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Chemically Reactive Intermediates and Pulmonary Xenobiotic Toxicity

THEODORE E. GRAM[a]

Chief, Section on Drug Interactions (retired), Developmental Therapeutics Program, National Cancer Institute, National Institutes of Health, Bethesda, Maryland

[a] Address correspondence to: Theodore E. Gram, 1036 Welsh Drive, Rockville, MD 20852-1202.

I. Introduction

A. Activation of Xenobiotics to Chemically Reactive Intermediates

The biochemical literature abounds with reviews of drug oxidation and conjugation by and toxic effects in liver (Gillette, 1974a,b). Although it was once believed that drug-induced hepatotoxicity was the result of some inherent property of the native drug, more modern data have implicated chemically reactive intermediates as being ultimate toxicants (Guengerich and Shimada, 1991; Conney, 1982; Hinson and Roberts, 1992). It was suggested that chemically inert drugs were activated in vivo to metabolic products that were capable of forming covalent bonds with proteins, nucleic acids, and other endogenous substances, and the adducts were capable of inducing carcinogenesis or tissue (and cellular) necrosis (Brodie, 1967). In fact the large research field, which is now known under the generic term "drug metabolism," was presaged by investigations in the field of chemical carcinogenesis. Pioneer work initiated by the Millers and their associates (reviewed with verve and humanity by J.A. Miller, 1994) with the aminoazodyes revealed that dye residually bound to rat liver microsomes could not be removed by extraction with hot or cold mineral acids or alkali or organic solvents but were released upon digestion of liver proteins by proteases. It was thus concluded that the dye must be bound in liver by covalent bonds. After withdrawal of rats from N,N-dimethylaminoazobenzene (DAB[b]), dye disappeared from livers with a time required for plasma (or tissue) drug content to decline to 50% of peak value ($t_{½}$) of approximately 4 days, which was in accord with the turnover times of most rat liver proteins. In addition, DAB is selective in causing only liver tumors, and no covalently bound dye could be found in other organs. Moreover, very low or

[b]Abbreviations: 3-AB, 3-aminobenzamide; 3-MC, 3-methylcholanthrene; 3-MI, 3-methylindole; ACE, angiotensin converting enzyme; AT, adenine-thymine base pairs; atm, atmosphere; ATP, adenosine triphosphate; β-NF, β-naphthoflavone; BHT, butylated hydroxytoluene; BHT-QM, butylated hydroxytoluene-quinone methidine; BLM, bleomycin; BP, benzo[a]pyrene; BP 7,8-dihydrodiol, (±)-trans-7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene; (+) BPDE2, (+)benzo[a]pyrene diol epoxide 2; BSO, buthionine sulfoximine; CCl_4, carbon tetrachloride; cDNA, complementary DNA; CO, carbon monoxide; DAB, N,N-dimethylaminoazobenzene; dBLM, desamido-bleomycin; DCE, 1,1-Dichloroethylene; DDPD, N,N′-diphenyl-p-phenylenediamine; DEM, Diethylmaleate; diol epoxide 1, (±)-trans-7β,8α-dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydrobenzo[a]-pyrene; diol epoxide 2, (±)-trans-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene; GC, guanine and cytosine base pairs; GSH, glutathione; GSH-Px, glutathione peroxidase; GTA, 2-S-glutathionyl acetate; H_2O_2, hydrogen peroxide; HOCI, hypochlorous acid; HPLC, high-performance liquid chromatography; HPRT, hypoxanthine (guanine) phosphoribosyntransferase; ip, intraperitoneal; LDH, lactate dehydrogenase; LI, labeling index; LT_{50}, time to 50% of deaths; MDA, malondialdehyde; mRNA, messenger ribonucleic acid; MS, mass spectrometry; NAD, nicotinamide adenine dinucleotide; NADPH, reduced nicotinamide adenine dinucleotide phosphate; NMR, nuclear magnetic resonance; NNK, 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, N-nitrosonornicotine; O_2^-, superoxide anion radical; O^6-MGMT, O^6-methylguanine-DNA methyltransferase; ·OH, hydroxyl radical; OOSTMP, O,O,S-trimethylphosphorothioate; OSSTMP, O,S,S-tri-methylphosphorothioate; PAP, nuclear poly (ADP-ribose) polymerase; PB, phenobarbital; PEITC, phenethyl isothiocyanate; RER, rough endoplasmic reticulum; ROS, reactive oxygen species; SEM, scanning electron microscopy; SER, smooth endoplasmic reticulum; SKF 525-A, 2-diethylaminoethyl-2,2-diphenylvalerate; SOD, superoxide dismutase; $t_{½}$, time required for plasma (or tissue) drug content to decline to 50% of peak value; TBA, thiobarbituric acid; TEM, transmission electron microscopy; TIDAL, time-integrated DNA adduct levels.

undetectable levels of bound dye could be found in livers of mice, guinea pigs, rabbits, or chickens, species that are essentially resistant to the carcinogenic effects of DAB (Miller, 1994).

In approximately 1968 or 1969, B.B. Brodie, while vacationing in Australia, was visiting a sheep station where oral doses of carbon tetrachloride (CCl_4) were routinely administered to the animals as an anthelmintic. He was told that almost all the sheep tolerated a standard dose of CCl_4 well, but an occasional animal died of hepatic necrosis. It was known that pretreatment of sheep with phenobarbital (PB) greatly increased the hepatotoxicity of CCl_4 (Costa et al., 1989). This information promptly ushered into existence, in pharmacology and toxicology, the enormously productive area of the activation of chemically inert drugs and their conversion in vivo into highly reactive metabolites that evoked cyto- and histotoxicity either by forming covalent bonds with critical cellular macromolecules or by stimulating the formation of highly toxic oxygen metabolites such as hydrogen peroxide (H_2O_2), superoxide anion radical (O_2^-), hydroxyl radical (·OH), or singlet oxygen ($^1O_2$). Reactive oxygen species (ROS) usually intitiate toxicity by stimulating lipid peroxidation of highly unsaturated fatty acids (e.g., 20:4, 22:6) present in phospholipids that form an integral part of the biomolecular leaflet present in virtually all biological membranes. Cytotoxins may also act by altering cellular glutathione (GSH), calcium, and energy homeostasis.

If, indeed, ontogeny recapitulates phylogeny, cytochrome P450 enzymes should be practically absent in newborn mammals as well as more phylogenetically primitive animals, such as reptiles and fish (Brodie and Maickel, 1962). This is, indeed, the case.

In evolutionary terms, as animals left sea water as a habitat and began to inhabit the land (fish to amphibians; frogs and reptiles to mammals), they encountered a new population of pernicious substances such as alkaloids, terpenes, and steroids, lipid-soluble compounds present in their new diet of aerial plants. It has been argued on teleological grounds that plants, in turn, evolved to produce these noxious and toxic substances to ward off their herbivorous intruders and thus insure their own survival. Many of these phytotoxins were lipid-soluble, and because the mammalian kidney behaves like a large lipid membrane, mammals had to evolve a mechanism for converting lipophilic compounds into hydrophilic ones that could be readily excreted. In liver, this occurred by evolution of a hydroxylating system, a hydrolytic system, and a conjugation system. In addition to converting a lipophile into a hydrophile, these reactions converted the substrate from one possessing significant biological activity (morphine, atropine, ethanol, and cocaine) to products that were essentially biologically inert. Oxidation (hydroxylation) requires cytochrome P450, nicotinamide adenine dinucleotide phosphate (NADPH), O_2, and NADPH cytochrome c reductase, is the most common primary detoxication mechanism, and occurs with hundreds of drugs. Hydrolysis totally inactivates esters, drugs such as procaine, cocaine, and succinylcholine. Finally, once a functional group (such as hydroxyl or primary amine) has been added or unmasked, conjugation occurs using cosubstrates such as glucuronic acid, sulfate, acetyl groups, GSH, or free amino acids. Many xenobiotics are pharmacologically active but toxicologically inactive. Some of these compounds are metabolized to biologically inert products whose physical properties (e.g., water solubility) allow them to be rapidly cleared from the body in the urine or bile. Biological inactivation of xenobiotics often occurs by oxidation, often hydroxylation, which is followed by conjugation to glucuronides, sulfates, GSH (or other amino acid) derivatives. Occasionally, however, xenobiotic substrates are converted to highly chemically reactive products that can react covalently with vital cellular macromolecules such as nucleic acids, proteins, and lipids and dramatically alter or inactivate the normal physiological (biochemical) function of the critical cellular macromolecule. Chemically reactive foreign compounds may produce the whole plethora of cytotoxicities including dedifferentiation, transformation to benign or malignant tumors, teratogenesis, mutagenesis, or necrosis. In most cases however, the mechanisms and the chemical nature of the adducts formed between reactive intermediates and the tissue macromolecules that produce these pathological changes are unknown.

Early work resulted in the facile conclusion that covalent binding of xenobiotics in tissues resulted a priori in necrosis. These conclusions, however, proved to be overly simplistic. In the case of bromobenzene, it was shown (Monks et al., 1982) that p-bromophenol, a metabolite of bromobenzene, binds covalently in both liver and kidney in vivo but is not toxic to either organ. Thus, it was evident that the amount of covalently bound metabolite, per se, in a tissue could not predict toxicity of any series of compounds. Further, it was found that the antioxidant N,N′-diphenyl-p-phenylenediamine (DDPD) prevented bromobenzene-mediated cell death in cultured hepatocytes without affecting the extent of covalent binding (Casini et al., 1982). Using acetaminophen (Labadarios et al., 1977; Devalia et al., 1982), it was reported that pretreatment of animals with certain chemicals could prevent acetaminophen hepatotoxicity but had little effect on its covalent binding in tissues.

Nonetheless, there is a mountain of data indicating a close correlation between covalent binding of several exogenous compounds and cellular necrosis (Hinson and Roberts, 1992; Conney et al., 1994; Cho et al., 1995). Thus, factors that reduce xenobiotic activation and binding such as $CoCl_2$, 2-diethylaminoethyl-2,2-diphenylvalerate (SKF 525-A), and piperonyl butoxide also reduce necrosis. In addition, factors that increase binding such as pretreatment with PB or 3-methylcholanthrene (3-MC) tend to increase toxicity. Moreover, covalent bind-

ing is highest over frankly necrotic regions and dramatically less over cells that are spared. Treatments that deplete cells of endogenous protectants such as GSH and α-tocopherol markedly enhance covalent binding and cellular and tissue toxicity, whereas repletion of tissues with GSH or α-tocopherol reduces toxicity.

Thus, it is given that, in general, there is a high degree of correlation between the demonstration of covalent binding of a xenobiotic in an organ or cell type within that organ and cellular necrosis. There exist, however enough discrepancies between covalent binding and toxicity that should be explored to allow the careful scientist to tease apart and to reveal more precisely the biochemical mechanisms of how and why these correlations exist.

The purpose of this review is, therefore, to reveal situations in which correlations between covalent binding and toxicity exist and situations in which the two may be divorced.

B. Pulmonary Cytology

The mammalian lung is an extraordinarily heterogeneous organ, consisting of more than 40 distinctive cell types, but generally speaking, pneumotoxins select only six of these as targets: type I alveolar epithelial cells, type II alveolar epithelial cells, pulmonary alveolar macrophages, capillary endothelial cells, ciliated bronchiolar epithelial cells, and nonciliated bronchiolar epithelial cells, also known as Clara cells.

Type I alveolar cells are elongated and flattened and have corresponding nuclei (fig. 1a). Figure 1a also shows a capillary that is enclosed by a vascular endothelial cell and an erythrocyte (original magnification $\times$ 13,000). Type I cells have a limited cytoplasmic volume and a distinct paucity of organelles; they are attached to the basal lamina that they share with the capillary endothelium. Approximately 95% of the rat lung alveolar surface is covered by type I cells, the remainder being covered by type II cells (Crapo et al., 1980).

Type II alveolar epithelial cells have a more complex cytoplasm consisting of rough endoplasmic reticulum (RER), free ribosomes, mitochondria, and Golgi and multivesicular bodies (Kuhn, 1976; fig. 1b; original magnification $\times$17,000). The dominant organelles are the electron-dense osmiophilic lamellar bodies, thought to be the biosynthetic or storage sites of pulmonary surfactant. RER and ribosomes are also present. The lamellar bodies consist primarily of the dipalmitoyl ester of phosphatidylcholine, which is highly surface-active, and probably keep the alveoli from collapsing during expiration. Also abundant are enlongated mitochondria, RER, ribosomes, a small Golgi apparatus, and multivescular bodies. Esterases and acid phosphatases have been reported; activities of CYP450 enzymes and conjugating enzymes are measurable in type II cells but are much lower than in Clara cells.

Pulmonary alveolar macrophages can be found in the interstitium or alveolar spaces and have numerous villous projections on their outer surfaces. They exhibit short segments of RER along with ribosomes and small mitochondria. Numerous lysosomal granules fill the cytoplasm and are endowed with a plethora of hydrolytic enzymes such as esterases, acid phosphatases, lysozyme, cathepsins, RNase, elastase, hyaluronidase, and β-glucuronidase, a complement to degrade almost any necrotic cell (fig. 1c).

Capillary endothelial cells (fig. 1a) consist of a large basal nucleus, a lumen, and an exquisitely thin double membrane that forms the barrier for diffusion of oxygen from the alveolar space to the blood capillary (Ryan and Li, 1993). Recent work has revealed metabolic and uptake functions for endothelial cells for endogenous and exogenous amines and angiotensin converting enzyme (ACE) is known to be localized on the vascular surface of the capillary endothelial cells.

Ciliated bronchiolar epithelial cells have been characterized physiologically, strangely by the fact that they are not Clara cells. Little experimental data have accumulated on the cytochemistry or enzymology of ciliated bronchiolar cells, and methods have not been devised to purify them in high yield (fig. 1d).

Nonciliated bronchiolar epithelial (Clara) cells (figs. 1e,f) are cytologically and enzymatically heterogeneous (see Plopper et al., 1991b; Cho et al., 1995). Clara cells exist in somewhat different functional forms throughout the respiratory tree (in nasal mucous, trachea, bronchi, and bronchioles). In most animal species, however, they tend to be concentrated in the bronchioles. The most conspicuous morphological characteristic of the Clara cell is its bulbous configuration, the bulb or apex projecting into the bronchiolar lumen. In most species, the apex is rich in smooth endoplasmic reticulum (SER), known to be the site of P450 isozymes. More than 40% of the cytoplasmic volume is composed of SER. CYP450 isozymes are concentrated 5- to 10-fold in Clara cells over other pulmonary cell types and enzymes that conjugate xenobiotics with GSH, glucuronic acid, or sulfate less so. RER is generally restricted to the basolateral portions of the cytoplasm surrounding the nucleus. The nucleus is centrally placed. The apical portion of the cytoplasm contains a small number of ovoid, electron-dense secretory granules whose function is not known. Typical mitochondria and Golgi apparatus are found. Glycogen may or may not be present (Gould et al., 1972).

II. Hyperoxic Lung Injury

Attempts to specifically delineate the causative agent responsible for O_2-induced lung damage have not been rewarding. In vitro, oxygen-free radicals (O_2^- and ·OH) and other metabolites [$^1O_2$, H_2O_2, and hypochlorous acid (HOCl)] are known to produce cytotoxic damage to isolated lung cells. Many of these oxygen-derived products are interconvertible in vivo and in vitro, and the wide-

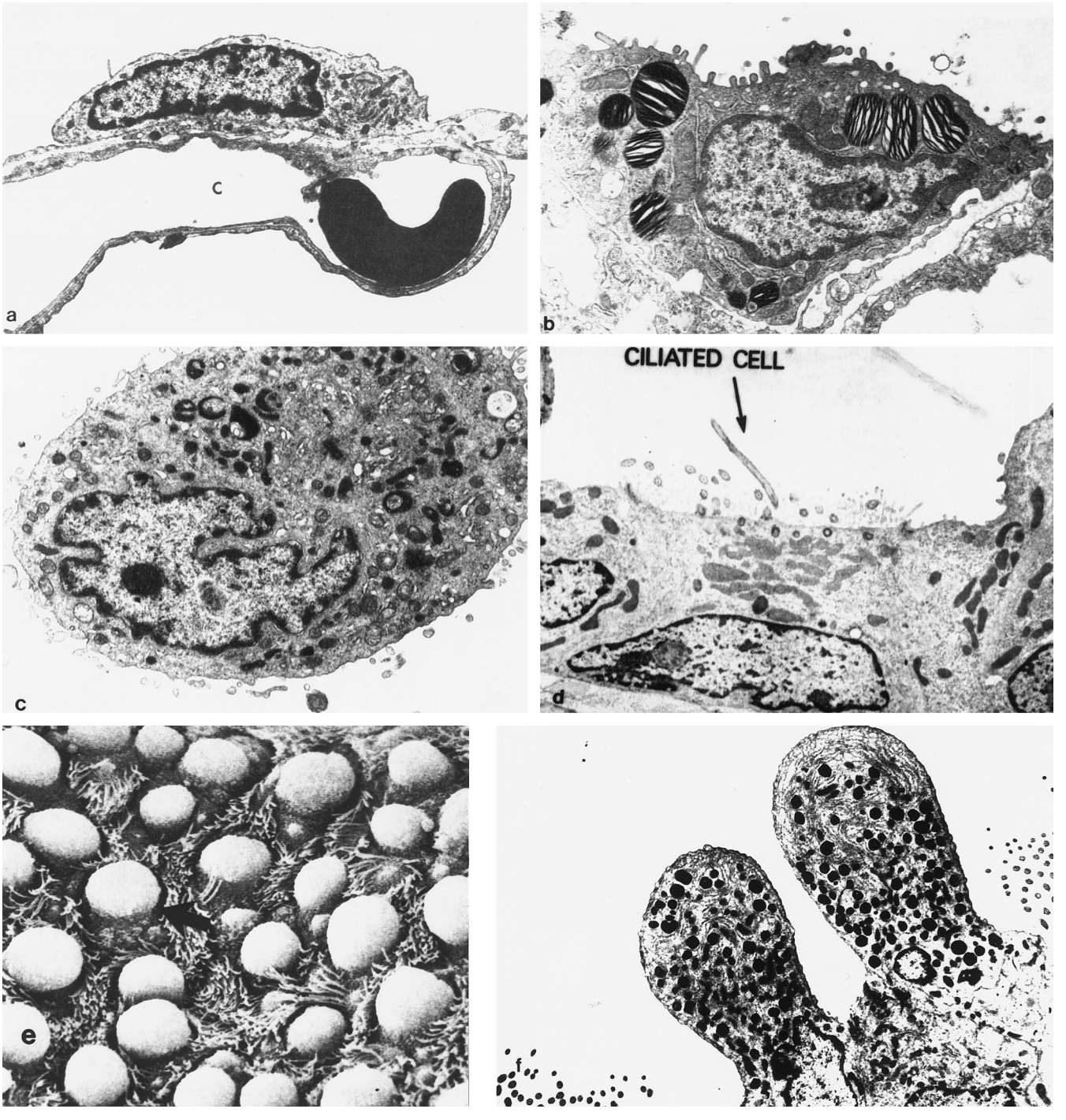


FIG. 1. (a) An electron micrograph showing a type I alveolar epithelial cell (original magnification × 13,800) and a capillary endothelial cell containing an erythrocyte. The type I cell is a long, flat cell with a large, flat nucleus containing sparse cytoplasmic organelles including few mitochondria, RER, ribosomes, and lysosomes. The endothelial cell cytoplasm is virtually devoid of organelles. Reproduced with permission from Kuhn (1976). (b) An electron micrograph showing a type II alveolar epithelial cell (original magnification ×17,000). The cytoplasm contains mitochondria, RER, numerous free ribosomes, and a Golgi apparatus. The most prominent feature of the type II cell is the electron-dense osmiophilic lamellar bodies thought to be involved in surfactant biosynthesis and/or storage. Reproduced with permission from Kuhn (1976). (c) An electron micrograph of a human pulmonary alveolar macrophage (original magnification × 13,500). The cytoplasm is richly endowed with lysosomes and contains numerous mitochondria, RER, and ribosomes. Reproduced with permission from Kuhn (1976). (d) An electron micrograph of a ciliated bronchiolar epithelial cell from mouse lung (original magnification × 12,000). Numerous mitochrondria and free ribosomes are seen. Reproduced with permission from Boyd et al. (1980b). (e) A scanning electron micrograph of a rat lung bronchiole (original magnification × 2,400) showing Clara cells (domes) as apical bulges amid the grain-like stalks of ciliated cells. Reproduced with permission from Gandy et al. (1983). (f) An electron micrograph of Clara cells of rat bronchiole (original magnification × 2,700). The most prominent organelle is the extensive network of (mainly tubular) SER particularly in the apex of the cells. This is known to be the site of CYP450 enzymes. Other characteristic organelles, particularly in the dome of Clara cells, are numerous electron-dense granules that are not mucin and do not contain phospholipid (surfactant). Reproduced with permission from Boyd et al. (1980b).

spread use of enzymes and their substrates do not inspire confidence in this area of investigation. Examples of such enzymes are substrates are: xanthine and xanthine oxidase thought to general O_2^-; glucose and glucose oxidase thought to produce H_2O_2; and a witch's brew of other substances, such as myeloperoxidase, lactoperoxidase, and galactose oxidase (which are thought to produce specific metabolic products that cannot be measured directly but rely on such crude measures as chemiluminescence) or by "specific radical scavengers," such as mannitol and thiourea.

Thus, until such time when methodology is available possessing both sensitivity and specificity for assays of single, discrete reactive oxygen metabolites, it is not considered useful to attempt to distinguish oxygen-free radicals and other metabolites (see last paragraph). Therefore, for purposes of this review, oxygen will be defined as O_2, O_2^-, ·OH, $^1O_2$, H_2O_2, and HOCl, plus any underdiscovered forms of oxygen formed in vivo.

