is opened, the "paraquat lung" fails to collapse, exhibits a solid, rubbery consistency, and sinks in water.

Nitrofurantoin is an agent used clinically to treat urinary tract infections. Its use, particularly when administered chronically, is accompanied by pulmonary reactions, once thought to be "hypersensitivity reactions" (55), ranging from cough and dyspnea to infiltrates, effusion, and pulmonary fibrosis, which can be confirmed by pulmonary biopsy. Administration of large doses of nitrofurantoin to rats produced severe respiratory compromise that caused death in 12–36 hours. Tachypnea and cyanosis were conspicuous symptoms; at autopsy the lungs were grossly distended, edematous, and hemorrhagic. Histologically, there was widespread interstitial and alveolar edema, vascular congestion, and hemorrhagic consolidation (56). Perhaps more importantly, the lethality of nitrofurantoin could be manipulated broadly by factors known to be involved in lipid peroxidation. For example, the lethality and pulmonary damage produced by nitrofurantoin were significantly enhanced in rats maintained in 100% oxygen after drug administration (56). Similarly, the lethality was greatly enhanced in rats maintained on a vitamin E deficient diet that was rich in polyunsaturated fat (corn oil). The LD_{50} of nitrofurantoin in this group was 35 mg/kg compared to 400 mg/kg in controls. If vitamin E–deficient rats were repleted with vitamin E, the lethality of nitrofurantoin returned to that of controls.

These findings should be integrated with those of Mason & Holtzman (57) that under aerobic conditions and in the presence of NADPH, microsomes catalyze a one-electron reduction of the nitro group of nitrofurantoin to yield a nitro free radical ($R\text{-}\dot{N}O_2^-$) that spontaneously reacts with oxygen to regenerate the parent nitro compound and simultaneously reduces oxygen to $O_2^{-\cdot}$. Although covalent binding of nitrofurantoin can be demonstrated under anaerobic conditions in vitro, under aerobic conditions little covalent binding occurs but large amounts of superoxide are formed in the presence of lung microsomes, NADPH, and nitrofurantoin. Thus it seems likely that the pneumotoxicity of nitrofurantoin is mediated through superoxide and its secondary metabolites, H_2O_2 and $\cdot OH^-$. The mechanism of toxicity therefore seems to bear a strong resemblance to that of paraquat and high concentrations of oxygen itself.

Pulmonary Toxicity of Covalently Bound Xenobiotics

Recent lengthy reviews (38, 58) have explored this area in exquisite detail, and space limitations do not permit reiteration here. Table 1 lists some of the xenobiotics that have been shown to undergo oxidation to reactive intermediates either in lung or elsewhere, to bind covalently in lung, and to produce toxicities. Some xenobiotics that produce lung injury by as yet unknown mechanisms are noted as well.

Table 1 Some xenobiotics that undergo metabolic activation and bind covalently in lung and/or produce pulmonary injury.

Agent	Covalent binding in lung demonstrated	Reference
Polycyclic aromatic hydrocarbons (benzo[α]pyrene, 3-methylcholanthrene)	+	62
Naphthalenes (naphthalene, 2-methylnapthalene)	+	64, 65
4-Ipomeanol	+	66
3-Methylfuran	+	67
Bromobenzene	+	68
3-methylindole	+	69
Butylated hydroxytoluene (BHT)	+	70
Carbon tetrachloride	+	71
1,1-Dichloroethylene (DCE)	+	72
0,0,S-Trimethylphosphorothioate (contaminant of malathion)	+	73
p-Xylene	+	74
α-Naphthylthiourea (ANTU)	+	58
Pyrollizidine alkaloids (monocrotaline)	+	58
Methylcyclopentadiethyl Mn tricarbonyl		
Bleomycins		
Nitrosoureas		
Mitomycin C		
Melphalan		
Cyclophosphamide	+	75, 76

Space limitations preclude discussion of all the agents in Table 1; because so much information is available on 4-ipomeanol and because many of the agents are thought to act through similar mechanisms, 4-ipomeanol is discussed here as a prototype of metabolically activated pulmonary toxins.

4-Ipomeanol [1-(3-furyl)-4 hydroxy-pentanone] is a natural product secreted by moldy sweet potatoes. Cattle consuming such sweet potatoes developed severe, occasionally fatal pulmonary insufficiency; necropsy revealed severe pulmonary edema and hemorrhage. Most of the published data on the biochemical toxicology of 4-ipomeanol are from the laboratory of Boyd and his coworkers (58).

Administration of 4-ipomeanol to rats, rabbits, guinea pigs, and hamsters results in an organ- and cell-specific lesion, viz, selective necrosis of the nonciliated bronchiolar (Clara) cells of the lung. Larger doses cause less specific effects in which ciliated bronchiolar cells are also involved and which eventually affect the alveolar epithelium and vascular endothelial cells. Larger

doses evoke massive intra-alveolar edema and hemorrhage, together with pleural effusion. Higher doses produce hepatic and renal lesions. In contrast to rabbits, rats, and other species that display pulmonary lesions in response to 4-ipomeanol, adult male mice exhibit renal cortical necrosis as a primary lesion; female mice and immature mice of either sex are remarkedly resistant to this renal insult. Taking advantage of an interesting phylogenetic peculiarity, Buckpitt et al (59) demonstrated that birds (Japanese quail, chickens), whose respiratory tracts lack Clara cells, fail to develop lung damage after 4-ipomeanol. Instead these species develop severe hepatic injury with no evidence of pulmonary involvement such as necrosis, edema, or hemorrhage. In accord with these findings microsomes prepared from lung and liver of rats, guinea pigs, hamsters, and rabbits catalyzed the metabolic activation and covalent binding of 4-ipomeanol in vitro as did microsomes from kidneys of adult male mice. In each case, metabolic activation in vitro correlated with target organ toxicity in vivo. Similarly chicken liver microsomes activated and covalently bound 4-ipomeanol in vitro, while chicken lung microsomes were devoid of activity.

Following parenteral administration of [^{14}C] or [^{3}H]-labeled 4-ipomeanol to rats or rabbits, covalently bound radioactivity is selectively concentrated in lung; smaller amounts are found in liver, kidney, and gut. For example, in rats 24 hr after i.p. administration of 4-ipomeanol (covalently bound material expressed per gram wet weight of organ), liver had 40% that of lung, kidney 25%, and ileum less than 10%. Cell fractionation studies showed that covalently bound material was not concentrated in any particular fraction; in the microsomal fractions, binding was to protein, not phospholipid or RNA. Autoradiography of lungs removed from animals treated 2–4 hours earlier with [^{3}H]-ipomeanol revealed that the covalently bound material was exclusively distributed in Clara cells; adjacent ciliated cells of alveolar epithelial cells were minimally labeled. The covalent binding by Clara cells preceded morphologic evidence of injury by about 12 hours; 16 hours after injection of 4-ipomeanol, Clara cells were undergoing pyknosis and massive cytoplasmic vacualization; necrotic cells were sloughing away from the basal lamina into the bronchiolar lumen.

In vitro studies revealed that when [^{14}C] 4-ipomeanol was incubated with NADPH and subcellular fractions of rat lung and liver, the microsomal fractions were clearly most active in catalyzing covalent binding (60). That metabolic activation of 4-ipomeanol was required before covalent binding could occur was demonstrated by the observations that heat denaturation of microsomes or incubation at 2° essentially abolished binding. Maximum binding occurred in the presence of microsomes, O_2, and NADPH; covalent binding was inhibited by CO, SKF 525-A, and piperonyl butoxide (which implies a role for cytochrome P-450), and also by the presence of cytochrome *c* or an antibody

to NADPH cytochrome *c* reductase (which clearly indicates participation of the latter enzyme). Covalent binding in vitro was also reduced by the presence of reduced glutathione (GSH), which suggests an active electrophile as a metabolic intermediate. Similarly, depletion of tissue stores of GSH in vivo by pretreatment with diethylmaleate markedly increased covalent binding of 4-ipomeanol. Interestingly, it was found that an antibody to cytochrome b_5 blocked the covalent binding of 4-ipomeanol to rat lung microsomes but not to rat liver microsomes. Relatively poor correlation occurred between total levels of cytochrome P-450 and covalent binding; for example, liver and kidney had higher cytochrome P-450 content then lung but lower covalent binding. On the other hand, lung damage and lethal toxicity correlated well with the amount of covalent binding. An interesting tolerance could be produced to 4-ipomeanol; administration of multiple small doses of the agent increased the acute LD_{50} by about threefold or more. This was not due to inactivation of the monooxygenase system, which activates 4-ipomeanol to the reactive electrophilic species as was shown to explain a similar tolerance to CCl_4 (61).

Knowledge as to the chemical nature of the covalent binding species of xenobiotics has been scanty. Only in the past five years has the dihydrodiol epoxide of benzo[α]pyrene been identified as the active species (62) of the hydrocarbon that actually binds to DNA. Studies on the mechanism of furan binding in lung have utilized the analog 3-methylfuran, which has many of the same biological effects as 4-ipomeanol itself, for example pulmonary toxicity and covalent binding. Recent work in Boyd's laboratory (63) showed that in the presence of NADPH and microsomes from rat lung or liver, 3-methylfuran undergoes ring opening at the oxygen with the formation of 2-methyl-2-butene-1,4-dial. The latter compound binds covalently to microsomes and presumably mediates the toxicity of 3-methylfuran. This finding confirms earlier work on structural studies in the 4-ipomeanol series showing that the presence of the furan ring was essential for metabolic activation and covalent binding to microsomes.

Until recent years it was widely believed that electrophilic metabolic intermediates were so reactive that they reacted with biomolecules at or very near their sites of formation. However, recent work has made it clear that active metabolites of bromobenzene, naphthalene, 1,1-dichloroethylene, and undoubtedly other compounds are sufficiently stable so that they may diffuse out of cells and be carried to organs throughout the body where they bind covalently and may exert toxic effects.

KIDNEY

A number of organic compounds are known to be metabolically activated and cause nephrotoxicity. They are discussed in detail elsewhere (77, 78).

Chloroform

Chloroform ($CHCl_3$) is both hepatotoxic and nephrotoxic in most animal species. $CHCl_3$-induced hepatic damage is manifested predominantly as centrilobular necrosis while the renal lesion is necrosis of the proximal tubules. Renal damage is reflected functionally by proteinuria, glucosuria, and increased blood urea nitrogen. In vitro, renal $CHCl_3$ toxicity is accompanied by reduced ability of renal cortical slices to accumulate organic ions such as p-aminohippurate and tetraethylammonium (77).

Administration of [^{14}C]-$CHCl_3$ to mice produced proximal renal tubular necrosis that was accompanied by extensive covalent binding of radiolabel in both kidney and liver. Autoradiograms revealed that in kidney the covalent binding was predominantly localized over the necrotic tubules. Covalent binding of $CHCl_3$ in liver of male and female mice was similar, whereas binding in kidney of male mice was nearly 10 times greater than in females (79).

Abundant experimental work has confirmed that, in mice, $CHCl_3$ nephrotoxicity occurs only in males while the hepatotoxicity is similar in both sexes. The sex difference in nephrotoxicity is apparently mediated by testosterone; immature or castrated male mice are not susceptible to $CHCl_3$ nephrotoxicity whereas female mice or castrated males injected with testosterone are susceptible (80). The high renal sensitivity of male mice to the toxic effects of $CHCl_3$ has been found to correlate with levels of renal cytochrome P-450 and P-450-linked monooxygenases that are 3–5 times higher in males than in females (80). An additional important clue to the possible involvement of metabolism in $CHCl_3$ nephrotoxicity was the observation that $CDCl_3$ was much less toxic than $CHCl_3$ when incubated with kidney slices from male mice. Organic ion uptake by slices was reduced 15–25% by $CDCl_3$ and 70–90% by $CHCl_3$ (81). Incubation of microsomes from the renal cortex of male mice in the presence of [^{14}C]-$CHCl_3$, NADPH, and O_2 resulted in the formation of [^{14}C]-CO_2 and covalently bound material. Microsomes from female mice were inactive.