A. Effects of Hyperoxia on Survival: Gross, Microscopic, and Ultrastructural Evidence of Pathological Changes

When adult rats are exposed to 100% O_2 at 1 atmosphere (atm) pressure, almost all the animals die within 60 to 72 h. Death is preceded by dyspnea, a bloody muzzle, alveolar hemorrhage, pulmonary edema and hypoxia. Prior exposure of rats to 85% O_2, however, allows animals to adapt, and after 5 to 7 days at 85%, they can be transferred to 100% O_2 and survive for long periods of time in pure O_2 (Crapo et al., 1980). Early work on the effects of pure O_2 on the lungs of monkeys (Macaca mulatta) for periods of up to 12 days (Kapanci et al., 1969) traced both the time course and fine structural changes that characterize O_2 toxicity. Electron micrographs of control animals (exposed to air) revealed that the alveolar wall is lined by two types of epithelial cells (type I and II) and the capillaries by endothelium. The endothelium overlies a basement membrane that fuses with the epithelial cells. Therefore, the alveolar membrane that separates blood from air consists of a "sandwich" composed of type I (or type II) cells, the basement membrane, and the capillary endothelial cells. Endothelial cells possess sparse cytoplasmic structures. The alveolar epithelium consists of type I cells that are broad and flat and possess few organelles and thin cytoplasmic extensions. Type I cells are cytologically similar to endothelial cells. Type I cells cover approximately 95% of the alveolar surface. Type II cells have extensive cytoplasm and organelles, mitochondria and osmiophilic lamellar bodies that are thought to be the biosynthetic precursor and storage form of pulmonary surfactant are prominent (Frank and Massaro, 1979).

Four days of O_2 exposure (Kapanci et al., 1969) severely damaged the alveolar walls. Ninety percent of the type I cells were necrotic, and some exfoliated, leaving a denuded basement membrane covered by fibrin strands. Seven days of O_2 exposure revealed a starkly different picture. Type I cells were replaced by primitive type II cells containing fewer lamellar bodies than controls. Type II cells now constituted 95% of the alveolar epithelium as compared with 5% in controls. Type II cells contained numerous mitotic figures. In essence, then, type I cells had undergone necrosis and were replaced by hyperplasia of type II cells that resulted in a thickening of the alveolar epithelium. Of equal importance, endothelial cells had been reduced to approximately 50% those of controls, suggesting massive endothelial cell necrosis.

Using a combination of morphometric and biochemical techniques, Crapo et al. (1980) reported that after 60 h of exposure of rats to 100% O_2, the most significant injury occurred to the capillary endothelium. The latter cell type was reduced in number by approximately 45%. In contrast to Kapanci et al. (1969), Crapo et al. (1980) found no significant changes in type I cells in total volume, total number, and mean surface area after exposure to 100% O_2 for 60 h (96% of a LT_{100}). Therefore, Crapo et al. (1980) attributed death to lethal changes in endothelial cells and alterations in type II cells, consisting of swelling of RER or granular endoplasmic reticulum and mitochondria, and ruputure of the plasma membrane and pynknotic nuclei. Lethality was not reflected in significant changes in total superoxide dismutase (SOD), SOD containing copper and zinc as part of the molecule, or levels of SOD containing manganese as part of the molecule.

Employing a different experimental design, Adamson et al. (1970) exposed adult male mice to 90% O_2 for 7 days. Most (90%) of the animals developed respiratory distress and died, but the remainder survived to make a complete recovery on return to air. The earliest ultrastructural change in poisoned animals was focal cytoplasmic swelling of capillary endothelial cells. Animals from the group that died displayed progressive cytoplasmic swelling of type I epithelial cells. This caused disintegration of the basement membrane. Type II cells were well preserved. Capillary distension was a prominent feature. In the 10% of mice that survived, destruction of type I epithelium did not occur. Type II cells were normal until the sixth day of 90% O_2, at which time no lamellar bodies were observed, and the mitochondria displayed swelling and polymorphism. Two days after return to air, focal hyperplasia of type II cells was observed. Surfactant in dying animals was either destroyed or inactivated.

Barry and Crapo (1985) reported that the pulmonary capillary endothelium was a primary target for injury in animals exposed to hyperoxia at normobaric pressure. A key factor in death or survival associated with exposure to rats to either 85% or 100% O_2 was maintenance of the pulmonary capillary bed (endothelium). Adult Swiss mice were exposed to 90% O_2 for periods ranging from 24 h to 14 days, and the only pathological lesions were consistent ultrastructural alterations in the capillary endothelium (Bowden et al., 1968). No consistent alter-

ations were noted in alveolar epithelium; the number of type I alveolar cells did not differ from controls.

In a carefully controlled study of the effects of 99% O_2 on the fine structure of adult male rat lungs (Kistler et al., 1967), it was reported that the most profound alteration was a 50% loss in capillary endothelial cells. Rats were exposed to O_2 for 6, 24, 48, and 72 h at 1 atm pressure. The basement membrane, i.e., the air-blood barrier, approximately doubled in thickness. In contrast to the striking destruction of capillary endothelium, the vast majority of epithelial type I and II cells revealed normal fine structure. A functional correlate of the dramatic loss of capillary endothelial cells, thickening of the basement membrane, and reduction of gas-diffusing capacity, the authors noted marked dyspnea and cyanosis of animals returned to room air. This study clearly identified capillary endothelium as the primary pulmonary target of hyperoxia. Similar findings were reported by Crapo et al. (1978), who investigated fine structural aspects of adaption to hyperoxia. When adult rats were exposed to 100% O_2, nearly all died within 60 to 72 h, whereas animals exposed to 85% O_2 survived and after 5 to 7 days could be transferred to 100% O_2 and survive for prolonged periods, i.e., they become O_2-tolerant. This study compared the morphometric fine structure of lungs from control rats maintained in room air with those from rats maintained in 85% O_2 for 7 days. The O_2-adapted animals had a normal number of alveolar type I epithelial cells, and a moderate increase (two-fold) in the number of type II cells. A large increase was noted in numbers of interstitial cells (five-fold). The major area of pulmonary damage occurred in the vascular compartment, where entire segments of the capillary bed were lost, and the total number of endothelial cells decreased by 45%. Corresponding reductions were noted in capillary volume.

Thet and coworkers (1983), reported that small doses of endotoxin protected rats against O_2-induced lung injury. Bacterial endotoxin (Salmonella typhimurium lipopolysaccharide) was injected into rats that were then exposed to 100% O_2 for 72 h. At the end of O_2 exposure, seven of the eight endotoxin injected rats were still alive as opposed to one of the eight saline-injected, O_2-treated rats, thus confirming the protective effect of endotoxin against O_2 toxicity. This protection against O_2 lethality was not reflected by alterations in pulmonary fine structure. Capillary endothelial cells were reduced approximately 40% by 100% O_2, and this change was not attenuated by endotoxin. Capillary surface area, increased cellularity, and marked edema of the interstitium produced by O_2 were not influenced by endotoxin. Types I and II epithelial cells in hyperoxic animals were not significantly altered by endotoxin. The authors concluded that although the gross toxicity (survival) produced by endotoxin against O_2, poisoning was not accompanied by dramatic changes in lung ultrastructure; this protection might be reflected in enzymatic changes that parallel O_2 toxicity.

Harrison (1971) reminded us that the air-blood barrier consists of three components: the alveolar epithelium (type I and type II cells), the basement membrane, and the capillary endothelium. She studied the effects of 100% O_2 (1 atm) in young rats (90–100 g) for 3 to 6 weeks (immature rats are relatively resistant to high O_2 tensions) and described ultrastructural changes. In the first stage of O_2 poisoning, the mitochondria of epithelial and endothelial cells appeared swollen, and the cristae were compressed or destroyed. The cisternae of the endoplasmic reticulum were also swollen. The basement membrane became edematous and thickened. At the same time, discontinuities occurred in the endothelium. As damage progressed, the endothelial lining disappeared, and the basement membrane began to disintegrate, leaving the capillary space in direct contact with the epithelial cells. Soon, all that remained was a strand of epithelium and a few shreds of basement membrane that ultimately ruptured, and the destruction of the alveolar-capillary wall was complete. In addition to total loss of this barrier, alveolar spaces coalesced and accumulated exudate that contained cellular debris and myelin figures. Gas diffusion became impossible. Thus, destruction of the air-blood barrier started with ultrastructural changes in the endothelial cells that line the capillary bed.

In an analysis of the literature up to 1979, Frank and Massaro (1979), concluded that the pulmonary capillary endothelial was the first lung cell type to be seriously damaged by hyperoxia. I concur with this analysis, and a predominant systemic effect should be hypoxemia.

B. Pulmonary Enzymes That Alter Oxygen Toxicity

1. Enzymes that promote formation of reactive oxygen species. In mitochondria, oxygen undergoes stepwise and sequential one-electron reduction with the formation of water. These reactions are catalyzed by a series of enzymes collectively termed "cytochrome oxidase," and the reactions are exquisitely sensitive to cyanide and other inhibitors. These reactions are tightly linked to oxidative phosphorylation. In addition to this normal function of oxygen in intermediary metabolism, it may undergo a second type of metabolism that produces ROS that range from slightly to enormously cytotoxic (Bishop et al., 1984; fig. 2). O_2^- and ·OH are radicals, and $^1O_2$ and H_2O_2 are not, but these four species collectively are known as ROS (Halliwell and Gutteridge, 1984). ROS can enter into a wide variety of toxic reactions, some functional (e.g., strand scission of DNA and enzyme inhibition) and some structural (lipid peroxidation of polyunsaturated fatty acids in phospholipids that constitute most biological membranes (Bishop et al., 1984). Table 1 shows the cellular consequences of oxygen-centered free radicals. ROS can react with protein, lipid (membranes), DNA base pairs, and the deoxyribose phosphate backbone of DNA. Lipid peroxidation can be cytotoxic as chain reactions can be initiated (Comporti, 1987).

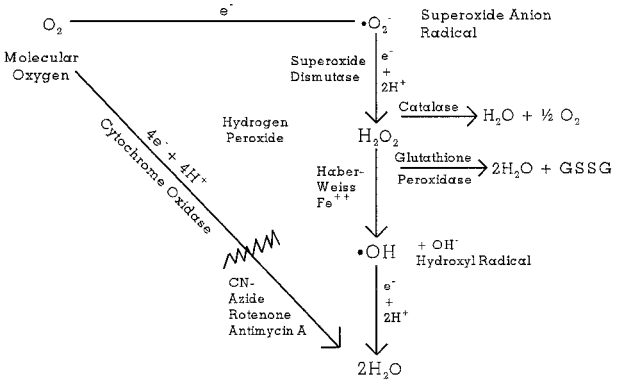


FIG. 2. Reduction of oxygen to water. Tight coupling to oxidative phosphorylation is not presented. Adapted with permission from Bishop et al. (1984).

TABLE 1
Some cellular effects of reactive free radicals

Target	Results
Macromolecules	
Protein	Denaturation, chain scission strand
Scission	
DNA	Base modification, cell cycle disruption
Smaller molecules	
Carbohydrates	Cell surface receptor changes
Nucleic acid	Cell cycle changes, mutations
Unsaturated lipids	Fatty acid oxidation organelle and cell membrane permeability changes
Amino acids	Enzyme inhibition, cross linking
Antioxidants	Reduce availability of GSH, α-tocopherol, β-carotene and ascorbate
Cofactors	Reduced availability of nicotinamide and flavin-containing cofactors

2. Enzymes that catalyze inactivation of reactive oxygen species. The chemistry and biochemistry of ROS have been discussed in numerous reviews (Freeman and Crapo, 1982; Kehrer, 1993; Doelman and Bast, 1990; Halliwell and Gutteridge, 1986; Halliwell and Gutteridge, 1984; fig. 2) and will not be detailed further here. Some of the biological effects of ROS are presented in table 1. Many of these effects result in cytotoxicity.

a. LIPID PEROXIDATION. The interaction of ROS with a variety of biomolecules results in toxic changes. Because of the importance of polyunsaturated fatty acids in virtually all biological membranes, these interactions will be treated now. The ·OH can abstract a hydrogen atom from polyunsaturated fatty acids in membranes with the formation of a conjugated diene radical, resulting in the formation of epoxides, peroxides, and lipid peroxyradicals. Subsequently, this radical may abstract another hydrogen atom from another polyunsaturated fatty acid, and a chain reaction of propagation may begin. The oxygen-oxygen bond of the lipid hydroperoxide (R-O-O-R′) may be cleaved homolytically by some proteins such as cytochrome P450, thus forming alkoxy and hydroxyl radicals (see Doelman and Bast, 1990). In addition, lipid peroxy radicals can be formed. The lipid peroxidation proceeds, and alkanes such as ethane and pentane, alkenes, aldehydes (including the reactive 4-hydroxy-23-transnonenal), ketones, and hydroxyacids are produced (Benedetti and Comporti, 1987). Destruction of the lipid bilayer causes calcium influx in bovine pulmonary artery endothelial cells. In addition, membrane phospholipase A_2 is activated during liver microsomal lipid peroxidation. This in turn, facilitates further deterioration of the lipid bilayer by free radical processes (Kehrer, 1993).

b. DNA DAMAGE. The hydroxyl radical is a powerful oxidant that can cause DNA damage (Trush et al., 1982). The deoxyribose sugar is probably the main target of radical damage and is thought to be the source of malondialdehyde, which is a rough index of DNA damage. In addition the ·OH has been shown to hydroxylate guanine in calf thymus DNA. Lipid peroxidation products such as lipid radicals or aldehydes may cause DNA strand breaks, DNA adducts, and DNA-protein cross links. In particular, 4-hydroxynonenal causes DNA fragmentation and sister-chromatid exchange (Wiseman and Halliwell, 1996). In a very clever set of experiments, Agarwal and Sohal (1994) studied the effects (100%) of hyperoxia on oxidative DNA damage and life span in houseflies. The life span of houseflies can be manipulated by the amount of physical activity they perform by restricting their living space (walk versus fly). The time to 50% of deaths (LT_{50}) was 21 days in high-activity flies versus 58 days in low-activity flies. The relative amount of 8-hydroxydeoxyguanosine, an indicator of oxidative damage, in DNA increased steadily with age, exhibiting 70% higher levels in 16-day-old flies as compared with 5-day-old flies. Mitochondrial DNA showed 2- to 3-fold higher 8-hydroxydeoxyguanosine concentration than total DNA. Exposure of 5-day-old flies to 100% O_2 for 3 days increased the amount of 8-hydroxydeoxyguanosine in total DNA by 37% and by 100% in mitochondrial DNA as compared with the air-exposed control group. Therefore, high physical activity shortens life span and increases the concentrations of 8-hydroxydeoxyguanosine in total DNA and mitochondrial DNA of houseflies. Hyperoxia has the same effects. The authors concluded that an inverse relationship exists between oxidative DNA damage and the life span of flies.

O_2^-, H_2O_2, O_2, and especially the highly reactive ·OH are individually and collectively toxic to tissues and cells. Normally, tissues have defense mechanisms that protect them against the toxic effects of oxyradicals. Among these are SOD, catalase, and glutathione peroxidase (GSH-Px), together with tissue stores of α-tocopherol, reduced GSH, and ascorbate. However, when these defenses are overwhelmed as in oxygen toxicity or paraquat poisoning, toxic levels of oxyradicals may accumulate in tissues.

That oxyradicals are directly capable of evoking tissue damage was cleverly demonstrated by Johnson et al. (1981). Xanthine and xanthine oxidase, which together generate O_2^- 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^-) but not 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 alveolar 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.

3. Role of cytochrome P450 and glutathione in pulmonary oxygen toxicity. A major tool in understanding the mechanism of cytochrome P450 has been the use of various mouse strains that are either "responsive" or "unresponsive" to its inducers, benzo[a]pyrene (BP) or 3-MC (Okey et al., 1989). Lungs of "responsive" animals usually respond with 2- to 4-fold induction of cytochrome P450 but are unresponsive to the inductive effects of PB as are lungs of most other species that have been examined (Gelboin, 1993). Gonder et al. (1985) studied pulmonary O_2 toxicity in genetically responsive (to aromatic hydrocarbons) and unresponsive mouse strains. Hydrocarbon responsive (C3H/HeJ) and unresponsive mouse (DBA/2J) strains were tested for their susceptibility to O_2 toxicity by exposing these and other strains to O_2 (>98%) for periods up to 96 h. DBA/2J mice lived significantly longer than C3H/HeJ animals in hyperoxia (122 h versus 92 h). Lung microsomal cytochrome P450 increased approximately 3.5- to 4-fold in responsive mice after 72 h of hyperoxia but was not induced in unresponsive animals. Histological evaluation of lungs revealed no significant alterations in DBA/2J animals, whereas lungs of C3H/HeJ mice displayed profound septal thickening and pulmonary edema at 96 h. The authors concluded that O_2 toxicity parallels the genetic control of cytochrome P450 induction and that O_2 toxicity develops at a time when O_2 has induced cytochrome P450.

In later work, Mansour et al. (1988) studied rats pretreated with β-naphthoflavone (β-NF), 3-MC, or PB before being exposed to hyperoxia (>95% O_2) at 1 atm pressure for up to 7 days. All (15) untreated control rats were dead within 5 days of O_2 exposure; 4 of 10 treated with PB survived, whereas 7 days after pretreatment with 3-MC, 11 of 15 rats survived and 9 of 10 treated with β-NF survived O_2 exposure. As a rough index of pulmonary toxicity, in animals exposed to hyperoxia for 60 h, pleural fluid volumes for control animals were 8.5 mL, which was not significantly altered by PB (7.8 mL) but was dramatically reduced by 3-MC (1.9 mL) and β-NF (1.7 mL). Similarly, lung wet weight: dry weight ratios were 5:8 in controls, 5:6 in PB-treated animals (not different), but 5:1 in both 3-MC– and β-NF–treated animals (significant reduction). At gross autopsy, lungs from 3-MC– and β-NF–treated rats had normal appearance, whereas PB-treated animals had large, apparently confluent hemorrhagic areas on the pleural surface. As an index of lipid peroxidation, pulmonary malondialdehyde levels (expressed per whole lung) were not altered by PB but were reduced approximately 50% by 3-MC and β-NF. Pulmonary cytochrome P450 in hyperoxic and air-exposed animals was not altered by PB but was tripled by both 3-MC and β-NF. Thus, the pulmonary P450 inducers 3-MC and β-NF afforded protection against acute pulmonary injury produced by lethal hyperoxia in adult rats, whereas PB was without effect.

Cytochrome P4502E1 was known to be induced in rat liver and lung by acetone and ethanol. Tindberg and Ingelman-Sundberg (1989) reported that exposure of rats to 95% O_2 induces this isozyme approximately three-fold in lungs and livers of rats. Induction by hyperoxia was more profound in lung (0.07–0.23 nmol/mg protein) than in liver (approximately 50%).

It has been repeatedly demonstrated (DeLeve and Kaplowitz, 1991) that treatment of animals with buthionine sulfoximine (BSO) blocks the biosynthesis of GSH. Fasted mice exposed to 100% O_2 had more lung damage and died sooner than fed mice. Smith and coworkers (1990) studied the effects of starvation and hyperoxia on lung injury in mice. In air-exposed mice, 3 days of starvation reduced pulmonary GSH levels by approximately 40%. When starvation and hyperoxia were studied together, only 10% of the fed mice but 89% of the starved mice were dead by day 4 of hyperoxia. Treatment of fed mice for 14 days with BSO followed by hyperoxia for 4 days revealed that BSO-treated mice died sooner and had more severe lung damage than controls. Hyaline membranes and pulmonary edema were more profound in the former group. Thus, the increased susceptibility of mice to 100% O_2 correlated with earlier death, enhanced lung pathology, and reduced lung GSH levels.

Diethylmaleate (DEM) depletes tissues of preformed GSH and enhances the toxicity of hyperbaric oxygen. After DEM administration to air-breathing rats, lung GSH levels declined to <5% of controls, and their survival time in 100% O_2 was reduced from 319 minutes to 204 minutes. Supplementation of DEM-treated rats with exogenous GSH reversed the toxic enhancement of hyperoxia and repleted pulmonary GSH (Weber et al., 1990).

4. Age and species differences in the susceptibility to oxygen toxicity. An interesting ontogenetic pecularity has been revealed in that neonatal rats are quite resistant to the pneumotoxic effects of prolonged hyperoxia compared with adults. This was explained by Stevens and Autor (1980) with the observation that lung tissue of

neonatal rats was capable of a rapid, oxygen-induced enhancement of SOD activity (see fig. 2). This response was age-dependent, the maximum effect occurring in 10-day-old animals, and the enzyme change resulted from increased oxygen-mediated protein synthesis. Accordingly, cycloheximide, an inhibitor of protein synthesis, inhibited the oxygen-enhanced incorporation of [^{3}H]leucine into semi-purified SOD. The activities of both mangano-SOD and the cupro-zinc SOD were induced in lungs of oxygen-exposed neonates. Catalase and GSH-Px were also rapidly induced in the lungs of these animals during hyperoxia. The enzymatic response to hyperoxia thus consists of induction of four antioxidant enzymes. A correlation was found between age-dependent tolerance to hyperoxia and enzyme induction in young rat lungs. In hyperoxic animals, SOD activity peaked abruptly at 10 days of age, whereas both GSH-Px and catalase activities increased slowly from birth to peak at 20 to 25 days of age and then fell abruptly. By 34 days of age, little or no oxygen-mediated enzyme responses were detectable; similarly, rats older than 34 days were no longer resistant to oxygen-provoked lung damage. These observations provided compelling evidence for the hypothesis that the resistance of neonatal rats to hyperoxia may result from rapid induction of pulmonary enzymes that catalyze the detoxication of ROS.

Phylogenetic differences to hyperoxia exist as well. Somayajulu et al. (1978) reported that exposure of chickens to 100% oxygen at 1 atm pressure for prolonged periods produced no pathological pulmonary damage, although H_2O_2 did accumulate in their airways. Rabbits and chickens were exposed to hyperoxia, and all the rabbits died within 5 days, whereas the chickens remained unaffected at 19 days. At death, rabbit lungs showed generalized edema and patchy hemorrhage; chickens showed no edema or hemorrhage after exposure to 100% oxygen for 19 days. Because H_2O_2 occurred in considerable amounts in the airways of both rabbits and chickens, it appeared that H_2O_2 might not be the source of oxygen damage in rabbits unless a large species difference to H_2O_2 was evident. It is of importance to note that the airways of chickens and other birds are devoid of nonciliated bronchiolar epithelial (Clara) cells, the lung cells richest in cytochrome P450 and enzymes that catalyze drug oxidation and conjugation (Boyd, 1980). This following question presents itself. Does the deficiency of pulmonary cytochrome P450 in bird lungs somehow protect them from pulmonary oxygen toxicity or are other factors involved?