That the reaction in male kidneys required NADPH and O_2 and was inhibited by CO, SKF 525-A, and metyrapone suggested a cytochrome P-450–dependent mechanism (82). Later work revealed that renal homogenates or microsomes from male mice oxidize $CHCl_3$ in a P-450–dependent reaction to trichloromethanol ($HO\text{-}CCl_3$) that is unstable and spontaneously dehydrochlorinates to yield phosgene ($Cl_2C{=}O$) (83). This step is rate-limiting, and since the C-D bond is stronger than the C-H bond, $CDCl_3$ is less toxic than $CHCl_3$. Phosgene, a highly toxic electrophile, may react with tissue components or with water to give CO_2 and HCl. It can also react with two moles of GSH to form diglutathionyl dithiocarbonate, which in the presence of renal γ-glutamyltranspeptidase and other factors can be converted to the cyclized product 2-oxothiazolidine-4-carboxylic acid (OTZ). Renal glutathione levels are de-

pleted in the process, and pretreatment with diethylmaleate markedly potentiates $CHCl_3$ nephrotoxicity in male mice but not in female mice (84, 85).

Thus, available data suggest that the nephrotoxic species resulting from $CHCl_3$ administration to animals may be phosgene, which is likely formed within the kidney.

Hexachloro-1,3-butadiene

1,1,2,3,4,4-hexachloro-1,3-butadiene (HCBD) is a relatively selective nephrotoxin which when administered to rats causes marked diuresis (i.e. reduced urinary concentrating ability), glucosuria, proteinuria, and increased urinary excretion of alkaline phosphatase and N-acetyl β-glucosaminidase (77). Microscopically one observes selective necrosis of the straight segment (S_3 cells) of the proximal tubules located in the outer zone of the medulla that are characterized by loss of the brush border. Although HCBD is a potent nephrotoxin in rats, it has little morphologic or functional effect in liver. In rats, there is a dramatic sex difference in response to HCBD. For example, a marked depletion of hepatic GSH was observed in male rats after HCBD, whereas no depletion was found in kidney (86). In contrast, female rats, which are much more susceptible to the nephrotoxic effects of this compound, display minimal depletion of hepatic GSH but significant depletion in kidney. Finally, pretreatment of male rats with diethylmaleate, which reduces both hepatic and renal GSH, markedly enhanced the nephrotoxicity of HCBD.

Studies in vitro (87) revealed that incubation of HCBD with glutathione and microsomes or cytosol from either male or female rats resulted in a marked reduction in the GSH concentration. Addition of NADPH to this mixture did not enhance the rate of GSH consumption, which suggests that prior activation of HCBD by a cytochrome P-450 mechanism was not required. Chromatographic and mass spectral analysis of the incubation media allowed characterization of a metabolite, S-(1,1,2,3,4-pentachloro-1,3-butadienyl) glutathione, which indicates formation of an adduct between HCBD and GSH with the elimination of one chlorine. This conjugate was formed at a much faster rate by hepatic microsomes than cytosol (in the presence of exogenous GSH); hence microsomal glutathione S-transferase, as opposed to one of the several cytosolic forms, may predominate in the formation of this adduct, at least in vitro. Adduct formation was uninfluenced by N_2, CO, or the absence of NADPH; the only cofactor requirement was GSH. Glutathione adducts were also formed by renal cytosol from male and female rats but at much slower rates than hepatic fractions.

The toxicology of HCBD and its GSH conjugates was investigated in vivo by Nash et al (88). A nephrotoxic dose of HCBD (200 mg/kg) was administered to male rats, and its excretion into bile was monitored; the major biliary metabo-

lites were a HCBD-glutathione conjugate and smaller amounts of the cysteinylglycine conjugate. The biliary metabolites of HCBD were found to be reabsorbed from the gut and excreted by the kidneys. The glutathione conjugate, its synthetic mercapturic acid derivative, and lyophilized bile containing HCBD metabolites were all nephrotoxic when administered orally to rats. Rats prepared with biliary cannulae that were exteriorized, thus diverting bile from the intestine, were completely protected from renal damage when dosed with HCBD. Thus, it appears that the GSH conjugate of HCBD or one of its degradation products (such as the cysteinylglycine or cysteine conjugate or the mercapturic acid excreted in bile and reabsorbed from the gut) is responsible for the renal toxicity of HCBD. Reabsorption of the conjugate from the gut is more likely after degradation to the lower molecular weight, more lipophilic cysteinylglycine or cysteine conjugates.

Recent work has revealed the presence of an enzyme in renal tubular cells known as cysteine conjugate (C-S) or β-lyase that cleaves the cysteine but not GSH or N-acetylcysteine (mercapturic acid) conjugate to form a reactive fragment containing sulfur (89). The nature of the sulfur is not presently known but it could be a mercaptan (R-C-SH), a sulfenic acid (R-C-SOH), or methylation of the mercaptan followed by oxidation at sulfur could result in products such as R-C-S-CH_3, R-C-SO-CH_3 (methylsulfinyl), or R-C-SO_2-CH_3 (methylsulfonyl) (90). Conjugates of other xenobiotics and GSH are toxic: incubation of 1,2-dibromothane (a carcinogen) with DNA in the presence of GSH and glutathione S-transferases results in formation of the adduct S-[2-(N^7-guanyl)ethyl]glutathione (91). Furthermore, sulfur-containing metabolites that could arise from S-(2-chloroethyl) glutathione or the nephrotoxic S-(2-chloroethyl) cysteine have been isolated from the urine of rats treated with the nephrotoxin 1,2-dichloroethane (91). Thus, although the precise metabolic product of HCBD responsible for its nephrotoxicity is not known, its S-substituted cysteine conjugates or their further metabolism by β-lyase to yield reactive sulfur-containing moieties are likely to be involved in the renal toxicity. Green et al (92) reported that the cysteine conjugate of HCBD is cleaved by β-lyase to the mercaptan that is oxidized to the sulfenic acid (R-C-S-OH), which is both nephrotoxic and highly mutagenic.

It is worthy of note that Miyajima et al (93) have compared the utility of histological and functional criteria in evaluating the nephrotoxicity of $HgCl_2$, $K_2Cr_2O_7$ and cephaloridine utilizing relative kidney weight, uptake of the organic ions p-aminohippurate and tetraethylammonium by slices, BUN, and histology. The authors concluded that renal cortical accumulation of organic ions appeared to be the most sensitive of the functional parameters but that quantitative histological evaluation was equally sensitive as an indicator of nephrotoxicity.

Cephaloridine

Cephaloridine, in contrast to various other cephalosporins and structurally related penicillins, undergoes little or no net secretion by the mammalian kidney (94). Nonetheless, cephaloridine is highly cytotoxic to the proximal renal tubules—the outer stripe of the cortex—which is the segment of the nephron responsible for secreting organic anions such as p-aminohippurate. The cytotoxicity of cephaloridine correlates with high renal cortical drug levels and is reduced or completely prevented by probenecid and other inhibitors of organic ion transport that also reduce the cortical concentration of cephaloridine. The massive accumulation of the drug in the proximal tubular cells appears to result from a paradoxical combination of two factors: active secretion of the drug from the peritubular blood into the cells of the proximal tubule, and a highly limited capacity of the tubular cells to transport the drug into the tubular urine. The very high cortical content of cephaloridine appears to represent and result from a relatively trapped cellular pool in the proximal tubules that is larger than that of p-aminohippurate.

There appears to be a good correlation between renal cortical accumulation of cephaloridine and nephrotoxicity. Thus, administration of the drug to rabbits, guinea pigs, and rats results in highest cortical concentration in rabbit and lowest in rat kidneys. This correlates with the susceptibility of each species to cephaloridine nephrotoxicity. In addition, young animals that did not concentrate cephaloridine to the same extent as older animals, were not as susceptible to cephaloridine nephrotoxicity. Further, as pointed out above, probenecid reduces cortical concentration of cephaloridine and also protects against its nephrotoxicity.

Kuo & Hook (95) studied cephaloridine nephrotoxicity in three species using morphologic and functional criteria. They found that the toxicity was rabbit > rat > mouse and also that cephaloridine produced a dose-related depletion of GSH in renal cortex but not in medulla. The relative susceptibility of the three species to renal GSH depletion paralleled species differences in cephaloridine nephrotoxicity. Pretreatment of animals with diethylmaleate potentiated cephaloridine nephrotoxicity; this strongly suggests a correlation between GSH depletion and cephaloridine toxicity. Kuo et al (96) demonstrated differential effects of phenobarbital pretreatment on cephaloridine toxicity in kidneys of rats unresponsive to the inductive effects of phenobarbital and rabbits that exhibit phenobarbital induction. Phenobarbital induced monooxygenase activities in rabbit kidneys and also potentiated cephaloridine toxicity. Similar treatment of rats had no effect on renal enzymes nor did it affect cephaloridine toxicity. These data suggested that cephaloridine may have been bioactivated in kidney, thus enhancing its toxicity. However, further analysis showed that phenobarbital differentially increased cephaloridine concentration in renal cortex of rabbits (approximately twofold) but had no effect in rats. Thus, the

potentiating effect of phenobarbital on cephaloridine nephrotoxicity in rabbits was more likely due to the increased renal cortical accumulation of cephaloridine, the parent drug, rather than an effect on bioactivation. This experiment also emphasizes the obvious fact that microsomal enzyme inducers frequently have multiple effects.

Bromobenzene

Reid (97) demonstrated that [^{14}C]-labeled bromobenzene administered to mice or rats produced necrosis of the proximal renal tubules that was associated with substantial covalent binding of radiolabel in the necrotic cells. Prior treatment of animals with piperonyl butoxide blocked both the covalent binding and the necrogenic effects of bromobenzene. It was concluded that an activated metabolite of bromobenzene (not the parent compound) was responsible for the covalent binding and the renal tubular necrosis and that the metabolite was formed in the liver and transported to the kidney. Bromobenzene is now known to be initially activated by a cytochrome P-450 mechanism to its 2,3- and 3,4-oxides, which may then undergo enzymatic and nonenzymatic conversion to a number of products, among them o- and p-bromophenol, which in turn can be converted to 4-bromocatechol, 4-bromoquinone or hydroquinone, or 2-bromoquinone or hydroquinone (98). o-Bromophenol caused a 50% reduction in renal GSH in rats within ninety minutes, whereas hepatic GSH levels were reduced by only 20% after five hr. Thus, renal GSH was far more susceptible to the initial depleting effects of o-bromophenol than hepatic GSH. In rats, o-bromophenol caused severe renal necrosis associated with elevations in BUN levels (2–3-fold). Liver microsomes converted o-bromophenol to covalently bound material while kidney microsomes did not (99). However, in vivo, o-bromophenol covalently bound to kidney protein in amounts four times greater than to liver protein. Phenobarbital treatment increased covalent binding in vivo to kidney and also increased BUN levels but did not increase covalent binding to liver, which indicates that the nephrotoxic metabolite of o-bromophenol may be generated in liver and transported to kidney.

2-Bromohydroquinone was identified as a metabolite of both bromobenzene and o-bromophenol in the rat (98). Administration of 2-bromohydroquinone to rats caused a dose- and time-dependent decrease in hepatic and renal GSH levels, an increase in BUN, and histopathological changes in kidney (proximal tubular necrosis) without causing alterations in liver. The histologic changes in kidney were indistinguishable from those produced by either bromobenzene or o-bromophenol, but the dose of 2-bromoquinone required to produce similar nephrotoxicity was $< 10\%$ that of bromobenzene.

Following administration of bromobenzene to mice (100), p- and o-bromophenol were the major urinary metabolites, although m-bromophenol and 4-bromocatechol were also excreted, all as conjugates. 4-Bromocatechol

and 3-bromophenolic isomers were all nephrotoxicants, measured in vivo as increased BUN or in vitro as impaired accumulation of organic anions by renal cortical slices; but they were not hepatotoxicants at equal doses. 4-Bromocatechol was the most nephrotoxic of the four compounds both in vitro and in vivo, resulting in a dose-dependent decrease in renal function while hepatic function was altered only slightly at the higher doses. The renal cortical necrosis produced by 4-bromocatechol administration to mice could not be distinguished histologically from that evoked by bromobenzene.