III. Paraquat and Nitrofurantoin-Induced Lung Damage

A. Paraquat Lung Toxicity

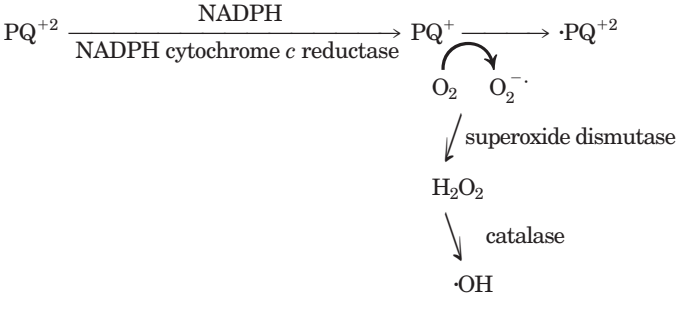
1. Morphological effects. The morphological effects of a single dose of paraquat have been reported and are well documented (Smith and Heath, 1976). They occur in humans and in most common laboratory species except rabbits; to produce "paraquat lung" in rabbits requires subacute or chronic dosing (Smith et al., 1979). The lung is the primary target of paraquat toxicity, but the kidney, liver, and thymus are also affected. The dose-response curve for the pneumotoxicity of paraquat in rats is quite steep: a dose of 30 mg/kg was lethal to all animals, whereas 20 mg/kg produced no pulmonary lesions (Vijeyaratnam and Corrin, 1971). Thus, 25 mg/kg administered intraperitoneally produced the following effects. Lungs of rats dying within 4 days were from grossly dyspneic animals. They were markedly congested and plum-colored. There was perivascular edema and hemorrhage into the alveolar spaces. Frequent mitoses were observed in alveolar cells and large amounts of interstitial infiltration with large mononuclear cells and lymphocytes were noted. At 14 days, the cellularity of the alveolar walls was further increased by profibroblasts and fibroblasts, the cells were now arranged in whorls, and large increases in interstitial and intraalveolar reticulin and especially collagen were noted. Ultrastructurally, 3 days after paraquat, there was complete loss of alveolar epithelium in both type I and II cells. In contrast to these dramatic changes in epithelial cells, the capillary endothelium was unaltered. Therefore, large areas of the alveolar surface consisted merely of basement membrane and endothelium. In animals injected with paraquat and sacrificed shortly antemortem, lungs did not collapse upon thoractomy, appeared nearly solid and rubbery, and sank when placed in water. Upon cross-section, normal lung architecture was not recognizable, interstitium was markedly hypercellular and thickened, alveolar spaces obliterated mainly by the influx of fibroblasts, with their abundant RER and their extensive forest of secreted collagen fibers. Incubation of lung explants from paraquat-intoxicated rats with [^{3}H]hydroxyproline revealed a 10- to 20-fold increase in collagen biosynthesis (Greenberg et al., 1978). In those animals that survived, type II epithelial cells repopulated the alveoli, and some of these cells redifferentiated into type I cells (Smith and Heath, 1974). BALB/c mice were exposed to a paraquat aerosol and killed 1 to 28 days later. Initial necrosis and sloughing of the bronchiolar and alveolar epithelium with intact endothelium were followed by type II cell hyperplasia, fibroblast proliferation, and increased synthesis of collagen. Thus, inhaled paraquat produces pulmonary fibrosis that resembles that administered systemically (Popenoe, 1979).

Therefore, the pathological changes in lung produced by paraquat consist of wide-spread necrosis of type I and II cells followed by a massive influx of primitive fibroblasts that elaborate enormous quantities of collagen and obliterate pulmonary alveoli into an airless, nonfunctional bag of cells that cannot participate in gas exchange. This collection of cells is so primitive that they have been termed "tumorlets" (Klaff et al., 1977).

2. Biochemical pharmacology. Paraquat is selectively concentrated in mammalian lungs as are many basic amines (Bend et al., 1985). After intravenous administration to rats, lung/plasma ratios for paraquat were approximately 17:1 at 5 h, 31:1 at 12 h, and 42:1 at 24 h (Witschi et al., 1977). Similarly, incubation of lung slices with paraquat reveals that slice/medium ratios ranging from 5:1 to 15:1 can readily be obtained. Accumulation does not occur by covalent binding (Rose and Smith, 1977). Paraquat uptake by lung is an active energy-dependent process that occurs against a concentration gradient and can be blocked by anaerobiosis, cold, iodoacetate, etc., and also by a variety of other amines that are themselves accumulated by lung slices such as 5-hydroxytryptamine, norepinephrine, spermine, propanolol, imipramine, chlorpromazine, diphenhydramine, and chlorphenterine (Bend et al., 1985). It is interesting to note that the administration of most of these amphiphilic amines does not produce the pulmonary fibrosis characteristic of paraquat but instead causes pulmonary phospholipidosis. In this disorder, apparently not a disease, per se, one observes a dramatic proliferation of osmiophilic lamellar bodies in the cytoplasm of pulmonary epithelial type II cells. This increased synthesis of lamellar bodies is followed by their secretion, exocytosis, into the alveolar space where it is attended by some blockade of the alveolar space and a dramatic increase in pulmonary surfactant that can be removed by lavage. These lamellar bodies are thought to be precursors of pulmonary surfactant that prevent the alveoli from collapsing upon expiration. Pulmonary surfactant is composed of dipalmitoyl phosphalidyl choline with some associated proteins.

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 (Witschi et al., 1977). Associated with this oxidative burst, one discovers a marked increase in O_2^- formation. These reactions are not blocked by carbon monoxide (CO) and are therefore not cytochrome P450-dependent (Ilett et al., 1974).

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 (Bus and Gibson, 1984). 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 futile reduction-oxidation cycle is set up in which paraquat catalytically functions, NADPH is consumed (NADPH/NADP falls; Witschi et al., 1977), and most importantly, enormous quantities of $O_{2\cdot-}$ are generated. Because 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 (Benedetti et al., 1980), it is not possible at this time to identify the more toxic of these coincidental events.

Mammalian organisms have evolved to possess 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, SOD degrades $O_{2\cdot-}$ catalase degrades H_2O_2, and GSH-Px converts fatty acid hydroperoxide to alcohols (Bus et al., 1976b). In addition, tissue stores of α-tocopherol, reduced GSH, and ascorbate scavenge oxyradicals (Bus et al., 1976b). The toxicity of paraquat, defined either in terms of LD_{50} or LT_{50}, can be manipulated with knowledge of these oxidant protective mechanisms. For example, hyperoxia (e.g., 100% O_2) markedly increases paraquat toxicity, whereas hypoxia (10% O_2) reduces it (Smith and Rose, 1977). Depletion of tissue GSH stores by pretreatment with diethylmaleate or vitamin E deficiency dramatically increases paraquat toxicity.

In vitro incubation of rat lung alveolar type II cells with paraquat causes a concentration- and time-related loss of viability. Addition of GSH to this system provided dramatic protection of the cells against the toxic effects of paraquat (Hagen et al., 1986). Similarly, selenium deficiency produced by dietary means increases paraquat toxicity (Omaye et al., 1978). Selenium is a component of GSH-Px. Selenium deficiency increases lung-lipid peroxidation (Burk et al., 1980; Combs and Peterson, 1983).

Hyperoxia dramatically reduced the LT_{50} of paraquat in rats (Kehrer et al., 1979; Fisher et al., 1973), whereas hypoxia reduced the toxicity (Rhodes, 1976). SOD inhibited lung lipid peroxidation (Trush et al., 1981) and vitamin E deficiency augmented it. SOD increased paraquats' LT_{50} dramatically in intact rats (Autor, 1974). Both percent survival and duration of survival were significantly reduced in vitamin E deficient rats (Block, 1979). Ogata and Manabe (1990) reported a poor temporal correlation between ultrastructural demonstration of lung damage and lipid peroxidation. These workers found severe alveolar epithelial damage long before (third day postadministration versus fifth day) malondialdehyde (MDA) concentrations in lung increased. They suggested that lipid peroxidation might result from the

phagocytic activities of macrophages rather than toxic cell injury.

Incubation of purified rat lung type II alveolar cells with paraquat, in vitro, confirmed earlier findings that the cytotoxicity (as evidenced by ^{51}Cr release) increased with increasing concentrations of paraquat, was inhibited by catalase and SOD and α-tocopherol and enhanced by 95% oxygen (Skillrud and Martin, 1984).

The reader is referred to numerous well-written reviews on paraquat pneumotoxicity (Autor, 1977; Bus and Gibson, 1984; Smith, 1985; Smith, 1986; Bus et al., 1976a).

B. Nitrofurantoin Lung Injury

Nitrofurantoin is an agent that has been used clinically to treat urinary tract infections. Its use, particularly when administered chronically, is accompanied by pulmonary reactions, once thought to be "hypersensitivity reactions" (Sovijarui et al., 1977), ranging from cough and dyspnea to infiltrates, effusion, and pulmonary fibrosis, which were confirmed by pulmonary biopsy. Administration of large doses of nitrofurantoin to rats produced severe respiratory compromise that caused death in 12 to 36 h. 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 (Boyd et al., 1979). 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 (Boyd et al., 1979) and in rats maintained on a vitamin E deficient diet that was rich in polyunsaturated fat (corn oil). The LD_{50} of nitrofurantoin in the oil group was 35 mg/kg compared with 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 and Holtzman (1975) that under aerobic conditions and in the presence of NADPH and microsomes catalyze a one-electron reduction of the nitro group of nitrofurantoin to yield a nitro free radical ($R\text{-}NO_{2-}$) that spontaneously reacts with oxygen to regenerate the parent nitro compound and reduces oxygen to O_{2-}. Although covalent binding of nitrofurantoin can be demonstrated under anerobic 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 (Martin, 1983).

IV. Pulmonary Neoplasia Associated with Benzo[a]pyrene

BP is but one of many polycyclic aromatic hydrocarbons that rely on metabolic activation to produce ultimate carcinogens in vivo (Conney et al., 1994). Administration of BP to mice produces neoplastic changes in lung which may be benign (adenomas) or malignant (adenocarcinomas).

A. Covalent Binding of Benzo[a]pyrene to Total DNA

The basic hypothesis of chemical carcinogenesis is the formation of a covalent bond between an exogenous chemical and DNA and is the essential first step in the tumor initiation process (Conney, 1982). Most chemicals require metabolic activation to bind covalently to cellular macromolecules, and the ultimate reactive species, which are electrophilic in character, react with nucleophilic groups of cellular macromolecules to form adducts.

Among the earliest clear demonstrations of the metabolic activation of a xenobiotic to a chemically reactive intermediate was that by Miller (1951), who applied [^{3}H]BP to mouse skin and found covalent binding of its metabolites to skin proteins. Similarly, Brooks and Lawley (1964) painted [^{3}H]BP onto the shaved skin of mice and recovered the radiolabel covalently bound to DNA. The extent of binding of [^{3}H]BP to DNA but not to protein correlated with carcinogenic potential. Later, Grover and Sims (1968) reported that rat liver microsomes incubated with exogenous DNA, NADPH and [^{3}H]BP resulted in covalent binding of radioactivity to DNA. Gelboin (1969) confirmed this finding and further reported that pretreatment of rats with 3-MC increased the binding of [^{3}H]BP to DNA by 2- to 4-fold. He also reported that although metabolic activation of [^{3}H]BP was required, the metabolites of BP were sufficiently stable so that the same level of DNA-bound radioactivity was found whether the DNA was added before or after the incubation. Purification of the DNA in CsCl gradients revealed that the [^{3}H] BP-derived radioactivity was, in fact, bound covalently to DNA.

B. Activation of Benzo[a]pyrene In Vivo to the Ultimate Carcinogen

The efforts of many workers have focused on the chemical nature of the ultimate carcinogenic species. Kapitulnik et al. (1978) studied the pulmonary tumorigenic activities of BP (±)-trans-7β,8α-dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (diol epoxide 1), (±)-trans-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (diol epoxide 2), (±)-trans-7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene (BP 78-dihydrodiol), and the tetraols derived from the hydrolysis of diol epoxide 2 in newborn Swiss-Webster mice. Animals were injected intraperitoneally with 4 nmol of each compound on day 1 of life, 8 nmol on day 8 and 16 nmol on day 15, and were killed

at 28 weeks of age. Diol epoxide 1 was highly toxic in newborn mice, and most of the animals treated with this compound died before weaning (25 days). Histological examination failed to reveal the cause of death. Diol epoxide 2 and BP 7,8-dihydrodiol were approximately 40 and 15 times more active than BP in causing pulmonary adenomas. The tetraols derived from diol epoxide 2 did not induce pulmonary adenomas (table 2). These data showed that BP diol epoxide 2 derived from BP 7,8-dihydrodiol is a highly active pulmonary carcinogen in newborn mice.

C. Stereochemistry of the Reactive Metabolite

Because the studies of Kapitulnik et al. (1978) were conducted with optical racemates, the next logical step was to investigate the tumorigenicity of the pure stereoisomers. This study was conducted by Buening et al. (1978). BP and each of the enantiomers of the diastereoisomeric BP 7,8-diol-910-epoxides derived from trans-78-dihydroxy-78-dihydrobenzo[a]pyrene were tested by intraperitoneal (ip) administration to mice of 12 and 4 nmol or 24 and 8 nmol of each compound on days 18 and 15 of life. The animals were killed at 34 to 37 weeks of age. (+)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo-[a]pyrene [(+) diol epoxide 2; compound No. 6] had exceptional tumorigenicity, whereas BP and the other optical isomers had little or no activity (table 3).

More recently (+)diol epoxide 2 has been found (Jeffrey et al., 1977) as a major adduct bound to DNA and RNA of bronchial explants after treatment of the cultures with BP. Also (+)diol epoxide 2 is the major enantiomer formed stereoselectivity in vivo from BP. It is concluded that (+)-BP-7β,8α-diol-9α,10α epoxide 2 is a major ultimate carcinogenic metabolite of BP in mice (fig. 3). These oxidative steps are thought to be catalyzed by cytochrome P450 1A1 and others (Foth, 1995).

D. Formation of the Metabolite-DNA Adduct

Ross et al. (1995) injected male mice with BP in doses ranging from 5 to 200 mg/kg and sacrificed them 1 to 21 days later. DNA was purified from whole lung homogenates and lung adenomas were counted 240 days after BP injection. DNA adduct analysis was conducted using ^{32}P postlabeling; time-integrated DNA adduct levels (TIDAL) were determined at each dose level. A strong correlation was found betwen lung ademona induction and the TIDAL levels. After BP administration, the major adduct isolated from lung was N (2)-(10β,7β,8α,9α-trihydroxy-7,8,9,10-tetrahydrobenzo[a]pyrene]yl deoxyguanosine, from the binding of the diolepoxide of BP to deoxyguanosine. The induction of adenomas as a function of TIDAL values suggests that the formation and persistence of DNA adducts determines the carcinogenic potency in a series of PAH. This relationship had been predicted earlier (Harvey, 1982) with deoxyguanosine as the DNA adduct. Earlier (Feldman et al., 1980) work had reported that reaction of the racemic ± BP diolepoxide with cultured human lung cells (A549) resulted predominately in the formation of the deoxyguanosine adduct.

TABLE 2
Pulmonary adenomas in newborn mice treated with BP and some of its metabolites

Compounds	Number of mice with pulmonary adenomas	Number of adenomas	Adenomas/ mouse
Control (DMSO)	8	9	0.13
BP	12	15	0.24
BP 7,8-dihydrodiol	41	110	1.77
(±) BP diol epoxide 1	2	3	0.14
(±) BP diol epoxide 2	55	283	4.42
BP tetraols	1	1	0.05

Data excerpted with permission from Kapitulnik et al. (1978). DMSO, dimethyl sulfoxide.

TABLE 3
Pulmonary tumors in newborn mice treated with BP and stereoisomers of BP-7,8-diol-9,10 epoxides

Compound number	Treatment	Total doses (nmol)	Mice with tumors (%)	Tumors/ mouse
1	Control (DMSO)	0	11	0.12
2	BP	7	18	0.22
3	(−)-BP-7β,8α-diol 9β,10β epoxide 1	7	13	0.15
4	(+)-BP-7α,8β-diol 9α,10α epoxide 1	7	14	0.15
5	(−)-BP-7α,8β-diol 9β,10β epoxide 2	7	9	0.09
6	(+)-BP-7β,8α-diol 9α,10α epoxide 2	7	71	1.72
2	BP	14	14	0.15
3	(−)-BP-7β,8α-diol 9β,10β epoxide 1	14	22	0.25
4	(+)-BP-7α,8β-diol 9α,10α epoxide 1	14	15	0.34
5	(−)-BP-7α,8β-diol 9β,10β epoxide 2	14	12	0.13
6	(+)-BP-7β,8α-diol, 9α,10α epoxide 2	14	100	7.67

Data excerpted with permission from Buening et al. (1978). DMSO, dimethyl sulfoxide.

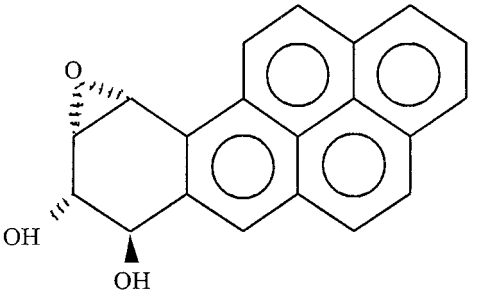


FIG. 3. (+) BP-7β,8α-diol-9α,10α-epoxide 2, an ultimate carcinogenic metabolite of BP formed in mouse lung. Reproduced from Buening et al. (1978).

E. Mutagenicity of the Reactive Metabolite of Benzo[a]pyrene

Recently, the distribution of BP diol epoxide adducts along exons of the p53 gene were examined in HeLa cells and bronchial epithelial cells was mapped at nucleotide resolution (Denissenko et al., 1996). Strong and selective adduct formation occurred at guanine positions in codons 157, 248 and 273. These same positions are the

major mutational hotspots in human lung cancers. Thus, targeted adduct formation rather than phenotypic selection appear to shape the P53 mutational spectrum in lung cancer.

Only (+) diol epoxide 2 (also referred to as the R, S,S, R enantiomer) demonstrated exceptional mutagenic activity in Chinese hamster V-79 cells, approximately eight times that of the other three isomers (Conney et al., 1994) which was in general accord with its higher tumorigenicity in newborn mouse lung (50 to 100 times) (Buening et al., 1978). The mutagenicity of three concentrations of (+) BP diol epoxide 2 [(+)BPDE2] was evaluated at the hypoxanthine (guanine) phosphoribosyntransferase (HPRT) locus in V-79 cells. Exposure of these cells to low (0.01 μM), intermediate (0.04–0.10 μM) or high (0.30–0.48 μM) concentrations of (+)BPDE2 resulted in 97%, 100% and 32% cell survival and mutation rates were increased 9-, 51- and 513-fold respectively compared with solvent (dimethyl sulfoxide) controls. Surviving colonies were isolated, complementary DNAs (cDNAs) were prepared, amplified by the polymerase chain reaction, and the coding region of the HPRT cDNA was sequenced (Conney et al., 1994). Base substitution mutations in the coding region of the HPRT gene were most common and were found in approximately 70% of the mutant clones. Exon deletions were observed in approximately 25% of the mutants and frame shift mutations were noted in approximately 5% of the mutant clones. Reducing the concentration of (+) BPDE2 decreased the proportion of base substitutions at guanine and cytosine base pairs (GC) and increased substitutions at adenine-thymine base pairs (AT). At the high concentration, 7 of 120 base substitutions occurred at AT (6%) and 113 at GC (94%). At the intermediate concentration, 20 of 82 base substitutions occurred at AT (24%) and 62 at GC (76%). At the low concentration, 27 of 76 base substitutions were at AT (36%) and 49 were at GC (64%). An evaluation of the frequency of mutations at specific bases in the coding region of the HPRT gene indicated differences in the profile of hot spots induced by different concentrations of (+) BPDE2. Eleven hot spots were observed at high concentrations, 7 hot spots at the intermediate concentration and 6 hot spots were noted at the low concentration. Thus, at a high cytotoxic concentration of (+) BPDE2, most base substitutions were at GC (predominantly GC → AT transversions), with only an occasional mutation at an AT. As the concentration of (+) BPDE2 was reduced, there was an increase in the base substitution at AT and a corresponding decrease in substitutions at GC (Conney et al., 1994).

V. Pneumotoxicity of 4-Ipomeanol and Other Furans

4-Ipomeanol [1-(3-furyl)-4-hydroxypentanone] is a natural product produced by the fungus Fusarium solani when it infects 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 (Boyd, 1976, 1980a).

A. Species Differences

Administration of 4-ipomeanol to rats, rabbits, guinea pigs and hamsters (Dutcher and Boyd, 1979) produces an organ- and cell-specific lesion, viz, selective necrosis of the nonciliated bronchiolar epithelial (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 and 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 hamsters, 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 markedly resistant to this renal insult. Taking advantage of an interesting phylogenetic peculiarity, Buckpitt et al. (1982a,b) 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. 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, whereas chicken lung microsomes were devoid of activity.

Dutcher and Boyd (1979) looked for possible species and strain differences in the covalent binding of 4-ipomeanol. Three strains of rat (Lewis, Sprague-Dawley, and Fisher-344), Hartley guinea pigs, albino New Zealand rabbits, Golden Syrian hamsters and six strains of mouse (BALB/CJ, C3H-HeJ, NIH Swiss, DBA/2J, C57BL/6J, and A/J) were examined. In all species tested, the lung was the major target for 4-ipomeanol covalent binding and toxicity as determined histologically. In the hamster and the mouse, 4-ipomeanol caused liver and kidney necrosis in addition to pulmonary damage. Corresponding high levels of covalent binding of 4-ipomeanol occurred in liver and kidney in these species.

The lung was the major site of damage in rats, guinea pig, and rabbit and this selectivity correlated with covalent binding. All six mouse strains exhibited some lung damage and striking renal toxicity: renal cortical necrosis occurred together with bronchiolar lesions. Covalent binding of 4-ipomeanol in the six mouse strains was roughly twice as high in kidney as in lung.

B. Covalent Binding

Boyd et al. (1975) showed that after administration of [^{14}C]4-ipomeanol to rats, radioactivity was concentrated in lung (per gram of organ), 90% of which was covalently bound. In this paper, the term "covalent binding" refers to material remaining bound to tissues or cell homogenates after their extraction with strong mineral acids and alkali, numerous hot and cold organic solvents ranging in polarity from methanol to hexane, boiling, and lyophilization. Employing electron microscopic autoradiography, Boyd (1977) demonstrated that after the administration of [^{14}C]4-ipomeanol to rats, mice and hamsters, electron-dense granules were found specifically localized over Clara cells which later became necrotic. In contrast, the adjacent ciliated bronchiolar cells and other major pulmonary parenchymal cells were neither radiolabeled nor became necrotic. Pretreatment of animals with piperonyl butoxide, an inhibitor of cytochrome P450 produced a striking reduction in covalently-bound radioactivity in the Clara cells and a total absence of necrosis. Boyd (1978) also pointed out that the covalently bound 4-ipomeanol was most heavily concentrated over the apical cap of Clara cells which is also the principal localization of the agranular endoplasmic reticulum in which the cytochrome P450 enzymes are known to be concentrated. Later work would confirm (Serabjit-Singh et al., 1988) that the Clara cells and particularly the apical tips which were rich in agranular endoplasmic reticulum contained rich concentrations of cytochrome P450 enzymes.