Therefore, depending on the species, it would appear as if 2-bromohydroquinone or 4-bromocatechol or some metabolite—oxidative or conjugative—is the proximate nephrotoxin derived from bromobenzene. This question remains open (see note added in proof at end of text).

Acetaminophen

About the same time that the hepatotoxicity of large doses of acetaminophen was being studied vigorously, McMurtry et al (101) reported that a mechanism similar to that being described in liver was apparently involved in a nephrotoxicity of acetaminophen. Thus, a single large dose of acetaminophen (~1 g/kg) administered to Fischer 344 rats elicited a dose-dependent acute renal necrosis confined primarily to the distal portions of the proximal tubule (pars rectus). Glomeruli and early proximal convolutions were largely unaffected. These doses of acetaminophen caused both hepatic and renal necrosis, markedly depleted target organ GSH, and resulted in large amounts of radiolabeled metabolite being bound to renal and hepatic protein. It was concluded that this tissue injury resulted from the activation of acetaminophen in situ to a chemically reactive species capable of covalently binding to tissue macromolecules. That the metabolic activation occurred in situ was demonstrated by Breen et al (102), who showed, that after administration of ^{3}H-acetaminophen to rats, covalent binding occurred in both kidney and lung, and that the binding in these tissues was unaffected by total hepatectomy.

Newton and co-workers (103) demonstrated a marked strain difference in rats in susceptibility to acetaminophen-produced renal damage. Acetaminophen produced renal necrosis—restricted to the straight segment of the proximal tubule—in Fischer 344 rats but not in Sprague-Dawley rats. Acetaminophen-induced renal functional changes such as elevation in BUN and impairment in the accumulation of p-aminohippurate by renal cortical slices also correlated with strain-dependent histopathologic changes. However, covalent binding of ring-labeled acetaminophen to renal and hepatic microsomal protein in vitro was the same in the two rat strains as was deacetylation of acetaminophen to p-aminophenol. Thus the strain differences in acetaminophen could not be attributed to differences in P-450-linked activation of parent drug or in the deacetylation to the nephrotoxic metabolite, p-aminophenol. Administra-

tion of p-aminophenol to Fischer and to Sprague-Dawley rats resulted in renal lesions in both strains indistinguishable from the acetaminophen-induced renal lesion in Fischer rats. However, covalent binding of p-aminophenol to renal microsomes in vitro was much greater in the sensitive Fischer rats than in the resistant Sprague-Dawley strain. In addition, covalent binding of p-aminophenol or a metabolite thereof was 5–10 times greater in kidney than in liver. It was concluded that strain differences in acetaminophen nephrotoxicity in rats might result from strain differences in the intrarenal metabolic activation of the metabolite p-aminophenol to an arylating species (104).

TESTIS

1,2-Dibromo-3-chloropropane

The literature reveals a pronounced dichotomy in our knowledge of the biological properties of 1,2-dibromo-3-chloropropane (DBCP). A rather great deal is known about the clinical effects of the compound both in humans and experimental animals but little information is available at the molecular level.

Other than renal tubular necrosis observed at high doses (105), the predominant effect of DBCP is gonadal hypofunction, seen only in males. DBCP is an effective nematocide when applied to soil or a variety of edible plants such as peaches, citrus fruits, tomatoes, bananas, and pineapples. Male workers employed in the manufacture of this compound were observed to have a severe impairment of spermatogenesis, azoospermia, and elevated plasma FSH and LH levels (106). Oligospermia was found in workers with lower exposure levels. Testicular biopsy showed selective atrophy of the germinal epithelium (spermatocytes and spermatids); Sertoli cells, from which spermatocytes are generated, were histologically normal as were Leydig (androgen-secreting) cells. No necrotic or inflammatory changes were observed in the seminiferous tubules, merely an absence of spermatogenic activity (107). In humans, the severe oligospermia resulting from DBCP exposure was slowly reversible, requiring 18–21 months following discontinuation of exposure; recovery occurred histologically and in sperm counts (108). Rao et al (109) exposed rabbits to concentrations of 0.1, 1, and 10 ppm DBCP by inhalation for 8–14 weeks. Sperm counts and breeding success followed a dose-response curve. Testicular atrophy was noted in the two highest dose groups (the intermediate dose produced 50% loss of testicular weight at 14 weeks).

Serum testosterone levels were comparable to controls or slightly elevated in exposed animals. Reversibility of these testicular alterations was proportional to dose. Animals exposed to the highest concentration of DBCP had oligospermia and abnormal testicular histology 38 weeks after cessation of exposure. It is worth noting that male mice have been reported to be resistant to the testicular effects of DBCP (110).

DBCP might be expected to undergo conjugation with GSH in the presence of glutathione S-transferase, and this reaction has indeed been demonstrated in vitro (111). Jones and co-workers (112) administered DBCP to rats and reported formation of 1-chloro-2,3-propanediol (α-chlorohydrin) and 1-bromo-2,3-propanediol (α-bromohydrin), formed through the intermediacy of the 2,3-epoxide. They also isolated S-(2,3-dihydroxypropyl)cysteine and 1,3-(bis-cysteinyl)propan-2-ol from urine. Sixteen days after i.p. administration of DBCP to male rats (50 mg/kg), testicular weight was reduced by nearly 50%; body weight and kidney weight were unaffected. The isolation of α-chlorohydrin was of great importance since earlier work (113) had shown this compound to be a potent chemosterilant in male rats. Low doses of α-chlorohydrin (10 mg/kg for 14 days) had been shown, based on serial matings, to have pronounced antifertility activity in male rats, activity apparent within 3–6 days after first administration; cessation of exposure was followed by return to normal fertility. This effect was also shown to occur in guinea pigs, hamsters, rhesus monkeys, rams, and boars and was not accompanied by alterations in sperm count, motility, or morphology. The mechanism of this antifertility effect was unexplained. Higher doses of α-chlorohydrin were accompanied by testicular atrophy and a blockage of the efferent ducts of the epididymis resulting in spermatoceles and sperm-granulomata.