Boyd and Burka (1978) confirmed a close relationship between covalent binding of 4-ipomeanol and pulmonary toxicity (edema and histological changes). They found that binding was localized in lung proteins and that the formation of a reactive metabolite was a prerequisite to covalent binding. Pretreatment of animals with piperonyl butoxide reduced pulmonary covalent binding of 4-ipomeanol and increased the LD_{50} by 3- to 4-fold. In addition, pretreatment of rats with PB and 3-MC, inducers of cytochrome P450, reduced covalent binding in lung and doubled the LD_{50}. Diethylmaleate pretreatment, which depletes tissues of GSH, increased covalent binding of the reactive metabolite of 4-ipomeanol in both lung and liver and reduced the LD_{50} from 24 mg/kg to 6.3 mg/kg.

It should be pointed out that the 'vital macromolecules' to which the reactive metabolite of 4-ipomeanol is covalently bound are tissue proteins and not nucleic acids. Previous studies had revealed that hot trichloroacetic acid or perchloric acid (PCA) removed nucleic acids and the insoluble material left behind was predominantly protein (Brooks and Lawley, 1964; Boyd and Burka, 1978; Miller, 1951; Boyd, 1980c), which was assayed for radioactivity.

C. Enzymatic Requirements for Covalent Binding: Cytochrome P450 and Glutathione

Incubation of subcellular fractions from rat lung and liver with NADPH and 4-ipomeanol revealed that covalent binding occurred exclusively in microsomal fractions, activities being negligible in crude nuclear, mitochondrial, and cytosolic fractions. Covalent binding required NADPH, was inhibited by incubation under N_2 and CO and heat inactivation of microsomes. As noted previously, binding was abolished by the addition of piperonyl butoxide. The Km for covalent binding was ten-fold lower in lung than in liver. Inclusion of GSH (1 mM) in incubations essentially abolished covalent binding. Finally, the furan moiety of 4-ipomeanol was obligatory for covalent binding; its substitution by phenyl or methyl groups abolished binding. These findings indicated that the furan ring of 4-ipomeanol was activated in lung and liver microsomes by an NADPH and O_2-dependent enzyme system which was cytochrome P450-dependent to an active metabolite(s) which covalently bound to tissue proteins. The reactive intermediate was electrophilic and could be trapped by conjugation with GSH (Boyd, 1980c).

Incubation of rat lung or liver microsomes, NADPH, ipomeanol and reduced GSH resulted in the formation of two conjugates as determined by high-performance liquid chromatography (HPLC) analysis (Buckpitt and Boyd, 1980). The same conjugates were isolated whether the radiolabel was in the ipomeanol moiety or the GSH. More importantly, the formation of these conjugates did not require cytosol. Therefore, the GSH S-transferases were not required and conjugation of the activated 4-ipomeanol with GSH occurred nonenzymatically.

Work performed with isolated, purified cell types from rabbit lung confirmed earlier autoradiographic findings that Clara cells were the primary site of activation and covalent binding of 4-ipomeanol (Devereux et al., 1981). Covalent binding of [^{3}H]4-ipomeanol was completely inhibited by piperonyl butoxide (1 mM) indicating that binding was dependent on cytochrome P450 catalyzed activation. No measureable covalent binding of [^{3}H]4-ipomeanol was detected using alveolar macrophages: at V_{max} concentrations of substrates, the binding of 4-ipomeanol to protein in Clara cells was 135 pmol/10^6 cells/minute whereas in type II cells it was 13 pmol/10^6 cells/minute, a ratio of approximately 10:1. Addition of GSH to incubations containing sonicated Clara or type II cells resulted in the formation of two (uncharacterized) GSH conjugates.

Prior administration of small doses of 4-ipomeanol over a seven day period to mice, rats, and rabbits produced tolerance to subsequently administered lethal doses of 4-ipomeanol (Boyd et al., 1981). In all three species, tolerance was accompanied by a 2- to 3-fold increase in the LD_{50} and in rats this tolerance was associated with a two-fold reduction in pulmonary covalent binding of [^{3}H]ipomeanol across a range of doses from 10 to 80 mg/kg. Similar tolerance to large doses of CCl_4 by small multiple doses of CCl_4 have been reported (Recknagel and Glende, 1973).

The susceptibility of adult male mice to renal damage by 4-ipomeanol was noted. This suggests the possible appearance of a specific isoform of CYP450 in kidneys of adult male mice. Adult female mice and immature male and female mice however, exhibited only pulmonary damage. These differential susceptibilities correlated with covalent binding. In kidneys of male mice, the covalent binding of 4-ipomeanol was localized predominantly in the proximal tubules of the renal cortex and this binding and nephrotoxicity could be prevented by pretreating the animals with piperonyl butoxide (Boyd and Dutcher, 1981). Toxicity and covalent binding was examined in "inducible" (C57BL/6J) and "noninducible" (DBA/2J) male mice. The terms inducible and noninducible refer to the strains responsiveness or lack thereof of their hepatic "benzo[a]pyrene hydroxylase" to pretreatment with 3-MC. At the highest dose of 4-ipomeanol examined (80 mg/kg, ip), 3-MC pretreatment increased covalent binding in liver, reduced it slightly in lung, and dramatically reduced it in kidneys of inducible mice: pretreatment with 3-MC was without detectable effects on covalent binding in liver, lung and kidney in noninducible mice. Similarly 3-MC treatment of inducible (C57BL/6J) mice increased the LD_{50} for 4-ipomeanol from 24 mg/kg to 125 mg/kg and increased necrosis in liver, lung and kidney. 3-MC treatment of noninducible (DBA/6J) mice did not affect the LD_{50} or tissue necrosis.

Toxic doses of 4-ipomeanol preferentially depleted rat lung GSH. Indeed, covalent binding of 4-ipomeanol metabolites and lung levels of GSH changed in a reciprocal fashion (Boyd et al., 1982). Pretreatment of rats with piperonyl butoxide, an inhibitor of the metabolic activation of 4-ipomeanol prevented both the depletion of lung GSH and the pulmonary covalent binding of 4-ipomeanol. Similarly, prior treatment of rats with diethylmaleate to deplete lung GSH increased both the pulmonary covalent binding and the toxicity of 4-ipomeanol whereas the administration of cysteine and cysteamine reduced covalent binding and toxicity. It thus appeared likely that GSH played a protective role against lung alkylation and lung toxicity by reacting with the toxic intermediates to form nontoxic conjugates.

Pretreatment of rats with PB or 3-MC markedly reduced the amounts of covalently bound 4-ipomeanol (to ~30% of control) in lung but had variable efforts on binding in liver (Statham and Boyd, 1982a,b). Similarly, pretreatment of rats with diethylmaleate increased covalent binding of 4-ipomeanol derived radioactivity in lung by 50 to 75% but had no effect on binding in liver (Statham and Boyd, 1982a,b). The ratio of formation of reactive electrophilic metabolites from 4-ipomeanol was measured in rabbit liver and lung microsomes either by binding to protein or GSH (Wolf et al., 1982). The V_{max} for covalent binding was three times as high in lung as liver (2.11 versus 0.71) when normalized to microsomal protein and approximately 13 times higher (8.44 versus 0.65) when normalized per nmole of cytochrome P450. Two isozymes of cytochrome P450 were found in rabbit lung and both catalyzed the conversion of 4-ipomeanol to reactive products that covalently bound to microsomal protein and to exogenous GSH.

Metabolic activation of 4-ipomeanol by cDNA was measured with a DNA binding assay (Czerwinski et al., 1991). Twelve human P450s and two rodent P450s were expressed in human hepatoma G2 cells and examined for their abilities to activate this toxin to DNA binding species. Three forms, designated CYP1A2, CYP3A3 and CYP3A4 were able to catalyze significant production of DNA-bound metabolites of 20-, 8-, and 5-fold respectively over appropriate controls. These enzymes may be expressed in human or rodent lung. Interestingly, rabbit cDNA expressed CYP4B1 was the most active enzyme (180-fold over control) tested in producing DNA binding metabolites from 4-ipomeanol. Human CYP4B1 was only twice as active as controls in catalyzing covalent binding to DNA. Thus, striking differences exist in the ability of rabbit and human CYP4B1 to metabolically activate 4-ipomeanol to electrophilic species capable of binding to DNA. Again, these findings emphasize the importance of species differences in drug action, not an alien concept to pharmacologists' but seemingly so to certain biochemists. Confirmation of the role of CYP4B1 in rodent lung toxicity of 4-ipomeanol came from the finding that inhibition of CYP4B1 by treating rats with p-xylene specific for CYP 4B1 prevented the lung damage produced by 4-ipomeanol, increased the acute LD_{50} from 18 mg/kg to approximately 160 mg/kg, and reduced the covalent binding in lung by approximately 60%. Moreover, the CYP4B1 substrate 4-aminofluorene when administered to rats caused a six-fold reduction in 4-ipomeanol toxicity. In addition, a polyclonal antibody raised against CYP4B1 prevented the covalent binding of [^{14}C]ipomeanol to lung microsomal protein in vitro (Verschoyle et al., 1993a).

D. Biochemical Toxicity of Methylfurans

Inasmuch as 4-ipomeanol is a substituted 3-methylfuran (3-MF), it is not surprising that the toxicology of 2- and 3-methylfurans have been studied. Eight h after the administration of [^{3}H]3-MF to male Swiss mice, either intraperitoneally or by inhalation, autoradiograms revealed the intense and selective accumulation of radioactivity in terminal pulmonary bronchioles. After 24 h, alkylation and necrosis of Clara cells were most profound in smaller bronchioles and terminal bronchioles which were almost denuded of Clara cells, leaving behind abnormal ciliated cells. Intraperitoneal administration of piperonyl butoxide, prevented both the bronchiolar alkylation and bronchiolar necrosis produced by 3-MF inhalation. Incubation of mouse lung microsomes with NADPH and [^{3}H]3-MF in air revealed significant covalent binding to microsomal protein which was markedly inhibited by CO, incubation under N_2 or addition, in

vitro, of piperonyl butoxide (1 mM) (Boyd et al., 1978; Haschek et al., 1983, 1984).

The chemical nature of the reactive intermediates of 2-MF and 3-MF in rat lung microsomes was addressed by Ravindranath et al. (1984). Incubation of microsomes with NADPH and semicarbazide resulted in the trapping of the disemicarbazones of acetylacrolein (2-MF) or methylbutenedial (3-MF). Proof of the structures of these products was by mass spectral analysis. Covalent binding of [^{3}H]3-MF to lung microsomal protein was markedly inhibited by semicarbazide, presumably by successful competition for the reactive metabolite. It may thus be concluded that formation of the reactive metabolite of 2-MF and 3-MF involves opening of the furan ring at oxygen with the formation of highly reactive dialdehydes. This has not been demonstrated for 4-ipomeanol but is inferred.

Male and female rats (both Fischer derived and CD/CR) and male and female golden Syrian hamsters were exposed to 3-MF by inhalation. The LC_{50} for male Fischer rats was 81 (μmol/L), whereas that for male and female CD/CR rats was in the range of 200 to 220. By contrast, the LC_{50} for male and female hamsters was >322 μmole/L. In subsequent studies, rats were exposed to 148 μmol 3-MF/L (70% of the LC_{50}) and hamsters 322 μmol 3-MF/L for 1 h (Haschek et al., 1983). Pulmonary damage was observed in both species. In the hamster, selective necrosis of Clara cells was seen one day after exposure with virtually complete regeneration by 14 days whereas in the rat the bronchiolar epithelial damage was more extensive; both ciliated and nonciliated cells were necrotic and desquamated into the lumen. In the terminal bronchioles some Clara cells were spared. 3-MF also induced centrilobular hepatic necrosis in both species. Inhalation exposure of male BALB/c mice to a range of concentrations of 3-MF for 1 h (Haschek et al., 1984) resulted in necrosis of Clara cells within one day; this damage was most severe in the terminal bronchioles. Virtually complete regeneration of Clara cells was observed within 21 days. Cell kinetic studies using [^{3}H]thymidine revealed a peak in the bronchiolar labeling index (LI) of 5.0% at 3 days compared with 0.4% in controls. Cell proliferation returned to control levels at 21 days concomitant with morphological recovery. Increased labeling of alveolar type II cells and endothelial cells peaked at 10 days and coincided with a mild interstitial pneumonitis. 3-MF toxicity in mice was also manifested as centrilobular hepatic necrosis and lymphocyte necrosis in thymus and spleen. [^{14}C]2-methylfuran (2-MF) was incubated with microsomes from livers, lungs and kidneys obtained from male Sprague-Dawley rats with the formation of [^{14}C]4-keto-2-pentenal (acetyl acrolein). Metabolism was dependent upon NADPH and oxygen. Covalent binding of radiolabel was substrate and time-dependent and was inhibited by addition of piperonyl butoxide (Ravindranath and Boyd, 1985). Acetyl acrolein added in vitro completely inhibited the microsomal metabolism of 2-MF indicating that 2-MF is a suicide substrate for the enzyme. The sulfhydryl nucleophile cysteine was a better trapping agent of the reactive metabolite than N-acetylcysteine or GSH. Lysine reduced the covalent binding of 2-MF metabolites presumably by reacting with the aldehyde group of acetylacrolein. Ravindranath et al. (1986) reported that 2-MF administered to male Sprague-Dawley rats produced centrilobular hepatic necrosis and severe injury to the bronchiolar epithelium but at the highest dose tested no nephrotoxicity was noted. [^{14}C]2-MF resulted in covalent binding of radiolabel to protein in liver, kidney and lung and to DNA in liver and kidney and was dose-dependent. Slight depletion of tissue GSH was noted in liver (20 to 30%) and kidney (13%) but not in lung after 2-MF administration. Covalent binding of 2-MF-derived radioactivity in lung was only 14% of that observed in liver. Finally, Chen et al. (1995) incubated rat liver microsomes with furan, NADPH and semicarbazide and recovered a metabolite that coeluted (HPLC) with synthetic standards of the bis-semicarbazone adduct of cis-2-butene-14 dial. Structural assignment of the metabolite was by HPLC and nuclear magnetic resonance (NMR) spectrometry. The metabolic activation and pulmonary toxicity of 4-ipomeanol and 3 -MF have been reviewed extensively by Boyd (1980, 1984).

VI. Lung Toxicity of Naphthalene and 2-Methylnapthalene

A. Pulmonary Morphology and Monooxygenase Activities

Although frequently mis-cited as providing evidence for covalent binding of naphthalene in mammalian lung, the report of Reid et al. (1973) provided data only on bromobenzene and other related halobenzenes and their role in bronchiolar necrosis. To our knowledge, Mahvi et al. (1977) provided the first experimental evidence that the administration of naphthalene to C57BL/6J mice resulted in selective necrosis of pulmonary Clara cells. Using a range of doses of naphthalene from 0.05 to 2.0 mmol/kg (6.2–25.6 mg/kg) the authors reported that 12 h after 1 mmol/kg, the Clara cells were grossly dilated and in some areas had sloughed off; most bronchioles were clogged with debris. Twelve h after 2 mmol/kg there was extensive dilatation and necrosis of Clara cells and in some areas they had sloughed off from the basement membrane. Twenty-four h after naphthalene, the bronchioles of most animals were filled with cellular debris and a few Clara cells. Seven days after naphthalene administration, the structure of the bronchioles was restored and was indistinguishable from controls. Thus in the earliest morphological report on the pulmonary toxicity of naphthalene, Clara cells were found to dilate and exfoliate into the lumen and upon regeneration, the Clara cells and the cilated cells gradually regained their

normal structure. These morphological changes were pathognomic of naphthalene toxicity in mice.

Tong et al. (1982) reported on the effects of a single dose of naphthalene (225 mg/kg) on pulmonary morphology and monooxygenase activities in mice. Pulmonary monooxygenase activities were impaired significantly (30 to 70%) with a nadir at approximately 3 days after naphthalene without alterations in these activities in liver microsomes. No concomitant alterations in hepatic morphology were observed. NADPH cytochrome c reductase and cytochrome P450 were reduced to approximately 45% of control, whereas 7-ethoxyresorufin O-deethylase decreased to 30% of controls in lung microsomes. The remaining microsomal enzyme activities examined in lung clustered midway between the extremes at approximately 50% of control values. Inhibited enzyme activities remained at relatively constant levels between day 3 and day 8 and by day 15 there was a clear trend returning toward controls. Despite this trend, three of the six pulmonary enzyme activities remained significantly below control levels 15 days after a single dose of the hydrocarbon. Histologically, the pulmonary Clara cell was the primary target of naphthalene toxicity. At early time points it appeared as if the entire bronchiolar epithelium was undergoing necrosis and sloughing into the lumen but higher magnifications revealed residual intact ciliated epithelium. The distribution of Clara cell damage varied considerably; some bronchioles appeared completely denuded and in the same section, Clara cells had been spared, or, alternatively, were in the process of regeneration. No naphthalene-induced morphological alterations were noted in the alveoli. Bronchiolar damage had not achieved full repair even 15 days after naphthalene, at a time at which pulmonary monooxygenases were approaching control levels. In general, however, morphological alterations in lung bronchioles coincided with enzymic changes.

Hesse and Mezger (1979) studied covalent binding of [^{14}C]naphthalene and [^{14}C]naphthol to rat liver microsomes and reported that binding was exclusively to protein. Covalent binding was abolished by SKF 525-A, incubation under N_2 and reduced by 85% by incubation under 80% CO. With [^{14}C]naphthol as substrate, incubation under N_2 reduced binding by 75% but CO inhibited binding by only 40%. Addition of GSH (1 mM) impaired the binding to microsomal protein of both naphthalene and 1-naphthol by 60 to 70%. Covalent binding of [^{14}C]naphthalene was also blocked by omission of NADPH and by heat inactivation of microsomes. Binding was thought to be mediated by secondary metabolites of [^{14}C]naphthol.

Male C57BL/6J mice weighing 20 to 25 g were injected once ip with doses of 2-methylnaphthalene ranging from 0.1 mg to 1 g/kg and were sacrificed 24 or 48 h later. No renal or hepatic changes were observed by light microscopy in animals receiving up to the highest dose (Griffin et al., 1981). However, beginning at the 200 mg/kg dose, bronchiolar necrosis was observed which selectively involved the Clara cells; the alveolar parenchymal (type I and II) cells were unaffected. At higher doses of 2-methylnaphthalene (800 mg/kg) there was extensive involvement of both small and large airways and sloughing of epithelial cells into the lumen. Incubation of microsomes with 2-methylnaphthalene and NADPH produced three isomeric dihydrodiols as well as three monohydroxylated metabolites as determined by HPLC. Only trace amounts of these metabolites were produced by kidney.

Later work from this group (Griffin et al., 1982) used a fixed dose (400 mg/kg) of 2-methylnaphthalene administered to C57BL/6J male mice and investigated pathological changes in organs, covalent binding of [^{14}C]derived radioactivity and the effects of pretreatments known to modulate cytochrome P450. Twenty-four h after dosing, most of the Clara cells had sloughed off the bronchiolar basement membrane into the lumen. Ciliated bronchiolar cells generally remained intact but 20% of the animals experienced complete loss of bronchiolar epithelial cells. This dose of 2-methylnaphthalene did not produce hepatic or renal necrosis as determined by light microscopy. After administration of [^{14}C]2-methylnaphthalene, covalent binding was highest in kidney, followed by liver and lung. It was dose-dependent and, in general, at various doses followed the pattern kidney > liver > lung. Pretreatment of animals with either PB or 3-MC, produced variable and inconsistent effects in various organs. No clear pattern emerged. For example, treatment with diethylmaleate slightly reduced covalent binding in lung and kidney but had no effect in liver. The authors concluded that the covalent "binding of 2-methylnaphthalene metabolites to tissue macromolecules, per se, need not necessarily be synonomous with toxicity; metabolites may bind yet show no indications of toxicity" (Griffin et al., 1982).

B. Role of Tissue Glutathione Levels: Glutathione Conjugation and Lung Toxicity

Intraperitoneal administration of naphthalene to male Swiss mice resulted in severe bronchiolar epithelial necrosis at doses at which hepatic and renal necrosis were not observed. Pulmonary damage and mortality by naphthalene were increased by prior treatment with diethylmaleate and were reduced by prior treatment with piperonyl butoxide suggesting activation of naphthalene to a reactive electrophile and its detoxication by conjugation with GSH. Pretreatment with SKF 525-A had no effect on naphthalene-induced pulmonary damage. Administration of [^{14}C]naphthalene resulted in the covalent binding of radiolabel to tissue macromoles (Warren et al., 1982). The highest levels of binding occurred in lung, liver and kidney. Levels of covalent binding reached a maximum 2 to 4 h after treatment and corresponded to rapid depletion of GSH in lung and liver. Covalent binding was dose-dependent and showed a peak between 200 and 400 mg/kg which corresponded

to almost total depletion of endogenous GSH levels. Covalent binding of reactive metabolites was increased 3- to 4-fold by prior treatment with diethylmaleate and was reduced 3- to 4-fold by pretreatment with piperonyl butoxide. These results are consistent with the view that napthalene-induced lung damage is mediated by cytochrome P450-dependent reactive metabolites and that GSH plays an important role in the detoxication of the lung-toxic metabolite(s).

Incubation of [^{14}C]naphthalene with mouse lung microsomes, NADPH, GSH, and mouse lung cytosol (a source of GSH S-transferases), resulted in the production of three adducts (Smart and Buckpitt, 1983) which were distinguishable by HPLC analysis. These adducts were GSH conjugates of some reactive form of naphthalene as they were not formed in the absence of exogenous GSH. Earlier work had revealed a reciprocal relationship between covalent binding of [^{14}C]naphthalene in lung and GSH depletion in mice (Warren et al., 1982). Buckpitt and Warren (1983) confirmed these findings and also reported that the administration of p-xylene to mice which selectively impaired the metabolism of naphthalene to reactive metabolites reduced the in vivo covalent binding in liver and kidney to a similar extent as in lung. Pretreatment with buthionine sulfoximine preferentially depleted hepatic and renal but not pulmonary GSH levels but markedly increased covalent binding in all three organs. The severity of bronchiolar damage was increased by prior administration of buthionine sulfoximine. It was concluded that a portion of the reactive metabolites which deplete GSH and bind covalently in lung and kidney originate in the liver. Subsequent work (Buckpitt et al., 1984) revealed that incubation of [^{14}C]naphthalene with microsomes from mouse lung, liver and kidney resulted in the formation of 12-dihydro-12-dihydro-xynaphthalene (diol) and three GSH-derived conjugates that could be separated by HPLC. Addition of lung cytosol to liver microsomes or liver cytosol to lung microsomes changed the overall rate of conjugate formation but not the relative proportions of each of the conjugates. Addition of piperonyl butoxide or SKF 525-A to liver microsomes markedly reduced covalent binding of naphthalene metabolites but only slightly decreased GSH adduct formation. Dihydrodiol formation was increased by both inhibitors. These and other data indicate that the formation of conjugate peak 2 by lung microsomes in comparison to liver microsomes is due to the regio- or stereoselectivity of naphthalene metabolism by cytochrome P450 monooxygenases or epoxide hydrolases but not by the GSH transferases. Buckpitt et al. (1985) noted that the intermediate formation of phenols from several volatile aromatic hydrocarbons resulted in the formation of covalently bound metabolites and evaluated the involvement of 1-naphthol in the covalently bound metabolites and pulmonary bronchiolar necrosis produced by naphthalene. Marked differences were observed in the formation of 1-naphthol in mouse lung and liver microsomes without correspondingly large differences between the formation of covalently bound metabolites from naphthalene and 1-naphthol. GSH reduced covalent binding in liver microsomes containing [^{14}C]naphthol but did not change binding from [^{14}C]naphthalene. Lung and liver levels of covalently bound radioactivity in mice treated with [^{14}C]naphthol or [^{14}C]naphthalene were similar. In contrast to naphthalene, 1-naphthol administration did not deplete lung GSH nor mediate any bronchiolar damage. Thus, 1-naphthol was not an obligate intermediate in the covalent binding of naphthalene nor a proximate lung toxicant. O'Brien et al. (1985) reported on the relative toxicity of naphthalene in mice (male Swiss) and rats (male, Wistar derived). A dose of 200 mg/kg naphthalene administered to mice caused selective Clara cell damage. At higher doses (400 and 600 mg/kg), there was also necrosis of the epithelial cells of the proximal renal tubules. In contrast to the effects of mice, doses of naphthalene as high as 1.6 g/kg ip caused no pulmonary or renal damage in rats. This dramatic difference between mice and rats was reflected by the ability of naphthalene to more severely deplete GSH in the mouse lung and kidney than in those organs in rats. The metabolism of naphthalene was studied in lung and liver microsomes of mouse and rat (O'Brien et al., 1985). In all cases, naphthalene was metabolized to a covalently bound product(s) and to 1-naphthol and 12-dihydro-12-dihydroxynaphthalene. However, both the covalent binding and total metabolism of naphthalene were approximately ten-fold higher in microsomes from mouse lung than in those from rat. Thus the higher covalent binding of reactive metabolites and depletion of lung GSH levels may account for the differences in toxicity of naphthalene in the mouse and rat lung. Thus, in accord with the findings of Buckpitt et al. (1985), the toxicity of naphthalene does not appear to be mediated by 1-naphthol. Buckpitt and Bahnson (1986) investigated naphthalene metabolism in microsomes from human lung. In the presence of GSH and GSH S-transferases (purified from mouse liver) human lung microsomes catalyzed the formation of naphthalene dihydrodiol and three GSH conjugates. Interestingly, addition of human lung microsomes markedly inhibited mouse liver microsomal naphthalene metabolism, suggesting the possibility of an inhibitor in human lung. The human lung microsomes enhanced the formation of the dihydrodiol 3.5-fold, but reduced the formation of conjugates 12 and 3 by 50%, 75% and 75%, respectively.

Male Swiss-Webster mice were injected with a range of doses of naphthalene, 1-methyl-, 2-methyl, 1-nitro and 2-nitronaphthalene and their tissues were analyzed for cytotoxicity by light and electron microscopy and for [^{3}H]thymidine incorporation. In all cases, the first evidence of cytotoxicity was seen in the Clara cells and at the highest doses, toxic effects were found in Clara cells and adjacent ciliated cells (Rasmussen et al., 1986). Nei-

ther Type I or II alveolar epithelial cells showed evidence of morphological damage at any dose of naphthalene. Cytotoxic effects were confined to lung with minimal toxicity in liver and kidney. After naphthalene, [^{3}H]thymidine uptake increased 4-fold in bronchiolar epithelial cells at 3 and 7 days and returned to control in 14 days. No significant increase in labeling was found in alveolar cells. Richieri and Buckpitt (1988) reported that administration of (±) naphthalene oxide to male Swiss mice had no effect on hepatic or renal GSH levels but resulted in a dose-dependent depletion of this important tripeptide in lung at doses that exceeded 75 mg/kg, a threshold at which morphological alterations began to occur in lung. Studies employing isolated mouse hepatocytes incubated with [^{14}C]naphthalene revealed that naphthalene oxide, formed intracellularly, could be trapped extracellularly with[^{3}H]GSH and quantified by HPLC analysis. The oxide is an obligate intermediate in the metabolism of naphthalene (Richieri and Buckpitt, 1987). Between 17 and 35% of the naphthalene oxide formed within hepatocytes diffused out of the cell and effluxed into the medium and formed three GSH conjugates as had been reported previously. Therefore some of the covalently bound naphthalene radioactivity found in lung and in kidney may result from activation in liver.

C. The role of Stereochemistry in Naphthalene Pneumotoxicity

In a hallmark publication, Buckpitt et al. (1987) delineated the chemical structures of the three GSH conjugates formed during the pulmonary and hepatic microsomal oxidative metabolism of naphthalene in the presence of GSH and cytosolic GSH transferases. Products were identified by fast atom bombardment mass spectroscopy and by proton NMR spectroscopy and by comparison of metabolites with analogs prepared by chemical synthesis. The compounds were identified as monoglutathionyl derivatives of hydroxydihydronaphthalene. Adducts 1 and 2 were GSH conjugates of the diastereomers of 1-hydroxy-2-glutathionyl-12-dihydronaphthalene and adduct 3 was generated from the attack of GSH at the 1 position of naphthalene-12 oxide. Incubation of synthetic (1S, 2R)-naphthalene 12-oxide with GSH and GSH transferases resulted in the formation of adducts 1 and 3 in equal proportions. Under the same conditions, conjugate 2 was formed from (1R, 2S)-naphthalene 12 oxide. Incubation of naphthalene, GSH and GSH transferases with pulmonary, hepatic, on renal microsomes from mouse, rat and hamster resulted in the formation of all three GSH conjugates. It is important to note that (1R, 2S)-naphthalene oxide (adduct 2) was the predominant enantiomer (10:1 ratio) formed by microsomes from mouse lung, the major target organ in this species. In comparison, the rates of formation of (1R, 2S)-(adduct 2) to (1S, 2R)-naphthalene 12 oxide (adducts 1 and 3) in mouse liver and kidney and in rat and hamster tissues were 1:1 or less. These findings provide evidence for the formation of thio-adducts derived from attacks of GSH at the C-1 position of naphthalene 12-oxide and suggest that opening of the oxirane ring may proceed with both regio- and enantioselectivity in the GSH conjugation reaction. In addition, the comparative metabolism studies indicate that the stereoselectivity of naphthalene epoxidation varies considerably between species and organs and may be important in the pathogenesis of the acute toxicity of naphthalene (see also Buckpitt and Franklin, 1989). In addition, adduct 2 is clearly the metabolic product which accounts for the selective toxicity of naphthalene in mouse lung.

The work of Recknagel and his associates (Recknagel and Glende, 1973) showed that the administration of repeated small doses of the hepatotoxin CCl_4 results in the inhibition of the activation of CCl_4 to its proximate toxicant. Accordingly, O'Brien et al. (1989) reported that the administration of small daily doses of naphthalene to mice dramatically reduced the pulmonary changes (Clara cell necrosis) produced by a subsequently administered large toxic dose of the hydrocarbon. In addition, this apparent tolerance resulted in a selective decrease in the formation of 1R,2S-naphthalene oxide by mouse lung but not liver microsomes. However, alterations in the rate of formation of reactive covalently bound naphthalene metabolites in lung microsomes were not observed nor were there any differences in the levels of covalently bound reactive metabolites in vivo between tolerant and control animals. Thus, although the mechanism of this naphthalene tolerance remains unclear, these findings clearly separate necrosis from covalent binding.

The addition of 1R,2S- or 1S,2R-naphthalene oxide enantiomers to mouse hepatocytes resulted in a rapid depletion of intracellular GSH and this depletion correlated with rapid formation of naphthalene oxide GSH conjugates. Subsequent loss of GSH conjugates from incubations corresponded to the formation of N-acetylcysteine adducts (mercapturic acids), Buonarati et al. (1989) (fig. 4). In hepatocytes partially depleted of GSH by buthionine sulfoximine, addition of 1S,2R-naphthalene oxide resulted in significant losses in cell viability (75 to 100%). In contrast, changes in cell viability were slight (10 to 15%) with 1R,2S-naphthalene oxide. Both epoxides produced similar losses in cellular GSH. The differences noted in cytotoxicity of the epoxide enantiomers were considered to be related to differences in the rate of epoxide hydrolase-catalyzed dihydrodiol formation because substantially more diol was produced from the 1R,2S-epoxide than the 1S,2R-enantiomer. Buonarati et al. (1990) studied urinary excretion of isomeric naphthalene oxide-GSH conjugates in an effort to confirm earlier findings (Buckpitt et al., 1987) that revealed a high degree of stereoselectivity in the formation of (+)-1R,2S-naphthalene oxide from naphthalene in a target tissue (mouse lung) but not in a nontarget tissue such as mouse liver. Mercapturates (N-acetyl cysteine derivatives) accounted for approximately 70% of the

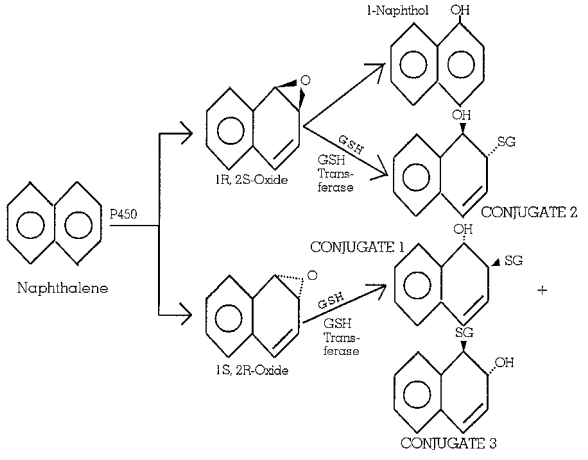


FIG. 4. Some routes of naphthalene metabolism in mice. Taken in part with permission from Buonarati et al. (1989). Reprinted from Chemico-Biological International 71:147–165, 1989, with kind permission from Elsevier Science Ireland Ltd., Bay 15K, Shannon Industrial Estate, Co. Clare, Ireland.

dose in 8 h urines of mice treated with trans-1-(S)-hydroxy-2-(S)-glutathionyl-12-dihydronaphthalene (adduct 1) and approximately 80% for trans-1-(R)-hydroxy-2-(R)-glutathionyl-12-dihydronaphthalene (adduct 2). Only 40 to 60% of the dose of trans-1-(R)-glutathionyl-2-(R)-hydroxy-12-dihydronaphthalene (adduct 3) administered to mice was excreted as a mercapturic acid derivative. However, two additional metabolites were detected which were not present in the urine of mice treated with adducts 1 and 2. The first (2 to 4% of the dose) was not identified. The second metabolite isolated by HPLC and identified by mass spectroscopy was (hydroxy-12-dihydronaphthalenethio) pyruvic acid accounted for 14 to 25% of the administered dose of adduct 3. Administration of a range of doses of racemic naphthalene oxide resulted in the excretion of all three mercapturic acid isomers in ratios that revealed no enantioselectivity in GSH conjugation of the (±) naphthalene oxide. Adducts 12 and 3 were all identified as mercapturic acid derivatives of naphthalene. Nagata et al. (1990) attempted to identify the isozyme(s) of cytochrome P450 in mouse liver and lung that catalyze the formation of both (1R, 2S) and (1S, 2R)-naphthalene 12 oxide but unfortunately used the trivial nomenclature then in use (e.g., P450m50a, P450m5b, etc). Their report is of limited utility.

A method was developed to investigate metabolic activation of naphthalene and Clara cell necrosis in the isolated perfused mouse lung (Kanekal et al., 1990). Using a synthetic perfusion medium, the lung was found to be viable for 4 to 5 h. There was evidence of interstitial and peribronchial edema and increased pulmonary arterial pressure at 5 h of perfusion. GSH levels remained constant for the first 3 h but fell to 57% of control at 5 h. Addition of naphthalene to the perfusion medium resulted in vacuolization of Clara cells followed by losses of this cell type from the bronchiolar epithelium. Clara cells lining the terminal airways were more sensitive morphologically to perfusion with naphthalene than those lining the bronchi. Clara cells comprised 63% of the bronchioles in control mice: 10 μmole of napthalene reduced this value to 30%. Perfusion with naphthalene resulted in concentration-dependent decreases in pulmonary GSH. Reactive metabolites were bound covalently to protein in the lung and were released into the perfusate.

The cytotoxic effects of naphthalene (0.5 mM) on bronchiolar epithelium in lung explants was identical with the vacuolization and exfoliation observed in Clara cells of bronchioles in mice 24 h after ip administration of naphthalene (100 or 300 mg/kg). The majority of the ciliated cells linining terminal bronchioles did not appear to be altered (Plopper et al., 1991a). Preincubation of the explants with piperonyl butoxide, prevented naphthalene-induced cytotoxicity. Naphthalene oxidation was easily measureable in all levels of airway including trachea, lobar bronchi and distal bronchioles. No metabolism was detected in alveolar epithelium or large blood vessels. A dihydrodiol and a GSH adduct (conjugate 2) derived from 1R,2S-naphthalene were the sole metabolites detected by HPLC. Formation of a single diastereomeric GSH conjugate indicated that the metabolic epoxidation of naphthalene was highly stereoselective.

D. Species Differences in Naphthalene Lung Toxicity

Buckpitt and his colleagues published a series of four papers on relations between cytochrome P450 and Clara cell toxicity after administration of naphthalene to three or more animal species. The first paper (Plopper et al., 1992b) compared the susceptibility of Clara cells in lungs of mice, rats, and hamsters at all levels of the tracheobronchial tree to varying doses of naphthalene administered ip. In mice, doses ranged from 0 to 400 mg/kg, in hamsters from 0 to 800 mg/kg and in rats from 0 to 1600 mg/kg; naphthalene was dissolved in corn oil, and the animals were sacrificed 24 h after dosing. In mice, naphthalene evoked Clara cell toxicity at 50 mg/kg characterized by edema and vacuolization in Clara cells in terminal bronchioles. At 100 mg/kg the lesions in these cells increased. At 200 and 300 mg/kg almost all the noncilated cells lining terminal bronchioles were necrotic and exfoliated. At 300 mg/kg lobar bronchus and trachea showed some edema and vacuolization of Clara cells in mice. In contrast, there was no apparent naphthalene effect on Clara cells or ciliated cells in rats treated with doses up to 1600 mg/kg. At 800 mg/kg, minor alterations in Clara cells in some terminal bronchioles were observed in hamsters. The trachea and lobar bronchus were unaffected in rats but showed cytotoxic changes in hamsters. Thus naphthalene injury to the tracheobronchial tree in mice was Clara cell specific, dose-related in the terminal bronchioles and involved more distal airways in a dose-dependent manner. The tracheobronchial epithelium of the rat lung was refrac-

tory to naphthalene-induced Clara cell damage even at the LD_{50} but proximal airways were more susceptible than distal airways in the hamster. The next paper in this series (Buckpitt et al., 1992) focused on stereoselectivity of naphthalene epoxidation in respiratory tissues of mouse, hamster, rat and rhesus monkey. Analysis of the three unstable chiral epoxide metabolites which undergo GSH transferase-dependent conjugation with GSH was conducted in nasal mucosa, lung, and liver in vitro. The highest rates of naphthalene metabolism were found in mouse lung and liver microsomes. Rat, hamster and monkey lung microsomes metabolized naphthalene at 12, 37, and 1% respectively of the rate observed in mouse lung. The three GSH conjugates were separated by HPLC and quantified by liquid scintillation counting. At high substrate concentrations (0.25 to 1 mM), the ratio of 1R,2S- to 1S,2R-naphthalene oxide measured by GSH adducts (adduct 2/adducts 1 plus 3) in mouse lung microsomes was 10:1 and at low substrate concentrations (0.062 mM and below) varied from 13.8:1 to 30:1. In contrast, the ratio of 1R,2S- to 1S,2R-naphthalene oxide produced in mouse liver microsomes varied from 1:1 at high substrate concentrations to 5:1 at low substrate concentrations. The ratios of naphthalene oxides were unaffected by the concentration of GSH. In contrast to the preferential formation of 1R,2S-naphthalene oxide observed in mouse lung microsomes, lung microsomes from rat, hamster, and monkey yielded 1R,2S- to 1S,2R-epoxide ratios of 0:48, 0:61, and 0:12 at naphthalene concentrations of 0.5 mM. Microsomes from the olfactory region of mouse, rat, and hamster showed the highest rates of substrate turnover and 1R,2S-naphthalene oxide was the predominant enantiomer formed in all three species. This study showed a good correlation between the rate and stereochemistry of naphthalene epoxidation with the species, tissue and regional toxicity of naphthalene in rodents.

Plopper et al. (1992a) described the comparative Clara cell toxicity of injected naphthalene in a range of doses in mice, hamsters, and rats. That there are marked species differences in the acute toxicity (LD_{50}) of naphthalene: 380 mg/kg in mice, 2.2 g/kg in rats, and 800 mg/kg in hamsters. There are accompanying marked species differences in pulmonary toxicity. Clara cells in mice are highly sensitive to naphthalene at very low doses (50 mg/kg), undergoing vacuolization, vesiculation, necrosis and sloughing. In mice, more vacuolated cells are lost from bronchioles than from bronchi. The Clara cells of the hamster exhibit a minimal response at 16 times the dose (800 mg/kg) and those of rats displaying no response at 32 times the dose (1.6 g/kg).

In the fourth paper in the series (Buckpitt et al., 1995) it was reported that the ratios of naphthalene metabolism in vitro were substantially higher in mouse airways, obtained by micro-dissection, than in comparable airways of hamsters and rats. At most airway levels, the rates of substrate turnover in mice were at least twice as high as in hamsters and were 3 to 5 times greater than in rats. The overall rate of metabolism was significantly higher in more distal airways then in proximal airways. Metabolism of naphthalene in mouse airways was highly stereoselective whereas in hamster and rat tissues it was not. Clara cells at all airway levels in mice were heavily labeled with an antibody to cytochrome P4502F2 whereas little labeling was observed in any portion of rat or hamster lung. The authors concluded that the rate and particularly the stereoselectivity of naphthalene metabolism to naphthalene 1R,2S-oxide catalyzed by cytochrome P4502F2 are critical determinants in the species-specific and region-selective cytotoxicity of naphthalene in mice.

E. Metabolism of Naphthalene in Purified Clara Cells from Mouse Lung

Clara cells were isolated and purified from mouse lungs (average purity of 72 ± 5%). The ability of these cells to metabolize naphthalene was analyzed under two conditions: in homogenized Clara cells supplemented with GSH and GSH S-transferases, and in intact Clara cells. In homogenized cells, naphthalene was metabolized primarily to a single GSH adduct (major metabolite) derived from the 1R,2S-epoxide and to a minor dihydrodiol metabolite. In intact cells, the formation of the GSH adduct was much lower and the dihydrodiol predominated (Chichester et al., 1994). Incubation of high concentrations (0.25 and 0.5 mM) of naphthalene oxide with cell homogenates favored the formation of the adduct derived from the 1R,2S-epoxide. Intact Clara cells were incubated with naphthalene and naphthalene metabolites to determine effects on cell viability. High concentrations of naphthalene (0.5 and 1.0 mM) reduced Clara cell viability by approximately 40% and this reduction was blocked by preincubation with piperonyl butoxide. In contrast 0.5 mM naphthalene oxide and 14-naphthoquinone, reduced Clara cell viability by approximately 85% of control and these losses in viability were not affected by piperonyl butoxide. Thus, it is clear that the viability of Clara cells isolated from mouse lung are more susceptible to naphthalene metabolites than to naphthalene itself.

In further work from the Buckpitt laboratory (Cho et al., 1994), employing isolated Clara cells from mouse lung as well as samples of mouse lung obtained by blunt dissection (trachea, lobar bronchus, major and minor daughter branches and distal bronchioles), reactive metabolite binding was 5 to 7 times higher in incubations of [^{3}H]naphthalene with distal bronchioles and isolated Clara cells then with explants of trachea or bronchus. Likewise, binding was substantially higher in incubations of murine Clara cells than in identical incubations with mouse hepatocytes (nontarget cells) or rat tracheal cells (nonsusceptible species). The data showed a good correlation between cellular susceptibility to toxicity and the amount of reactive metabolites bound in vitro. Concentrations of GSH adduct were highest in the me-

dium and the nuclear/cell debris fraction of isolated Clara cells incubated with naphthalene, and then homogenized. Covalent binding of reactive metabolites to proteins at all airway levels was 2- to 15-fold higher in the medium than in the tissue. The highest levels of bound metabolite were observed in the medium of incubations that contained the distal bronchi and was at least 2.5 times greater than in incubations that contained other lung subcompartments. In isolated Clara cell incubations, reactive naphthalene metabolite binding was 5 to 7 times higher than in isolated mouse trachea or mouse hepatocytes. Preincubation with piperonyl butoxide reduced covalent binding by 80 to 90% in cells and medium. Clara cells were incubated with [^{3}H]naphthalene, unbound radioactivity was removed and radiolabeled proteins separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). Labeling occurred mainly to a single protein, M_r 14 to 15 kDa with lesser amounts bound to proteins at 30 and 45 kDa. Binding in hepatocyte incubations was solely to a protein of 14 to 15 kDa. These experiments suggested that the amount of metabolite bound to the 14 to 15 kDa protein may be an important factor in susceptibility to naphthalene and that the major protein targeted in susceptible mouse Clara cells and unsusceptible hepatocytes maybe similar.

In a recently published abstract, Buckpitt's group (Chang et al., 1996) verified that naphthalene is metabolized by CYP2F2 in mouse lung to products that are cytotoxic to Clara cells. Mouse lung and liver microsomes were incubated with the suicide inhibitors 1- and 2-ethylnylnaphthalene, 9-ethynylphenanthrene and 5-phenyl-1-pentyne (PP). Inhibition by PP requires NADPH and is dependent on preincubation and inhibitor concentration. Concentrations of 5 μM PP resulted in 80% inhibition of the formation of the 1R,2S-epoxide by lung microsomes, less inhibition of 1S, 2R isomer but only 25% inhibition in liver microsomes.