Kluwe et al (113a) compared in male rats the gonadal toxicity of DBCP and its metabolites 3-chloro-1,2-propane epoxide (epichlorohydrin), 3-chloro-1,2-propanediol (α-chlorohydrin), and oxalic acid. Although a threefold difference in potency was noted (DBCP was most active, α-chlorohydrin least active), DBCP and its two halogenated metabolites produced similar impairment of testicular function while oxalic acid was inactive. DBCP, epichlorohydrin, and α-chlorohydrin produced progressive testicular atrophy and hypospermatogenesis; the latter two compounds also produced sperm granulomas, spermatoceles and an increased number of morphologically abnormal spermatozoa (113a). Following a single dose of DBCP, atrophic seminiferous tubules devoid of spermatazoa persisted for as long as 25 days and testicular atrophy (~50%) persisted as long as 75 days.

Metabolic studies with α-chlorohydrin (3-chloro-1,2-propanediol) in male rats revealed a number of products, among them: loss of chlorine resulting in the formation of 1,2-epoxy-3-propanol (glycidol) followed by conjugation with GSH at the epoxy function resulting in the 1-cysteine and N-acetylcysteine derivatives [S-(2,3-dihydroxypropyl)cysteine] and the mercapturic acid [N-acetyl-S-(2,3-dihydroxypropyl)cysteine] (113).

It should be noted that compounds chemically related to DBCP have been reported to provoke testicular atrophy, aspermatogenesis, epididymal lesions, and other reproductive abnormalities in male animals. Among these are chloromethane (methyl chloride) (114), 1,2-dibromomethane (115), tris[tris(2,3-

dibromopropyl)phosphate] (116), chlordecone, carbon disulfide, vinyl chloride, and 2-chlorobuta-1,3-diene (chloroprene) (117).

Benzo[α]pyrene

The metabolism of benzo[α]pyrene by the isolated perfused rat testis and by testicular homogenates has been described (118, 119) but no effort was made to relate this metabolism to toxicity.

OVARY

Benzo[α]pyrene

Mattison & Thorgeirsson (120) reported that aryl hydrocarbon hydroxylase (AHH) activity was localized in the microsomal fraction of murine ovary, was NADPH and oxygen dependent, and was inhibited by CO and by an antibody to NADPH cytochrome *c* reductase. Pretreatment of animals with 3-MC increased ovarian AHH activity in three rat strains (about threefold), and nearly doubled activity in C57B1/ 6N (B6) mice but had no affect in DBA/2N (D2) mice. Interestingly, the toxicity of 3-MC to oocytes in mice and rats was dramatically different. A histologic technique was devised that permitted quantitative analysis of all the oocytes in a whole ovary. It was found that 7 days after a single i.p. dose of 3-MC (80 mg/kg), 87% of the primordial oocytes were destroyed in the ovaries of B6 mice, 69% were destroyed in D2 mice, but there was no change in oocytes in the ovaries of Sprague-Dawley rats. The data were interpreted to suggest that the oocyte toxicity of 3-MC in mice was related to ovarian AHH activity whereas no association was apparent between ovarian AHH activity and oocyte toxicity in the rat. Moreover, in mice, there is a striking relationship between oocyte destruction and ovarian granulosa cell tumor formation (121). Later work confirmed that ovarian AHH activity in D2 mice was relatively unresponsive to pretreatment with 3-MC but was induced 2–3-fold in B6 mice; basal ovarian AHH activity was similar in both strains (122). However, primordial oocytes of both D2 and B6 mice were destroyed by the carcinogenic hydrocarbons 3-MC, BP, and 7,12-dimethylbenz[α]-anthracene (DMBA) but not by the noncarcinogens pyrene, and α- and β-naphthoflavone (Table 2). The rate of oocyte destruction was faster in responsive B6 mice than in nonresponsive D2 mice. Sprague-Dawley rats were consistently less responsive to the destruction of primordial oocytes by BP, 3-MC, and DMBA than either D2 or B6 mice (123).

Pretreatment of female DBA/2N mice with BP reduced their fertility in a dose-dependent manner; mice receiving a single i.p. dose of BP of 200 or 500 mg/kg were completely infertile when mated 14 days later (Table 3) (124). This was in accord with earlier suggestions that tobacco smoke, which contains BP, might decrease fertility and produce a premature menopause (125) in humans.

Table 2 Effect of various polycyclic hydrocarbons on residual primordial oocytes six days after administration to mice[a]

Treatment[b]	Number of primordial oocytes/ovary	
	DBA/2N mice (D2)	C7B1/6N mice(B6)
Control	5240	3700
β-Naphthoflavone	4945	3555
Pyrene	4240	3216
BP	4493	1233[c]
3-MC	2763[c]	33[c]
7,12-Dimethybenz[α]anthracene	897[c]	20[c]

[a]Taken in part from Mattison & Thorgeirsson, with permission (122).
[b]Compounds administered in a single dose, 80 mg/kg, i.p., 6 days prior to sacrifice.
[c]Significantly different from control ($p < .05$).

BP administered to mice also destroyed primordial oocytes in a dose-related manner but did not reduce the ovarian response to pregnant mare's serum gonadotropin, which suggests that BP acted upon primordial oocytes and not upon growing or preovulatory oocytes or follicles (124). Because α-naphthoflavone, a competitive inhibitor of BP metabolism, blocks the destruction of primordial oocytes, it was suggested that this destruction depends in part on the formation of reactive ovotoxic metabolites with destruction occurring more rapidly in mice with higher rates of ovarian metabolism.

Female C57B1/6N(B6) and DBA/2N mice were dosed with 80 mg/kg i.p. of several polycyclic hydrocarbons. BP, 3-MC, and DMBA destroyed primordial oocytes and the rate of destruction was proportional to the (induced) ovarian AHH activity. After treatment with BP or 3-MC only primordial oocyte destruction occurred with no evidence of toxicity in contiguous granulosa or ovarian stromal cells. DMBA was more generally toxic and destroyed large follicles and oocytes in addition to primordial oocytes and primary follicles. Seven weeks after 3-MC, the ovary had the afollicular histologic appearance of ovarian failure. All three of these hydrocarbons (BP, 3-MC, DMBA) are capable of producing premature ovarian failure in rodents (126).

BP in doses ranging from 10 ng to 10 μg was injected (1 μl corn oil) directly into the ovaries of B6 and D2 mice (126a). Bilateral intraovarian injection of BP was ovotoxic in a time- and dose-related manner. Corn oil alone was without effect. Primordial oocyte destruction was maximal eight days after injection in both mouse strains. Unilateral injection destroyed oocytes only in the treated ovary and i.p. administration of α-naphthoflavone inhibited the oocyte destruction by the highest dose (10 μg) of BP in both strains. Fourteen days after bilateral intraovarian injection, approximately 80% of the primordial oocytes had been destroyed in B6 mice and about 50% in D2 mice. Bilateral in-

Table 3 Effect of benzo[α]pyrene (BP) treatment on some reproductive parameters in female DBA/2N mice[a]

BP dose (mg/kg)	Reproductive performance		Primordial oocytes/ovary	Oocyte destruction (% control)
	Total offspring (12 weeks)	Pups/mouse week		
0	137	0.91	4791	0
5	—	—	3940	18
10	91	0.61	3908	19
50	. ..	—	2086	56
100	28	0.20	580	88
200	0	0	—	—
500	0	0	0	100

[a]Taken in part from Mattison et al with permission (124).

traovarian injection of BP at 10 μg/ovary had no effect on hepatic AHH or cytochrome P-450 content in either D2 or B6 mice (126a).

Cyclophosphamide

Cyclophosphamide is known to produce sterility in female animals exposed either pre- or postnatally (127). Seven days after a single dose of cyclophosphamide (100 mg/kg), 63% of the primordial follicles of C57B1/6N mice were destroyed. In young rats, destruction of primordial oocytes by cyclophosphamide decreased with age as ovarian GSH levels were increasing (128). Studies on oocyte and follicle destruction by cyclophosphamide in Sprague-Dawley rats and in C57B1/6N and DBA/2N mice revealed that primordial oocytes were most sensitive to destruction. Destruction depended on time, dose, strain, and species; B6 mice were most sensitive to this effect. These results are of particular interest in view of the fact that oocyte destruction and premature ovarian failure is a significant side effect in women treated with alkylating agents (129, 130).

BONE MARROW

Benzene

Exposure to chemicals in the plastics, chemical, and rubber industries and in other areas of the workplace remains a significant public health problem in developed areas of the world. Subacute and chronic benzene toxicity is usually manifested as bone marrow hypoplasia in the form of leukopenia, anemia, thrombocytopenia, and pancytopenia, which may progress to aplastic anemia or leukemia (131). Humans appear to be more susceptible to the leukemogenic action of benzene than most animal species; workers exposed chronically to

benzene appear to die in approximately equal numbers from aplastic anemia and leukemia (acute myelogenous or aleukemic) (132), while animals usually succumb to bone marrow aplasia.

Benzene exposure—unless otherwise stated, subacute or chronic exposure is to be assumed—elicits generalized bone marrow depression resulting in reduced numbers of circulating erythrocytes, granulocytes, thrombocytes, lymphocytes, and monocytes. There is a general agreement (131) that benzene is preferentially toxic toward progenitor cells of intermediate differentiation while sparing mature nondividing cell types. In the red cell series for example, early erythroblasts are more sensitive to benzene than stem cells, reticulocytes, or mature erythrocytes.

Irons and co-workers (133) reported that in male Fischer 344 rats exposed to benzene, peripheral lymphocytes and differentiating bone marrow precursor cells were the most sensitive cell populations. Benzene exposure resulted in an increase in the relative number of bone marrow precursor cells in G_2 or M phase of the cell cycle and a reduction in the uptake of ^{3}H-thymidine into bone marrow DNA. As noted above, analysis of bone marrow smears indicated an early cytotoxic effect of benzene on cells of intermediate differentiation (promyelocytes, myelocytes, basophilic and polychromatophilic erythroblasts) irrespective of cell line, with a relative sparing of blast forms and mature nondividing cell types.

Current evidence strongly suggests that a benzene metabolite mediates the myelotoxicity of benzene (132). Metabolites of benzene that have been isolated from biological sources such as urine or microsomal incubations include phenol, catechol, hydroquinone, benzoquinone, 1,3,5-trihydroxybenzene, *trans,trans*-muconic acid, 1,2-dihydro-1,2-dihydroxybenzene (1,2-dihydrodiol)(all isolated either free or as the sulfate or glucuronide conjugates), and phenylmercapturic acid. The evidence favoring a metabolite as the myelotoxic species, rather than benzene itself, has been reviewed elsewhere (132). Andrews et al (134) reported AHH activity in the bone marrow (femurs, tibia) of rabbits that was localized in the microsomal fraction, was NADPH dependent, was inhibited by CO, and was inducible by pretreatment with 3-MC. Fractionation of the bone marrow revealed that most of the total activity was localized in white cells, i.e. immature granular leukocytes.

Later work (135) showed that benzene could be hydroxylated to phenol and an unidentified metabolite by an analogous in vitro system from rabbit bone marrow that contained cytochrome P-450 and NADPH cytochrome *c* reductase. Irons et al (136) reported that benzene could be metabolized and covalently bound in bone marrow in situ. Fischer 344 rats received ^{14}C-benzene instilled directly into the bone marrow space of the distal head of the femur, and the hind limb was subjected to perfusion with whole rat blood for up to sixty minutes. Blood and bone marrow were analyzed for benzene and metabolites and the

authors found phenol, catechol, hydroquinone, and unknown metabolites covalently bound to bone marrow. Gollmer et al (137) compared the benzene monooxygenase system of bone marrow with that of liver from rabbits and demonstrated that pretreatment with benzene did not affect O-dealkylation in bone marrow but stimulated benzene metabolism and covalent binding of ^{14}C-benzene metabolites. It was also concluded that bone marrow and liver develop separate patterns of cytochrome P-450 isozymes.