The role of naphthalene and other xenobiotics in producing Clara cell toxicity has been recently reviewed (Cho et al., 1995).

VII. Pulmonary Neoplasia Resulting from the Tobacco-Specific Nitrosamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone

A. Reaction of 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone Metabolites with DNA and Persistence of the Adduct

More than 3000 specific chemical compounds, many of which are mutagenic or carcinogenic have been identified in tobacco smoke (Surgeon General's report, 1982). Among these are several nitrosamines, of which 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) has been studied extensively in animals.

Hoffmann et al. (1984) reported a high incidence of pulmonary tumors associated with the administration of the tobacco-derived nitrosamines N-nitrosonornicotine (NNN) and NNK but not N′nitrosoanatabine administered to rats three times weekly for 20 weeks at total doses of 13 and 9 mmol/kg (table 4). There was apparently a slight sex difference in lung tumor incidence, males being somewhat more susceptible than females. In addition, in male rats, there was no obvious dose-response relationship in lung tumors, at the doses employed. Although many of the lung tumors were benign adenomas, there were significant numbers of adenocarcinomas and squamous cell carcinomas, especially in males.

NNK was administered daily (Belinsky et al., 1986) to Fischer 344 male rats for up to 12 days, which resulted in the accumulation and persistence of the promutagenic adduct O^6-methylguanine (O^6-MG) in the DNA of the lung. The persistence of this adduct correlated with the inhibition (>95%) of the repair enzyme O^6-methylguanine-DNA methyltransferase (O^6-MGMT) which removes the methyl group from O^6-MG in DNA. These studies demonstrated that although O^4-methyldeoxythymidine was also formed, it was removed rapidly from NNK-treated lungs, whereas there was persistence of the promutagenic adduct O^6-MG-DNA in lung during repeated exposure to NNK.

Peterson and Hecht (1991) similarly demonstrated a strong correlation ($r = 0.98$) between lung tumor yield and levels of O^6-MG in A/J mouse lung. They suggested that the formation and persistence of O^6-MG were critical events in the initiation of lung tumors by NNK.

B. Reaction of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone Metabolites with Specific Pulmonary Cell Types

Belinsky et al. (1987) administered NNK to male Fischer 344 rats in doses ranging from 0.1 to 100 mg/kg ip, daily for up to 12 days and found that the ratio of lung O^6-MG/guanine, when plotted against the dose of NNK, an index of alkylation efficiency, decreased as the dose increased. This observation suggested a low-capacity readily saturable uptake mechanism in whole lung. Separation of pulmonary cell types revealed, especially at

TABLE 4
Incidence of respiratory tract tumors in Fischer 344 rats receiving subcutaneous injections of NNK for 20 weeks

Dose	Rats with nasal tumors (%)	Rats with lung tumors (%)
1 mmol/kg		
Males	74	85
Females	37	30
3 mmol/kg		
Males	87	87
Females	80	47
9 mmol/kg		
Males	93	93
Females	93	60

Data excerpted from Hoffmann et al. (1984).

lower doses of NNK, a marked concentration of the O^6-MG adduct in Clara cells (table 5).

Thus, when corrected for cross-conamination of cell types, the calculated concentration of O^6-MG in Clara cells was 19 to 25 times greater than in type II cells and 7.5 times greater than alveolar macrophages. The distribution of NNK within lung was determined in rats treated with 1 mg/kg [^{3}H]NNK and killed 4 h later. Autoradiograms revealed that NNK was much more concentrated in Clara cells than in other cell types in lung (Belinsky et al., 1987). Treatment of rats with NNK also produced a dose-dependent inhibition of O^6-MGMT, and this inhibition correlated with O^6-MG levels in lungs of animals treated with NNK.

Unusually high steady state levels of a xenobiotic in a cell type is generally considered to result from high influx, peculiar mechanisms for retention (e.g., covalent binding), or slow efflux. Excepting active or facilitated transport, influx is generally perfusion-related and might account for differences in xenobiotic accumulation between myocardium and bone. Well recognized are differences in efflux, which may reflect diffusion or metabolism (e.g., to glucuronides) and differences in metabolic rates of xenobiotics between cells and organs. Which of these factors account for the disparate distribution of NNK among cell types of the lung?

Male Fischer 344 rats were treated ip with NNK (10 mg/kg/day) for 4 days, and pulmonary cell types were purified (Belinsky et al., 1988). One day after the last injection, concentrations of O^6-MG (pmol O^6-MG/μmol guanine) in alveolar macrophages were 70, in type II cells 27, and in Clara cells 95. Moreover, the loss of O^6-MG over a period of 8 days differed markedly among pulmonary cell types. The disappearance of this adduct from DNA of alveolar macrophages was rapid ($t_{1/2}$ approximately 48 h) and followed first-order kinetics. In contrast, very little loss of O^6-MG was observed in Clara cells. The concentration of this adduct decreased only from 100 to 60 pmol O^6-MG/μmol guanine over the 8-day period. Assuming first-order kinetics, this reflects a $t_{1/2}$ in Clara cells of approximately 10 days. The disappearance of O^6-MG from DNA of type II cells was not linear. A rapid decline in adduct concentration was observed in type II cells for the first 3 days after discontinuing treatment (from 25 to 8 pmol O^6-MG/μmol guanine) followed by a further decline of 25% over the remaining 5 days. More importantly, as shown previously for whole lung (Belinsky et al., 1986), NNK treatment differentially affected O^6-MGMT among cell types: it had no observable effect on O^6-MGMT in alveolar macrophages, it reduced O^6-MGMT activity in type II cells by 82% and it abolished O^6-MGMT activity in Clara cells (table 6).

After a 4-day treatment of rats with NNK, the concentration of the promutagen O^6-MG was greatest in Clara cells and was 1 to 10 times greater than in other pulmonary cell types. Thus, Clara cells accumulate and preferentially retain O^6-MG relative to other pulmonary cell types. In addition, Clara cells clear O^6-MG much slower than other cells, and this difference correlates well with the inhibition by NNK of O^6-MGMT, the enzyme which converts O^6-MG to guanine in DNA (Belinsky et al., 1988). The accumulation and persistence of high concentrations of O^6-MG in Clara cells may result, in part, from saturation, inhibition, a selectively low rate of resynthesis of O^6-MGMT in this cell type during treatment with NNK. Moreover, the accumulation and persistence of high levels of O^6-MG in Clara cells suggest that this cell type may be the progenitor cell for NNK-induced rat lung tumors.

A study was conducted on the comparative biochemical toxicology of NNK and N-nitrosodimethylamine (NDMA) in rat lung, because it had been reported (Hecht et al., 1986) that NNK produced a 50% incidence of malignant lung tumors in rats, whereas treatment with equivalent doses of NDMA failed to induce any lung tumors. Male Fischer 344 rats were treated daily with a range of equimolar doses of NNK and NDMA for 4 days and killed 4 h after the last dose (Devereux et al., 1988). Purification of pulmonary cell types revealed no cell selectivity for DNA methylation (O^6-MG) with NDMA, whereas, at equimolar doses of NNK, the O^6-MG content in Clara cells was approximately 40 times that of Clara cells from rats treated with NDMA. At lower doses, whole lung DNA methylation was approximately 4 times greater with NDMA than with NNK, but O^6-MG content of Clara cells was 50 times higher with NNK than with NDMA. Similarly, pulmonary cells isolated from untreated rats and incubated in vitro with NNK revealed approximately eight-fold higher methylation in Clara cells as compared with type II cells (table 7).

TABLE 5
Accumulation of O^6-MG in rat lung cell types after administration of NNK

	O^6-MG (pmol/μmol guanine)	Alkylation efficiency (O^6-MG/dose)
Whole lung	0.9	3.0
Alveolar macrophages	3.8	12.7
Type II cells	1.1	3.7
Clara cells	28.2	93.2

Male Fischer 344 rats were injected intraperitoneally with 0.3 mg NNK/kg/day for 4 days and killed 4 h after the last dose. Data excerpted with permission from Belinsky et al. (1987).

TABLE 6
Effect of NNK treatment (10 mg/kg/day for 4 days) on O^6-MGMT activity in lung cells of Fischer 344 rats

	O^6-MGMT activity (pmol/O^6-MG/μmol guanine)	
	Vehicle control	NNK
Alveolar macrophages	31.1	28.2
Type II cells	32.5	6.0
Clara cells	15.2	not detectable

Data excerpted with permission from Belinsky et al. (1988).

TABLE 7
Activation of NNK in vitro by pulmonary cell types from rat lung

Cell types	Methylation index (pmol O^6-MG/μmol guanine)
Macrophages	Not detectable (<0.2)
Type II cells	2.9
Clara cells	21.7

Data excerpted with permission from Devereux et al. (1988).

To evaluate the relation between O^6-MG formation in Clara cells and pulmonary neoplasia (Belinsky et al., 1990), Fischer 344 male rats were treated with doses of NNK ranging from 0.1 to 50-MG/kg for 4 weeks. Purification of lung cell types revealed Clara cells to have 10- to 30-fold higher levels of O^6-MG than other cell types (table 8).

The earlier finding that there was no detectable O^6-MG formation in vitro by isolated macrophages (see table 7) suggested that the presence of this adduct in macrophages must derive from its formation in other cells and diffusion into macrophages. The efficiency of adduct formation was greatest in Clara cells where the content of O^6-MG increased markedly in the dose range from 0.1 to 1.0 mg/kg NNK. The dose-response curve for tumor incidence revealed a sharp increase in tumorigenicity as the dose of NNK increased from 0.3 to 1.0 mg/kg. When O^6-MG adduct concentration in specific cell types was plotted against tumor incidence, a linear relationship ($r = 0.99$) was found for Clara cells (Belinsky et al., 1990), whereas nonlinear plots with poor correlation coefficients were found for other cell types and whole lung. The authors concluded that the O^6-MG content of Clara cells appeared to be an excellent predictor of the carcinogenic potential of NNK in rat lung. The accumulation and persistence of DNA damage in Clara cells are compounded by the low basal activity or sluggish synthesis rate of the repair enzyme O^6-MGMT in Clara cells (table 6). It must be remembered that multiple doses of NNK reduced the activity of O^6-MGMT to below the limits of detection in Clara cells (table 6) and that there was little loss of O^6-MG from Clara cells over an 8-day period while this adduct was removed efficiently from type II cells. These factors make Clara cells a sensitive target for NNK-induced neoplasia.

TABLE 8
Relation between NNK dose and content of O^6-MG in specific pulmonary cell types of male Fischer rats

Dose (mg/kg)	O^6-MG (pmol/μmol guanine)			
	Whole lung	Macrophages	Type II cells	Clara cells
0.1	0.4	1.8	0.1	3.4
0.3	0.7	3.9	0.6	19.2
1.0	2.2	36.2	1.1	67.4
10.0	6.8	60.5	5.3	85.3

Data excerpted with permission from Belinsky et al. (1990).

C. Inhibition of 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone Activation and Carcinogenesis

In an amplification of the role of NNK activation in vivo with the formation of O^6-MG and its role in lung tumorigenesis, Morse et al. (1989) studied the effects of NNK activation on lung tumor formation in mice. Phenethyl isothiocyanate (PEITC), an inhibitor of NNK activation, was administered by gavage for 4 consecutive days. A single ip dose of NNK (10 umol/mouse) resulted in a 100% incidence of pulmonary adenomas (table 9), with an accompanying multiplicity of 10.7 tumors per mouse. PEITC clearly reduced both the number of mice with NNK-induced pulmonary tumors as well as the number of tumors per mouse in a dose-dependent manner. Neither dose of PEITC resulted in any overt toxicity. In addition, both doses of PEITC markedly inhibited the formation of O^6-MG in mouse lung after NNK administration (10 μmol/animal) (table 10).

Based on its ability to inhibit both NNK-induced tumorigenicity and O^6-MG formation, PEITC was tested for its ability to inhibit pulmonary microsomal activation of NNK in vitro (table 11). The total metabolic activation of NNK was reduced by greater than 90% at two doses of PEITC. This inhibition of NNK metabolism could readily account for the reduction of NNK-induced O^6-MG formation and tumor incidence in vivo by PEITC.

Thus, pretreatment of mice for 4 days with PEITC before the administration of a single dose of NNK markedly reduced the lung tumor incidence, the formation of O^6-MG, and the metabolic activation of NNK.

NNK is known to yield at least four metabolites in vivo, most of which are carcinogenic. 4-ipomeanol, although not carcinogenic, is known to be activated in, and cause necrosis of, Clara cells, thus, sharing a cellular site of metabolic activation with NNK. Accordingly, Lin et al. (1992) studied the effects of 4-ipomeanol and four chemical analogs on NNK metabolism and carcinogenicity in female A/J mice. The analogs reduced metabolite formation from NNK to varying degrees (10 to 50% reduction in vitro) by lung microsomes but had much less or no effect on formation of the same metabolites by liver microsomes. The same analogs that inhibited NNK metabolism in vitro reduced the number of NNK-induced lung tumors by approximately 50%. The authors concluded that inhibition of NNK metabolism in vitro correlated directly with NNK tumorigenesis in vivo.

TABLE 9
Effects of PEITC treatment on NNK-induced (10 μmol/animal) pulmonary adenomas in mice

Pretreatment	Daily dose (μmol) (PEITC)	Mice with adenomas (%)	Tumors/ mouse
None + NNK	—	100	10.7
PEITC + NNK	5	89	2.6
PEITC + NNK	25	30	0.3

Data excerpted with permission from Morse et al. (1989).

TABLE 10
Effects of PEITC treatment on NNK-induced 0^6-MG formation in mouse lung

Pretreatment	Daily dose (μmol)	O^6-MG (μmol/mol guanine)	
		2 h after NNK	6 h after NNK
None	—	33.1	30.9
PEITC	5	10.6	3.9
PEITC	25	12.8	<1.0

Data excerpted with permission from Morse et al. (1989).

TABLE 11
Effect of PEITC pretreatment on the metabolism of NNK by mouse lung microsomes

Pretreatment	NNK metabolized (%)
None	19
PEITC (5 μmol)	1.5
PEITC (25 μmol)	1.5

Data excerpted with permission from Morse et al. (1989).

Most research has concentrated on the chemistry of NNK metabolism as opposed to the enzymology. Crespi et al. (1991) suggested that NNK may be a substrate for the polymorphic human lung CYP2D6.

D. The Chemical Nature of the Ultimate Carcinogenic Metabolite of 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone

Unlike naphthalene, 4-ipomeanol and BP for which the structure of the ultimate toxicant has been determined with a fair degree of certainity, the extreme instability of nitrosamines, including NNK both in vitro and in vivo have not made facile identification of the moiety that binds to DNA. Indeed, in a recent review on the metabolic activation of NNK and the related NNN, a figure entitled "Metabolism of NNK and NNN in laboratory animals" occupies an entire journal page turned on its side (Hecht, 1994). This is because many of the intermediates are either highly labile and impossible to isolate or are purely theoretical. Hecht states "the overall pattern of P450 catalyzed α-hydroxylation of NNK is complex and not fully understood at present. However, it is clear that different forms of cytochrome P450 can catalyze the two α-hydroxylation pathways. α-Hydroxylation of the methylene group produces an intermediate which spontaneously decomposes to a keto alcohol and methane-diazohydroxide. The latter reacts with DNA to produce 7-methylguanine, O^6-MG and O^4-methylthymidine, all of which have been identified in tissues of animals treated with NNK: α-hydroxylation of the methyL group yields an intermediate which spontaneously decomposes to produce formaldehyde and 4-(3-pyridyl)-4-oxobutane-diazohydroxide which reacts with DNA forming adducts of unknown structures. . . . " To this reviewer this is a chemical problem not a biological one and will not be addressed further here.

VIII. 1,1-Dichloroethylene-Induced Lung Injury

A. Morphological Effects in Lung and Effect on P450-Related Oxygenases

1,1-Dichloroethylene (DCE), also known by its trivial name vinylidene chloride, is an important industrial compound which is widely used in the plastics industry. In 1976, the United States and Japan together produced approximately 150 million kilograms. Many early studies with DCE in animals revealed it to have liver and kidney toxicity and to have mutagenic and potentially carcinogenic activities. The major route of elimination of DCE was through the lungs (Bolt et al., 1982; Jones and Hathaway, 1978).

In 1982, Forkert and Reynolds (1982) reported that the administration of DCE to mice produced remarkable pulmonary injury. Oral doses of 100 mg/kg or 200 mg/kg selectively affected the bronchiolar epithelium with selective damage to Clara cells. After a 100 mg/kg dose of DCE, Clara cells revealed extensive dilation of the cisternae and degeneration of the endoplasmic reticulum. At 6 h after administration of 200 mg/kg of DCE, both ciliated and Clara cells were necrotic and the bronchiolar epithelial lining exfoliated. Pulmonary injury 24 h after the high dose produced significant hypoxia as demonstrated by a decrease in pO_2 of arterial blood (control of 100 mm Hg versus 73 after DCE; a dose-response relationship was noted). After 100 mg/kg DCE, despite the vacuolization, necrosis and sloughing of Clara cells the bronchioles underwent repair and at 48 h they displayed a normal and intact epithelium. After 200 mg/kg DCE despite the more severe pulmonary injury, complete recovery of the epithelium was accompanished by 7 days. It is of interest that after the low dose, the main bronchi and trachea did not exhibit any morphological alterations in the epithelium whereas the large dose produced degenerative changes and exfoliation in Clara cells in both main bronchi and trachea. Both doses of DCE produced hepatic necrosis as indicated by rather marked increases in liver enzyme activities (transaminases) in the serum.

In view of the toxic effects of DCE on pulmonary Clara cells which are known to possess high levels of cytochrome P450 and related oxygenases in mice, the effects of acute DCE treatment on these parameters was investigated (Krijgsheld et al., 1983). DCE was administered to male mice (125 mg/kg, ip) and they were killed 24 h later. Lung microsomal cytochrome P450 levels were reduced 50% by DCE, whereas other enzyme activities were reduced 30 to 60%. Corresponding enzyme activities in liver and kidney were unaffected by this dose of DCE. Morphological changes were found only in lung in which necrosis was found selectively restricted to Clara cells. No morphological changes were noted in liver or kidney. In a harbinger to later more sophisticated research, Hewitt and Plaa (1983) reported that pretreat-

ment of rats with acetone produced a dose-dependent effect on DCE toxicity. Low doses (5 and 10 mmol/kg po) markedly potentiated DCE hepatotoxicity whereas higher doses of acetone (15 and 30 mmol/kg) did not. Nephrotoxicity was not noted. Reynolds et al. (1984) published data indicating that the administration of DCE to fasted rats produced hepatic necrosis which resembled apoptosis. Krijgsheld et al. (1984) followed the time course of DCE administered to mice: animals were followed from 1 to 42 days after a single dose of 125 mg/kg and cytochrome P450 and related enzymes in lung were measured. The nadir of most microsomal enzymes was at either 1 or 4 days post-DCE and clustered at approximately 50% of control activities although coumarin hydroxylase activity declined to approximately 10% of control levels at 4 days after DCE. The rates at which enzyme activities returned to control varied greatly but most had returned to or near control 42 days after DCE. DCE administration produced no significant changes in monooxygenase activities in liver or kidney at any time point and no morphological changes were noted in livers and kidneys of DCE-treated mice at any time. In lungs however, most of the dome-shaped Clara cells had disappeared from the bronchioles and evidence of sloughed Clara cells were seen in lumens. The bronchiolar epithelium consisted almost entirely of flattened ciliated cells (24 h), sloughing of Clara cells occurred at all levels of the tracheobronchial tree. Seven days after DCE, Clara cells were once again identifiable although bronchiolar linings were not entirely normal in appearance. By 42 days posttreatment with DCE, the bronchiolar epithelium appeared to be identical with that of controls.

Mice were treated orally with 1,1-DCE (200 mg/kg) before a single pulse of tritiated thymidine ([^{3}H]Td). Necrosis and exfoliation of Clara cells of the bronchiolar epithelium were noted 1 day after DCE and were more severe by 2 days. A regenerative response was observed at 3 days after DCE and by 7 days the epithelium was essentially restored (Forkert et al., 1985). At 30 days after DCE re-epithelialization was achieved and areas devoid of epithelium were not observed.

Incorporation of [^{3}H]Td was inhibited 1 day after DCE and then peaked between 3 and 5 days. At 7 days after DCE, incorporation of [^{3}H]Td decreased to control levels. Autoradiographic examination of lung sections showed that the majority of the label was taken up by the Clara cells which correlated with repopulation of bronchioles. Therefore, DCE-induced damage to Clara cells was severe and rapid. Re-epitheliatization occurred rapidly whereas differentiation was a prolonged process.

B. Chemical Nature of the Reactive Metabolites of 1,1-Dichloroethylene

Metabolism of DCE in rat liver microsomes was reported by Costa and Ivanetich (1982). In a system supplemented with NADPH, DCE was converted to monochloroacetate and to dichloroacetaldehyde as follows:

$$Cl_2\text{-}C = CH_2 \longrightarrow \begin{cases} \nearrow Cl\text{-}CH_2\text{-}COOH \\ \searrow Cl_2\text{-}CH\text{-}CHO \end{cases}$$

CO or SKF 525-A inhibited these reactions by approximately 60%. It is noteworthy that no evidence for the formation of 2-mono- and 22-dichloroethanol or chloroacetaldehyde or dichloroacetic acid was obtained. Additional investigations on nephro- and hepatotoxicity of DCE have been published (Masuda and Nakayama, 1983; Oesch et al., 1983). A report on the metabolism of DCE by hepatic microsomes and isolated hepatocytes (Costa and Ivanetich, 1984) essentially confirmed that chloroacetate is a major isolate. Liebler and Guengerich (1983) found that oxidation of DCE by rat liver microsomal cytochrome P450 and in purified systems produced both $Cl\text{-}CH_2\text{-}CO_2H$ and $Cl_2\text{-}CH\text{-}CHO$ with concomitant suicide inactivation of three of the eight isozymes (trivial nomenclature) examined. Moreover, the proposed intermediary role of DCE oxide in the formation of monochloroacetic acid and dichloroacetaldehyde was examined. Kinetic studies of DCE oxide formation in a chemical system including P450 did not support its role as an obligate intermediate in the formation of the two compounds. Later work (Liebler et al., 1985) used rat and human liver microsomes and rat hepatocytes. Oxidation of DCE by microsomal P450 yields 2,2-dichloroacetaldehyde, 2-chloroacetyl chloride, 2-chloroacetic acid, and DCE oxide. The roles of these metabolites in covalent modification of proteins and GSH revealed that 2-chloroacetyl chloride reacted with model thiols (GSH and thiophenol) at least 10^3-fold faster than did DCE oxide and at least 10^5-fold faster than did 2,2-dichloroacetaldehyde or 2-chloroacetic acid. Microsomal covalent binding of [^{14}C]DCE was inhibited by GSH but not by lysine, suggesting that protein thiols rather than amino groups are major targets. Liver microsomes catalyzed the formation of three DCEs: GSH metabolite conjugates, identified as S-(22-dichloro-1-hydroxy) ethyl GSH; 2-(S-glutathionyl) acetate; and S-(2-glutathionyl) acetylglutathione, a novel conjugate containing both stable (thioether) and labile (thioester) linkages.

C. Covalent Binding of 1,1-Dichloroethylene Metabolites in Tissues

The tissue distribution and covalent binding of a single dose of [^{14}C]DCE (125 mg/kg ip) was studied in male mice (Okine et al., 1985). The chemical nature of the binding species was not determined. Covalent binding of radioactive material peaked at 6 to 12 h after DCE in all tissues and highest levels were found in kidney, liver, and lung, with smaller amounts in skeletal muscle, heart, spleen, and gut. Covalent binding in kidney, liver, and lung fell to approximately 50% of peak levels in 4

days. Between 12 h and 4 days after DCE administration, 70 to 100% of total radioactivity found in kidney, liver, and lung was covalently bound. Inducers of cytochrome P450 (PB and 3-MC) had variable effects on binding. Piperonyl butoxide and SKF 525-A reduced covalent binding in liver and lung. Diethylmaleate administration increased the covalent binding of DCE 2- to 3-fold in all three organs and also increased lethal toxicity. Thus, DCE was metabolized to a reactive intermediate(s) that was detoxified by conjugation with GSH. Forkert et al. (1986) using the same dose of [^{14}C]DCE (125 mg/kg ip) in mice reported selective necrosis of Clara cells that was accompanied by significant covalent binding of radiolabeled DCE (and metabolites) in lung. Lung injury as determined by electron microscopy and covalent binding were not modified by PB pretreatment, whereas 3-MC protected lung against morphological injury but failed to alter covalent binding to lung macromolecules. Pretreatment of animals with piperonyl butoxide aggravated bronchiolar injury by DCE, whereas covalent binding remained unaltered. In contrast, SKF 525-A protected against lung damage and significantly reduced (~50%) covalent binding. Degenerative changes were manifest in Clara cells as early as 1 h after DCE, necrosis was apparent at 2 h, by 8 h, areas of bronchiolar epithelium were devoid of Clara cells, and at 24 h, the majority of Clara cells were exfoliated and ciliated bronchiolar epithelial cells remained uninjured. Necrosis of Clara cells after DCE coincided with peak covalent binding of [^{14}C]DCE and significant impairment of components of the pulmonary cytochrome P450 system. In confirmation of the studies of Krijgsheld et al. (1984), lung cytochrome P450 levels were reduced maximally between 8 and 24 h after DCE (~50%). Therefore in murine lung, DCE evoked positive temporal correlations between structural damage, peak covalent binding, and alterations in cytochrome P450-dependent monooxygenases. Okine and Gram (1986; Okine et al., 1985) investigated the metabolism and covalent binding of [^{14}C]DCE in subcellular fractions of liver, kidney, and lung from mice in vitro. Covalent binding of DCE was NADPH- and cytochrome P450-dependent. Heat inactivation of microsomes, CO, addition of SKF 525-A, piperonyl butoxide, or GSH all inhibited (40–90%) covalent binding to lung and liver microsomes. The absence of O_2 (incubation under N_2) did not greatly affect covalent binding (Forkert et al., 1987).

It is of interest that Forkert and Troughton (1987), Forkert and Birch (1989), and Villaschi et al. (1991) have also reported Clara cell necrosis, covalent binding, and other characteristics of DCE pulmonary toxicity in mice after the administration of trichloroethylene. Forkert et al. (1990) prepared enriched fractions of Clara cells and type II cells from total lung digests: Clara cell fractions ranged from 56 to 65% Clara cells contaminated with 7% type II cells (Walker et al., 1989). Type II cell fractions ranged from 55% type II cells contaminated with 2 to 3% Clara cells to 51% type II cells contaminated with 1% Clara cells. Components of the CYP450 monooxygenase and related enzymes were measured in the two purified fractions and compared with activities in the total lung digest. As can be seen in table 12, enzyme activities in Clara cells of untreated mouse lungs are 3- to 7-fold higher than in type II cells. Other mice were injected with [^{14}C]DCE, and 1 h later, lung cells were isolated and the covalent binding of DCE measured. Covalent binding was highest in Clara cell fractions (480 pmol/10^6 cells) compared with fractions containing type II cells (126 pmol/10^6 cells) and mixed cells from whole lung (29 pmol/10^6 cells). Thus, the highest binding of [^{14}C]DCE metabolite(s) was in Clara cells, whereas much lower binding was found in type II cells and unseparated lung cells (table 13). The substantial binding of [^{14}C]DCE in Clara cells correlated positively with high monooxygenase capacity and preferential damage sustained by this cell population.

D. Role of Glutathione

Much work in the early 1970s dealt with the hepatotoxicity of DCE. Employing 1990s' technology, Forkert and Moussa (1993) reexamined the interaction of DCE and mouse liver in vivo. Employing doses of DCE ranging from 75 to 225 mg/kg, a nearly linear inverse relationship was observed between covalent binding and hepatic GSH depletion [whether the data were expressed per gram (wet weight), per milligram of protein, or per milligram of DNA]. Because DCE is known to produce hepatic vascular congestion, a correction was made for GSH in blood at the time of homogenization of the livers. The administration of DCE did not elicit any deaths but evoked hepatocellular injury that was dose-dependent. Whereas 75 mg/kg DCE caused no liver damage 24 h after dosing, 125 mg/kg elicited necrosis in small numbers of hepatocytes in the regional region, and at 225 mg/kg, centrilobular necrosis was observed in livers of all treated mice. These experiments established dose-dependent relationships between covalent binding of DCE metabolite(s), reduced levels of liver GSH, and hepatocellular necrosis.

Treatment of mice with a range of doses of DCE (75–225 mg/kg ip) elicited a significant dose-related reduction in GSH determined histochemically and a corresponding increase in covalent binding of DCE.

TABLE 12
Ratios of biotransformation activities in mouse lung fractions enriched in Clara cells and alveolar type II cells

Enzyme activity	Clara cell activity / Type II cell activity
NADPH cytochrome *c* reductase	7.3
7-Ethoxycoumarin deethylase	7.2
Glutathione S-transferase	3.0

Data excerpted with permission from Forkert et al. (1990).

TABLE 13
Covalent binding of [^{14}C]DCE in isolated mouse lung cell populations following in vivo administration

Cell	pmol [^{14}C]bound/10^6 cells
Cell digest	10
Type II cells	125
Clara cells	450

Data excerpted with permission from Forket et al. (1990).

Histochemical measurement of GSH revealed the highest staining intensities in Clara cells. Interestingly, heterogeneity in GSH staining was consistently noted in the Clara cell population in both control and DCE-treated mice (Moussa and Forkert, 1992). In addition, progressive increases in covalent binding and reductions in GSH content correlated with increasing severities of Clara cell injury. Later work from this group (Forkert and Moussa, 1993) confirmed that in mouse lung, the distribution of GSH, particularly in Clara cells, was heterogeneous and that subpopulations of Clara cells with high and low GSH levels were observed in the same segment of airway. Thus, cells with low GSH expression might be more susceptible to DCE-induced cytotoxicity. These concepts are in accord with more recent findings by Buckpitt's group (Duan et al., 1996) showing that in mouse lung, epithelial cells are differentially depleted of GSH and that the rates of resynthesis (from cysteine) may vary. It may be concluded that all Clara cells are not created equal.

E. Role of Pulmonary P450 Isozymes in 1,1-Dichloroethylene Metabolism

Forkert et al. (1991) reported that cytochrome CYP2E1 (a major band with M_r 51,000) was increased 1.8-fold in mouse liver in Western immunoblots by 10% ethanol for 1 week in the drinking water of mice, 4.7-fold by 1% acetone in the drinking water for 1 week, and 2.5-, 2.1-, and 6.8-fold by ethanol in a liquid diet for 9 days, 2, and 3 weeks, respectively. In mouse liver, CYP2E1 is preferentially localized in centrilobular regions constitutitively as well as in induced states. Forkert et al. (1994) reported that acute and chronic administration of acetone to mice produced a marked induction of hepatic CYP2E1 by 4.4- and 5.3-fold, respectively. This induction occurred in the absence of changes in CYP2E1 messenger ribonucleic acid (mRNA) levels. In vitro incubation of hepatic microsomes from untreated mice (Lee and Forkert, 1994) with DCE and NADPH resulted in inactivation of P450 (30%), whereas maximum reduction was produced by DCE incubated with microsomes from acetone-treated mice (50%). It was concluded, therefore, that DCE inactivates P450 by destruction or alteration of apoprotein rather than of heme moiety.

More recently, Lee and Forkert (1995) incubated lung microsomes from male mice, NADPH, and varying amounts of DCE (5–20 mM) and found corresponding decreases in 4-nitrophenol hydroxylase (a CYP2E1-dependent enzyme) activity (19–50%). Greater reductions were found in microsomes from female mice (26–70%) incubated under identical conditions. However, in the presence of an anti-CYP2E1 monoclonal antibody, the inhibition of 4-nitrophenol hydroxylation by DCE was abolished; CYP2E1 bioactivates DCE in mouse lung microsomes, leading to degradation of this P450 isozyme. The DCE-mediated degradation of P450 is due to destruction or alteration of the CYP2E1 protein as the heme of the enzyme remains unchanged. In male mice, DCE caused renal tumors. Renal microsomes from male mice converted DCE to chloroacetic acid, and this conversion correlated well with the ability of microsomes to oxidize 4-nitrophenol, a specific substrate for P4502E1 (Speerschneider and Dekant, 1995).

DCE requires cytochrome P450's catalyzed activation to the electrophilic metabolites DCE oxide, 2-chloroacetyl chloride, and 2,2-dichloroacetaldehyde (Dowsley et al., 1995) to exert its toxic effects. These investigators used ^{1}H-NMR, fast atom bombardment spectrometry, and electron impact mass spectrometry (MS), along with miscellaneous other spectroscopic and chromatographic technics. The results showed conjugation of GSH with DCE oxide, with the formation of the mono- and diglutathione adducts 2-S-glutathionyl acetate (GTA) and 2-(S-glutathionyl) acetyl GSH, respectively. 22-Dichloroacetaldehyde is thus unlikely to significantly contribute to GSH depletion, as GSH concentrations above physiological levels would be required to form significant amounts of S-(22-dichloro-1-hydroxy) ethyl GSH. See Dowsley et al. (1995) for chemical details of this rather complex system.

Employing in situ hybridization and immunohistochemical staining procedures, Forkert (1995) reported that CYP2E1 was localized prominently in the Clara cells but was not detected in the ciliated cells of the bronchiolar epithelium. Immunoreactivity for CYP2E1 was minimal in alveolar type II cells. The CYP2E1 mRNA was also predominantly localized in the bronchiolar epithelium and was most prominent in the Clara cells but was minimal in type II cells. CYP2E1 was revealed by protein immunoblotting as a single band having a M_r of ~51,000 in lung microsomes of CD-1 male mice. It is of interest, and confirmatory of findings described that there was variability of staining within the Clara cell population so that heterogeneity of staining intensities was apparent in the Clara cells located within a single airway.

A component of garlic, diallyl sulfone, was found (Forkert et al., 1996) to inhibit CYP2E1, suppress the bioactivation of DCE to reactive metabolites, and block DCE-induced Clara cell toxicity (table 14). DCE cytotoxicity is mediated by CYP2E1, which is highly localized in Clara cells. Bioactivation of DCE produced the primary metabolites 22-dichloroacetaldehyde that hydrolyzes to the acetal and DCE-epoxide, which reacts with GSH to

TABLE 14
DCE metabolites (pmol/mg/protein/min) in control and dialthyl sulfone-treated mice

Pretreatment	2,2-dichloroacetaldehyde	DCE epoxide
DCE	3.0	7.6
DCE + diallyl sulfone	1.8	4.2

Data excerpted with permission from Forkert et al. (1996).

form the conjugates 2-(S-glutathionyl) acetyl GSH and GTA (Dowsley et al., 1995). Treatment of mice with diallyl sulfone (100 mg/kg po) reduced CYP2E1-dependent 4-nitrophenol hydroxylation, which was apparent in 1 h. Enzyme activity reached its nadir of approximately 20% of control levels at 2 h, remained at this low level from 3 to 8 h, and returned to control levels at 24 h. Immunoreactivity of the CYP2E1 enzyme was reduced, parallel with hydroxylase activity in immunoblots of lung microsomes from diallyl sulfone-treated mice. Treatment with diallyl sulfone did not produce any morphological changes in lung tissue, but DCE (75 mg/kg) administered alone produced Clara cell damage. It is important to note that this lesion was not observed in mice treated with DCE in conjunction with diallyl sulfone. The lack of DCE cytotoxicity in vivo correlated with a reduction of approximately 45% in the levels of both acetal and DCE-epoxide-derived conjugates. These data indicated that diallyl sulfone significantly inhibited CYP2E1, reduced the production of DCE metabolites, and protected Clara cells against DCE-induced cytotoxicity.

Recent work revealed that DCE-epoxide (12.6 pmol/mg/protein/min) was the major metabolite formed from DCE in mouse lung and was identified as two GSH conjugates. Liver levels of the acetal of 22-dichloroacetaldehyde (3.6 pmol/mg/protein/min) were also detected. An antibody to CYP2E1 reduced the formation of these metabolites by approximately 50%. Therefore, mouse lung CYP2E1 metabolized DCE to reactive metabolites implicating DCE-epoxide as a major metabolite and an important toxic species in DCE-induced cytotoxicity (Dowsley et al., 1996).

In recently reported work (Forkert et al., 1997), incubations of mouse lung microsomes with DCE, NADPH, and GSH resulted in the formation of GTA and another metabolite that hydrolyzes to GTA. Products were identified by HPLC, ^{1}H-NMR, and mass spectroscopy. The data indicated that DCE-epoxide is the major metabolite generated from CYP2E1-dependent metabolism of DCE and that GTA may be an important product of this metabolic pathway. Also, they suggest that monochloroacetate and dichloroacetaldehyde are relatively unimportant intermediates. Forkert (1997) also proposed that the primary metabolites of DCE formed in vitro with mouse lung and liver microsomes are GSH conjugates of DCE-epoxide, 2,2-dichloroacetaldehyde, and 2-chloroacetyl chloride.

IX. 3-Methylindole–Induced Lung Toxicity

One of the first major publications describing pulmonary injury associated with 3-methylindole (3-MI) in goats was published in 1977 (Huang et al., 1977). The animals were given 160 mg/kg po and pairs were sacrificed at 0, 2, 4, 8, 24, and 48 h. Three animals were undosed controls. Grossly, the animals developed dyspnea at 4 h, which became increasingly severe at 15 h.

A. Morphological Evidence of Lung Damage After 3-Methylindole Administration

Four h after 3-MI, lungs were severely congested, and at 8 h, gross pulmonary edema was noted. Goat lungs exhibited diffuse reddish discoloration with a rubbery consistency. Frothy edema fluid could be expressed from cut surfaces, as well as trachea and bronchi. Animals sacrified at 8, 24, and 48 h were similar to those described at 4 and 8 h. At all time points, the edema fluid was markedly eosinophilic, indicating a high protein content. Structurally fine, type I epithelial cells were affected, and at 48 h, large areas of the alveolar surface were denuded. Also, much of the bronchiolar surface lacked Clara cells. Among lung cells, thus, cellular injury after 3-MI was observed in type I and Clara cells, whereas type II cells, endothelial cells, and interstitial cells were uninvolved. These observations were confirmed by Bradley and Carlson (1980), who administered a smaller dose of 3-MI (40 mg/kg iv) to goats and studied lung ultrastructure at times ranging from 30 minutes to 24 h later. They reported that 3-MI administration produced a rapid (first time point) cytotoxic effect on alveolar type I and Clara cells. The Clara cell effect was termed "most severe: sloughing of necrotic Clara cells into the bronchiolar lumen was apparent after 2 to 4 h" (Huang et al., 1977). The authors suggested that highly reactive intermediates formed from 3-MI were "probably responsible for the pneumototoxicity" (Bradley and Carlson, 1980). Turk et al. (1984) administered single oral doses of 100 mg/kg 3-MI to mature horses and ponies and animals were sacraficed at 30 minutes, 2, 3, 12 and 24 h, and at 3, 6, 13, 20, and 27 days after administration. Lung tissue was observed by light microscopy, transmission electron microscopy (TEM), and scanning electron microscopy (SEM). With TEM, terminal bronchioles normally displayed two characteristic structures: the dome-shaped Clara cells and the numerous wheat-stalk–shaped cilia emanating from ciliated bronchiolar epithelial cells. Both structures protude into the bronchiolar lumen (fig. 1e). From 30 minutes to 3 h after 3-MI, lesions were limited to Clara cells, which lost their dome-shaped apical caps, secretory granules, and had dilated SER. At 12 h after dosing, necrosis was evident in Clara cells, together with exfoliation and degeneration. By SEM, the bronchiolar surface was dramatically altered; the dome-shaped apical caps described above were replaced by pits, and the

numerous cilia remained, apparently intact, as were the ciliated cells themselves as viewed with TEM. Rare epithelial cells with hyperplastic SER appeared on the denuded basal lamina at 24 h. Reorganization resulted in a simple columnar bronchiolar epithelium with relatively normal ciliated cells and few fibroblasts. Clara cells still had a large nucleus and a flat (not dome-shaped) luminal surface. In addition, they were shorter with fewer granules than Clara cells from controls. Thus, mature Clara cells were rare at 27 days.

Weanling mice were injected with 3-MI (500 mg/kg ip) (Durham and Castleman, 1985), and their lungs were observed by light and TEM 2 to 360 h later. At early time points, interstitial edema and cytoplasmic swelling were prominent. In airways, Clara cells were the most prominant cells affected. Loss of secretory granules was followed by swelling of the endoplasmic reticulum and mitochondria. Necrosis of airway cells was most pronounced at 24 to 48 h after treatment. By 144 h after dosing, pulmonary repair was complete.

B. Relationships Between Activation of 3-Methylindole in Tissues, Covalent binding, and Pulmonary Cell Necrosis

Pretreatment of goats with PB-stimulated metabolism of 3-MI and accentuated lung toxicity, whereas piperonyl butoxide reduced lung toxicity. Covalent binding of [^{14}C]3-MI occurred both in vitro and in vivo; binding was highest in lung and was increased in vivo in goats depleted of GSH by diethylmaleate (Becker et al., 1984). Goats were infused with various doses (10–40 mg/kg) of [^{3}H]3-MI and killed immediately, after 2 and 6 h. Tissues were extracted in a Soxlet apparatus for 1 day with methanol, 1 day with acetone, and 1 day with methanol. This procedure had been shown to remove all the noncovalently bound [^{3}H]3-MI. Autoradiographic studies of tissues so extracted revealed that both ciliated and nonciliated bronchiolar cells were labeled pneumocytes, as were types I and II. Lung slices prepared from control goats and incubated with [^{3}H]3-MI were labeled in the same pattern. Inclusion of SKF 525-A (9% of control) or piperonyl butoxide (22% of control) slightly reduced the binding to both the alveolar and bronchiolar cells. Goats were pretreated with either PB or piperonyl butoxide before receiving an infusion of [^{14}C]3-MI (20–30 mg/kg). Piperonyl butoxide prevented the onset of acute pulmonary edema, whereas goats pretreated with PB followed by 3-MI had more severe lung lesions than those receiving 3-MI alone (Bray and Carlson, 1979). In another study (Hammond et al., 1979), goats were infused with [^{14}C]3-MI and excreted 87 to 92% of the radiolabel in the urine, 0.4 to 0.9% in the exposed air, and undectable amounts in the feces over a 3-day period. Urine samples yielded 10 peaks, and a major route of metabolism involved formation of 3-methyloxindole. Radiolabeled 3-MI become covalently bound to protein when incubated with bovine lung microsomes (Hanafly and Bogan, 1980). Covalent binding was dependent on time, temperature, oxygen, and NADPH and was inhibited by SKF 525-A, cytochrome c, CO, and cysteine. Thus, activation of 3-MI to an electrophilic metabolite may be involved in its pulmonary toxicity.

The effects of pretreatments and other manipulations on 3-MI covalent binding was studied in female Wistar rats and female goats (Merrill and Bray, 1983). Incubation of liver and lung microsomes (10,000 × g supernatant) was evaluated in the presence of NADPH. In goats, the covalent binding of [^{14}C]3-MI in liver was only approximately 40% of that in lung, and both were reduced by PB or 3-MC pretreatment. In rat tissues binding in lung was 60% of that of liver. Treatment with PB or 3-MC had no effect on binding in liver but reduced it in lung. Addition of GSH in vitro reduced 3-MI binding in lung by 40% but in liver by only approximately 10%. Merrill and Bray (1983) studied dietary manipulations for their effects on 3-MI pulmonary toxicity in goats. Compared with animals fed a normal protein diet, high-cysteine or high-sulfate diets increased lung GSH levels and reduced the severity of the lung lesion produced by 3-MI. Pretreatment with diethylmaleate increased the severity of the 3-MI lung lesion. Tissue GSH-transferase activities were not changed.

3-MI produces sometimes fatal pulmonary edema in cattle, goats and sheep. A study was conducted to determine whether these pathological effects of 3-MI could be evoked in mice. Animals were injected ip with 10, 100, 200, 300, 400, 600 and 800 mg/kg 3-MI dissolved in corn oil (Turk et al., 1984). One of the five mice that received 600 mg and all five that received 800 mg/kg 3-MI died between 10 and 20 h after injection. The experiment was terminated at 24 h after injection of 3-MI. All mice were free of gross pathology. Histological changes were not detected in the liver or kidneys in any mice nor were changes evident in the brain, heart, or spleen of mice that received 400 or 600 mg/kg 3-MI. In the lungs, however, bronchiolar epithelial damage occurred in all mice that received greater than 100 mg/kg 3-MI. In mice that received 200 mg/kg 3-MI, exfoliation was accompanied by reduced height, cytoplasmic vacuolization, and nuclear pyknosis of Clara cells in some bronchioles, whereas other bronchioles were histologically normal. Normal terminal bronchioles were rare in mice receiving 300 mg/kg 3-MI, and necrosis was more extensive with multifocal epithelial sloughing in mice that received 400 mg/kg 3-MI. With higher doses, epithelial damage extended proximally into the axial bronchi. It should be noted that in contrast to cattle, goats, and sheep, 3-MI did not produce pulmonary edema in mice.