Data presented to this point established that both liver and bone marrow possess monooxygenase systems that metabolize and activate benzene to reactive intermediates able to bind covalently to tissue macromolecules. The question remaining was the relative importance of liver and bone marrow in the production of myelotoxic benzene metabolites. This question was largely resolved in a series of publications from Snyders laboratory. Administration of benzene to control (sham-operated) rats caused a 50% inhibition in the incorporation of ^{59}Fe into erythrocytes, an index of erythrogenesis, and urinary excretion of benzene metabolites (138). Partial hepatectomy (75%) decreased the metabolism of benzene by 70% and completely protected animals against benzene-induced hemopoietic toxicity when compared to sham-operated animals that received benzene.

This finding that partial hepatectomy decreases whole body benzene metabolism and protects against benzene hemopoietic toxicity indicates that the liver may play a primary role in the development of benzene-induced bone marrow toxicity. Further support for this view came from the finding that benzene metabolism (covalent binding) and toxicity correlated well in mice of different strains (139). ^{14}C benzene administration to DBA/2N mice produced greater inhibition of ^{59}Fe incorporation into erythrocytes and higher amounts of covalently bound material (^{14}C) in bone marrow and liver than in the less sensitive C57/B6 strain. The concept relating metabolism of benzene to bone marrow hypoplasia was further supported by Andrews et al (140), who reported that the administration of ^{3}H-benzene to mice resulted in decreased incorporation of ^{59}Fe into erythrocytes and the accumulation of benzene and its metabolites in bone marrow and other tissues. Coadministration of toluene, shown to be a competitive inhibitor of benzene metabolism in vitro, protected mice against benzene-induced depression of red cell ^{59}Fe uptake, reduced the urinary excretion of benzene metabolites, and reduced by 50% or more the accumulation of ^{3}H-benzene metabolites in bone marrow, blood, liver, spleen, and fat without affecting levels of ^{3}H-benzene itself in these tissues. This and other work gives rise to some general principles regarding the link between benzene metabolism and myelotoxicity:

1. The good correlation between reduction in urinary benzene metabolites and reduced bone marrow suppression suggests that benzene metabolism is closely related to its toxicity.

2. Toluene markedly inhibited benzene myelotoxicity and reduced bone marrow levels of benzene metabolites, but it had no effect on those of benzene itself. Thus, benzene metabolites and not benzene per se must mediate bone marrow suppression.
3. Bone marrow accumulated four times more benzene metabolites than liver in the first eight hours after benzene administration. Metabolites in bone marrow persisted far longer than those in fat, spleen, liver, and blood [approximate tissue levels of total benzene metabolites in tissues eight hours after ^{3}H-benzene administration (nmoles/g): bone marrow, 550; liver 25; blood 20; spleen 15; fat 5].

The chemical mechanism of benzene activation (141) utilized a reconstituted system from rabbit liver microsomes. NADPH, cytochrome P-450$_{LM2}$ (form induced by phenobarbital), NADPH cytochrome P-450 reductase, and phospholipids were incubated with ^{14}C-benzene and its conversion to phenol and water-soluble metabolites studied. These reactions were inhibited by catalase, horseradish peroxidase, superoxide dismutase, and several hydroxyl radical scavengers, indicating the participation of H_2O_2, $O_2^{\cdot-}$, and ·OH in the process. Microsomal benzene metabolism was inhibited by six different ·OH scavengers. Biphenyl was formed in the reconstituted system indicating the cytochrome P-450–dependent production of a hydroxycyclohexadienyl radical as a consequence of interactions between ·OH and benzene. The formation of benzene metabolites covalently bound to protein was efficiently inhibited by free radical scavengers but not by epoxide hydrolase.

Returning to the morphological aspects of benzene-induced myeloid cytotoxicity, Wierda et al (142) reported that treatment of mice with benzene produced a dose-dependent inhibition of splenic T- and B-lymphocyte responsiveness to mitogens and a reduction in the capacity of antigen-reactive precursors of B-lymphocytes to generate antibody-producing cells. In addition to studies of the effects of benzene on bone marrow precursors of various circulating blood cells, Gaido & Wierda (143) studied its effects on bone marrow stromal cells since the latter form a supporting matrix for the developing cells and may influence and regulate hemopoetic cell development. Benzene metabolites were examined for their toxicity on mouse bone marrow stromal cell colony formation, and their TD_{50}s (given as μM concentration) were as follows: hydroquinone was most toxic (2.5 μM), followed by p-benzoquinone (17 μM), 1,3,5-benzenetriol (59 μM), catechol (124 μM), and phenol (189 μM). Finally, the benzene metabolites, hydroquinone and p-benzoquinone, inhibited mRNA synthesis (measured as RNA polymerase II) by rabbit bone marrow in a concentration dependent manner; they were equipotent and showed IC_{50} values in the range of 6 μM. Catechol and 1,3,5-benzenetriol were less potent with IC_{50} values near 0.1 mM; phenol did not inhibit mRNA synthesis even at concentrations of 1 mM (144).

In a preliminary outline for this review, we included skin, urinary bladder, gastrointestinal tract, adrenal, uterus, placenta, breast, pancreas, and thymus, various white blood cells, and other reticuloendothelial tissues. Space did not permit their inclusion but it is hoped that a subsequent review will do so.

CONCLUSIONS

Because of the attempted scope of this review, it is spotty, incomplete, and in some places less than thorough. We hope to have made the point that in most instances our knowledge of the precise biochemical mechanisms involved in toxic responses at the cellular or tissue level are scantily understood at best. A prominent feature of this ignorance is the current lack of reliable criteria by which we can determine, in cells in situ, cell death in biochemical terms. It is hoped that these many opportunities to gain insight into interesting and important biological problems will attract and appeal to enthusiastic young investigators.

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NOTE ADDED IN PROOF A recent report showed that glutathione conjugates of 2-bromophenol or 2-bromoquinone are the most nephrotoxic compounds yet discovered in the series (> 300 times more toxic than bromobenzene) (Monks, T. J., Lau, S. S., Highet, R. J., Gillette, J. R. 1985. Glutathione conjugates of 2-bromohydroquinone are nephrotoxic. *Drug Metab. Dispos.* 13:553–59).