Nocerini et al. (1985a) reported that radiolabeled 3-MI binds more extensively to goat lung protein than to liver protein. Both pulmonary toxicity and covalent binding of 3-MI were greatly enhanced by pretreatment of the animals with diethylmaleate. [^{14}C]3-MI was incubated with goat lung microsomes, an NADPH-generating sys-

tem, and GSH, and under these conditions, an adduct was formed between an oxidative metabolite of 3-MI and GSH, which was purified by HPLC. Adduct formation was increased by approximately 60% by increasing GSH in the medium from 2 to 4 mM and covalent binding to microsomal protein was reduced by 50% by including GSH in the medium. Bray et al. (1984) reported that 3-MI itself was not responsible for pulmonary toxicity, because inducers and inhibitors of P450 could alter 3-MI metabolism and severity of lung damage. Also, 3-methoxyindole and indole-3-carbinol, presumed metabolites of 3-MI, failed to induce lung injury. In goat organs, lung microsomes were found to covalently bind radioactivity from [^{14}C]3-MI at the rate of 33.0 (nmol/10 mg/protein/min), whereas corresponding values were 11.2 for liver and 2.4 for kidney. Inclusion of piperonyl butoxide in incubations reduced lung binding by 90%, liver by 70%, and kidney to below limits of detection. Addition of GSH reduced covalent binding in lung from 32.1 to 5.2 and that in liver from 9.0 to 2.0 and abolished it in kidney. As is the case with other species (Litterst et al., 1975), ratios of cytochrome P450 in lung/liver range from 0:1 to 0:4; in goats, Bray et al. (1984) reported that the ratio was approximately 0:4. Therefore, if the amount of covalently bound [^{14}C]3-MI was expressed per nanomole of P450, it was calculated to be approximately 7.4 in liver and 72.7 in lung. Autoradiographs prepared from horse lung incubated with [^{3}H]MI revealed 8× greater labeling over bronchiolar epithelial cells than over interalveolar septa. Incubation of horse lung microsomes with [^{14}C]3-MI, NADPH, and GSH produced a metabolite (not formed if one component was omitted), which was isolated by HPLC. Doubling the concentration of GSH in the incubations increased the amount of conjugate formed and reduced the amount of [^{14}C]3-MI covalently bound to microsomal protein. Addition of SKF 525-A or piperonyl butoxide to the incubations dramatically reduced the covalent binding to microsomal protein (Becker et al., 1985).

Abundant prior work had established that P450 oxidases are responsible for the bioactivation of 3-MI to a toxic intermediate. Although oxidation of 3-MI is required for tissue injury to occur, the precise nature of the toxic intermediate has not been established. It has been demonstrated that 3-methyloxindole and indole-3-carbinol, major metabolites of 3-MI in ruminants, are not toxic to goats (Hammond et al., 1979). Also, an inverse relationship is recognized between GSH status and both 3-MI toxicity and covalent binding in vivo. Nocerini et al. (1985b) incubated goat lung microsomes with[^{14}C]3-MI, an NADPH generating system, and GSH and analyzed the extracts by HPLC, liquid chromotography-mass spectrometry, and NMR. It was unequivocally determined that the ultimate metabolite was 3-[(glutathion-S-yl)-methyl] indole. The authors suggested that the postulated imine methide electrophile is the toxic intermediate in 3-MI–mediated pulmonary toxicity (fig. 5).

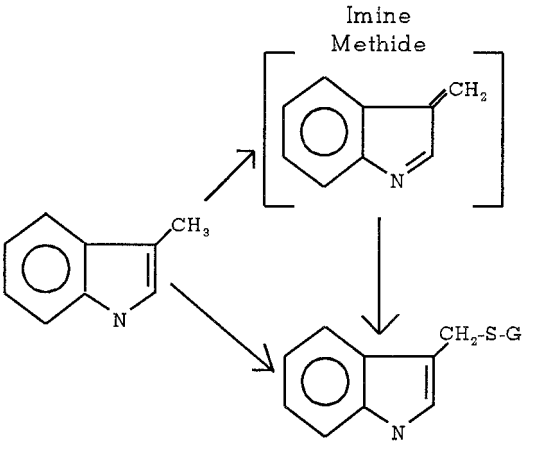


FIG. 5. Activation of 3-MI with NADPH, GSH, and goat lung microsomes results in the formation of 3-[(glutathion3-S-yl)-methyl indole]. Taken in part with permission from Nocerini et al. (1985b).

Nocerini et al. (1985a) expanded this work to investigate the ability of lung and liver microsomes of several species to form activated metabolites of 3-MI. Acute lung injury results from the cytochrome P450-catalyzed activation of 3-MI to electrophilic intermediates. Both formation and alkylation by electrophilic 3-MI metabolites occur in situ in the lung. If tissue stores of nucleophiles such as GSH are depleted, then the metabolites react covalently with tissue nucleophiles and evoke toxicity. When microsomes were incubated with [^{14}C]3-MI, GSH, and NADPH, electrophilic 3-MI metabolites were quantified as GSH adducts by HPLC and the amounts of activated intermediates that became covalently bound to microsomal protein. The highest rates of 3-MI-GSH adduct formation by the lung was in microsomes of the goat, followed by microsomes from horse, monkey, mouse, and rat. In contrast, hepatic 3-MI-GSH adduct formation was highest in microsomes from the rat followed by mouse, monkey, goat, and horse (Nocerini et al., 1985a). These data suggest that the species and organ-selective toxicity of 3-MI are related primarily to differences in oxidation of 3-MI to the electrophilic intermediate.

The bioactivation of the pneumotoxin 3-MI has been suggested to proceed via the formation of an imine methide (Nocerini et al., 1985b; fig. 5). To test this hypothesis, the toxicity of 3-MI in mice has been compared against the toxicity of its perdeuteromethyl analog. Deuteration of the methyl group should slow the rate of production of the corresponding imine methide and reduce the toxicity of deutero-3-MI if C-H bond breakage occurs before or during the rate-limiting step. In accord with this view, deutero-3-MI was found to be significantly less toxic (LD_{50} = 735 mg/kg) than 3-MI (LD_{50} = 578 mg/kg). Both compounds produced the same lesion at the LD_{50} dose, bronchiolar damage and mild alveolar edema indicating that deuteration did not alter the pathological process (Huijzer et al., 1987). However, at a much lower dose (25 mg/kg), 3-MI produced a mild bron-

chiolar lesion and mild pulmonary edema, whereas deutero-3-MI did not damage the lung. Finally, the depletion of pulmonary GSH by deutero-3-MI was much slower than depletion by 3-MI. Because the electrophilic imine methide is thought to bind with and deplete GSH, the evidence presented is in accord with an imine methide as the primary reactive metabolite in 3-MI-mediated pneumotoxicity (fig. 5) (Kubow et al., 1984)

An acute role for lipid peroxidation in 3-MI-induced lung injury had been proposed in light of the presumed free radical intermediacy of the imine methide (Adams et al., 1987). Instead of stimulating lipid peroxidation, as measured by the commonly used thiobarbituric acid (TBA) assay, in a system consisting of goat lung microsomes, NADPH, and 3-MI, lipid peroxidation was completely quenched by 3-MI. Addition of 3-MI to actively peroxidizing microsomes supported by NADPH caused an immediate cessation of lipid peroxidation. A combination of ascorbate and ferrous iron also dramatically stimulated lipid peroxidation (nonenzymatically) in the absence of NADPH. Again, 3-MI totally suppressed peroxidation in a system (Laegreid and Breeze, 1985) containing goat lung microsomes, ascorbate, and ferrous ammonium sulfate. Thus, lipid peroxidation is not likely involved in 3-MI lung injury. Using specific inhibitors of cytochrome P450 isozymes, Huijzer et al. (1989) reported that 3-MI is metabolized in goat lung microsomes to an electrophilic intermediate by CYP2B4; the inhibitor 1-aminobenzotriazole rapidly destroys the enzyme by alkylation of the prosthetic heme of CYP2B4 without effecting cytochrome b_5. Nichols et al. (1990) attempted to clarify an untidy situation in which fine structural analysis had shown that 3-MI causes lung lesions in Clara and alveolar epithelial cells in ruminants and rodents. Alveolar macrophages, type II epithelial cells, and Clara cells were isolated from rabbit lungs and incubated with several concentrations of 3-MI in vitro. It was not stated whether NADPH was added. Under these conditions, the order of susceptibility to 3-MI–induced cytotoxicity was Clara cells > type II cells > alveolar macrophages. Thus, 3-MI produced a dose-dependent cytotoxicity (viability was determined by trypan blue exclusion) to Clara cells detectable within 1 h of incubation at 37°, which reached a nadir at 3 h. Concentrations of 0.25 and 0.5 mM were cytotoxic to Clara cells, whereas type II cells and alveolar macrophages required 1 mM 3-MI before cytotoxicity was observed. The P2B4 suicide inhibitor, 1-aminobenzotriazole, inhibited 3-MI cytotoxicity in Clara cells, type II cells, and alveolar macrophages to roughly equal degrees. Studies with a trideuteromethyl analog of 3-MI demonstrated a much reduced cytotoxicity to Clara cells, type II cells, and macrophages alike. The deuterium isotope effect suggested that C-H bond breakage at the 3-methyl group is required in the bioactivation of 3-MI to a selective lung cell toxin.

Ruangyuttikarn et al. (1992) reported that 3-MI is oxidized by P450 enzymes to 3-methyleneindolenine (the imine methide), a methylene imine electrophile, the postulated reactive intermediate, which binds to proteins, a reaction that probably initiates the pneumotoxicity of 3-MI. Thioether adducts of this electrophile are formed with GSH in vivo. The formation of the GSH adduct was inversely related to covalent binding of 3-MI to goat lung microsomal proteins, and the 3-MI-induced lung injury was inversely proportional to the GSH status of lung tissues. 3-MI was incubated with NADPH and goat lung microsomes, and the proteins were hydrolyzed. 3-(cystein-S-yl) methyl indole was isolated and identified as the major amino acid adduct of 3-methyleneindolenine, demonstrating that cysteine thiols preferentially linked the exocyclic methylene position and resulted in a covalently (thioether) attached 3-MI residue to these pulmonary proteins. These findings demonstrate that the putative methylene imine (imine methide) intermediate is indeed the active electrophile that binds to proteins and presumably initiates the toxic events.

Twelve purified human cytochrome P450s and one mouse P450 were produced in HepG2 cells using vaccinia virus with cDNA expression and analyzed for their ability to bioactivate 3-MI to an electrophilic metabolite that could bind to cellular macromolecules. The P450 isozymes were incubated with HepG2 cellular lysate, [^{14}C]3-MI, and NADPH at 37°. Binding to lysate protein catalyzed by most isozymes was at or near background levels, but binding was catalyzed by CYP2A6 at approximately 4.7 pmol/mg/protein/min and by CYP2F1 at approximately 15 pmol/mg/protein/min (Thornton-Manning et al., 1991). Bioactivation of 3-MI by human CYP1A2 was negligible, whereas binding by cDNA expressed mouse P4501A2 was highest of any isozyme tested, 75.4 pmol/mg/protein/min. Thornton-Manning et al. (1991) state, "[b]ecause 1A2 has not been reported to be present in mouse extrahepatic tissues, the metabolite formed by this P450 is most likely not responsible for the pneumotoxicity elicited by this compound." What is the relationship between molecular biology and the real world? The reader is further referred to three excellent reviews on the pneumotoxicity of 3-MI written by experts in the field (Bray and Emmerson, 1994; Yost, 1989; Bray and Kirkland, 1990).

X. Butylated Hydroxytoluene and Pulmonary Toxicity

Butylated hydroxytoluene (BHT) (35-di-tertiary-butyl-4-hydroxytoluene) is a synthetic antioxidant that prevents oxygen-induced lipid deterioration by acting as a chain-breaker in a series of free radical reactions (Witschi et al., 1989).

A. Histological and Fine Structural Changes Produced by Butylated Hydroxytoluene in Lung: Incorporation of [^{3}H]Thymidine into DNA

Female mice were injected intraperiotoneally with doses of BHT ranging from 0.004 to 2.5 g/kg (Marino and Mitchell, 1972). Because all the animals receiving the highest dose died, the dose was reduced, and most of the

findings reported were of animals receiving 830 mg/kg. Tissues were examined by light microscopy. Grossly, lungs were markedly hemorrhagic. Microscopically, all other organs examined appeared normal, but lungs from BHT-treated mice were hyperplastic and hypertrophic. Alveolar septa were heavily infiltrated by inflammatory cells and were thickened and showed increased numbers of pyknotic nuclei. Proliferation of alveolar cells was accompanied by edema and dramatic capillary engorgement. Blebbing of alveolar epithelium together with a marked increase in septal cells and macrophages in the alveolar spaces were noted. Despite these dramatic histological changes, changes in animals receiving doses as high as 400 mg/kg were reversible within 7 days.

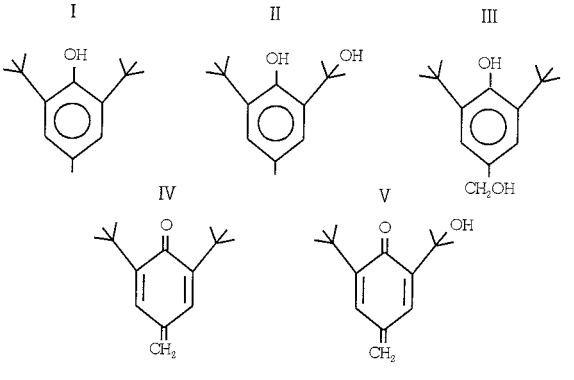
Witschi and Cote (1976) injected mice with a single dose of 400 mg/kg BHT intraperitoneally and studied the animals for 9 days. Total lung weight reached a peak of 75% above controls at days 4 and 5 and remained significantly above control levels at 9 days. Total lung DNA increased gradually and was still peaking (~50% above control) at days 7 and 9. Thymidine incorporation into total lung DNA increased significantly 2 days after BHT, peaked at days 4 and 5 (ten-fold above controls), and returned toward control at day 9. The biosynthesis of lung protein (from leucine) and RNA (from orotic acid) were not affected by BHT. Fine structural changes produced by the administration of 400 mg/kg BHT to mice were studied with time (Hirai et al., 1977). Type I epithelial cells were first to be altered. From days 1 to 3, type I cells revealed alterations in cytoplasmic organelles. Breaks in the plasmalemma and complete necrosis of type I cells occurred on days 2 and 3, leaving a basal lamina and the capillary endothelium intact. It is important to note that this damage to type I cells was not uniform throughout the lung. After type I cells had become necrotic, type II epithelium divided and transformed (as early as day 2) into a primitive cell resembling type I to replace the type I epithelium. The third temporal event after BHT administration was that capillary endothelium showed signs of damage on days 3 and 5. Interstitial cells began to proliferate on days 4 and 5 and invaded empty alveolar spaces, and concurrently, macrophages migrated in. It must be noted that from days 7 to 9, type I and type II cells were normal morphologically. Witschi and Saheb (1974) injected mice with BHT and [^{3}H]thymidine at various times thereafter. Among the organs examined, lung was the only one in which DNA synthesis was increased by BHT. [^{3}H]Thymidine incorporation into pulmonary DNA was enhanced by BHT at all times between days 2 and 7 but reached a peak at days 4 and 5 during which the stimulation was 3- to 4-fold. Adamson et al. (1977) confirmed that after 400 mg/kg BHT to mice, initial perivascular edema was followed by necrosis of type I alveolar epithelial cells and differentiation of type II cells, which repopulated the alveolar wall. This produced unusually large epithelial cells, containing abundant cytoplasm and DNA synthesis as revealed by increased [^{3}H]thymidine incorporation. These factors increased by 2 days (after BHT), peaked at 4 days, and returned to control levels by day 9. Changes in endothelial cells followed behind these changes, peaking (in [^{3}H]thymidine uptake) at 6 days and returning to control at 9 days. Late in the lesion, fibroblasts invaded the alveolar wall (8 to 10 days) and collagen was elaborated.

B. Covalent Binding of Butylated Hydroxytoluene in Tissues, Its Amelioration, and the Nature of the Reactive Metabolite(s)

Nakagawa et al. (1979b) studied the covalent binding of [^{14}C]BHT in vivo and in vitro. In the in vivo study (Nakagawa et al., 1979b), [^{14}C]BHT was administered to rats orally, and tissue homogenates were washed with trichloroacetic acid and a series of hot and cold organic solvents for up to a total of 8 washes. The residue was considered covalently bound. Covalently bound radioactivity was approximately equal in lung and liver when normalized to tissue protein at both 24 and 168 h after BHT administration. Binding studies in vitro (Nakagawa et al., 1979a) revealed that covalent binding [^{14}C]BHT in rat liver required NADPH and oxygen and was significantly inhibited by CO, SKF 525-A, and GSH. Normalized to protein, binding in liver was approximately 2.5-fold higher than in lung.

Although the structure of BHT is not complex, the chemical nature of (some theoretical) 'active intermediate' is not known. Some likely candidates are noted in figure 6.

Malkinson (1979) reported that the usual effects of BHT in mice [increase in lung weight and cellularity, necrosis of type I alveolar cells, and increased incorporation of thymidine (into lung DNA)] could be mitigated



Key

I	BHT
II	6-t-Butyl-2-(Hydroxy-t-Butyl)-4-Methyl Phenol
III	2,6-Di-tert-Butyl-4-Hydroxyl-Methyl-Phenol
IV	2,6-Di-tert-Butyl-4-Methylene-2,5-Cyclohexadieneone
V	6-tert-Butyl-2-(Hydroxy-tert-Butyl)-4-Methylene-2,5-Cyclohexadieneone (Quinone Methide) (BHT-QM)

FIG. 6. Chemical structures of BHT and some of its possible metabolites reponsible for lung toxicity. Taken in part with permission from Bolton and Thompson (1991) and Bolton et al. (1993).

by certain factors. Housing animals, particularly mice, on cedar shavings is known to induce certain CYP450 isozymes and to reduce the toxicity of some of the substances on which they act. Housing mice on cedar shavings as opposed to a standard ground corn cob bedding blocked the increase in relative lung weight produced by BHT even at doses up to 2.5 g/kg, a dose 5 times greater than the median effective dose. Mice of 13 different strains were raised on corn cob bedding or cedar shavings and then received a standard lung-toxic dose of BHT (400 mg/kg) (Malkinson, 1979). In all cases, cedar shavings blocked the lung weight gain produced by BHT, and in some cases reduced it by 100%. Young and old mice were injected with BHT, and the relative lung weight (lung weight/body weight) was determined. Young mice (2 to 3 weeks) metabolize drugs poorly and they did not exhibit changes in relative lung weight in response to BHT. The same was true of old mice (52 weeks). A biologically active terpene present in cedar shavings called "cedrol" similarly blocked the lung weight gain produced by BHT in a dose-dependent manner.

The effects of deuteration of the 4-methyl group of BHT were studied for isotope effects on metabolism and toxicity. Mice were injected with either BHT or BHT-d_3, and the deuterated compound produced significantly less relative lung weight gain and dry lung weight gain. The metabolism to the quinone methide (V; fig. 6) was reduced by 40%. By contrast, its conversion to the alcohol (III) was stimulated by 70%. The data were interpreted to support the view that butylated hydroxytoluene-quinone methidine (BHT-QM) (V) mediates the lung damage produced by BHT (Mizutani et al., 1983). Kehrer and Witschi (1980) chose to tease apart the microsomal cytochrome P450 system to further understand BHT toxicity. Administration of SKF 525-A or piperonyl butoxide in varying doses to mice treated with BHT prevented the increase in thymidine incorporation into pulmonary DNA. This effect was dose-dependent. Pretreatment of mice with $CoCl_2$, which inhibits CYP450 biosynthesis, diminished the BHT-induced increase in thymidine incorporation into pulmonary DNA. After the administration of [^{14}C]BHT, radioactivity was covalently bound to lung, liver, and kidney macromolecules of both mice, which exhibited BHT-induced lung damage, and rats, which did not. The greatest amount of radioactivity was bound to lungs of mice. This binding was prevented by the administration of SKF 525-A and was a linear function of the BHT dose from 50 to 600 mg/kg. Binding to other tissues of the mouse and all tissues of the rat was minimal and unaffected by SKF 525-A. These data accord with the concept that a CYP450-mediated formation of a reactive metabolite of BHT rather than the parent compound produces lung damage in mice.

When BHT is incubated with mouse lung or liver microsomes, a major metabolite formed is the t-butyl-hydroxylated derivative (II; fig. 6). Malkinson et al. (1989) found that II is 4 times more potent than BHT in increasing the lung weight/body weight ratio. Lung damage occurs earlier and is repaired more rapidly at lower doses of II than with BHT, but the nature of the damage (type I cell necrosis) and regenerative response (type II cell hyperplasia and differentiation) is identical. Neither BHT nor II cause damage to liver, kidney, and heart assessed microscopically, so they are both specific pulmonary toxicants. Thus, II formation is required in the conversion of BHT to the ultimate pneumotoxin, which may be the corresponding quinone methidine (BHT-QM).

Bolton et al. (1990) and Bolton and Thompson (1991) found that mouse lung microsomes generate two quinone methides (QM and QM-OH), whereas rats produce QM almost exclusively. In addition, they found that V reacted approximately six-fold faster with GSH than II, and the enhanced biological activity of V may not only account for the mouse/rat species difference in toxicity, but supports the view that V maybe the ultimate pneumotoxic metabolite resulting from BHT administration.

Bolton et al. (1993) incubated mouse lung Clara cells with BHT and NADPH. Most of their data compared the biological activity of BHT with that of hydroxylated metabolites and the inhibition of those reactions by SKF 525-A. Bolton et al. (1993) concluded, "[f]urthermore, the results support III as an intermediate in the P450-catalyzed oxidation of BHT to a cytotoxic species, most likely V." Nakagawa et al. (1984), used dietary supplementation with cysteine and produced a dose-related inhibition of pulmonary weight gain in mice. Supplementation with other amino acids did not inhibit the gain in lung weight produced by BHT. Dietary supplementation with 1% cysteine reduced by 50% the level of radioactivity bound in lung following administration of [^{14}C]BHT to the mice receiving a control diet.

C. Species Differences in Butylated Hydroxytoluene-Induced Lung Injury

As was pointed out (Malkinson, 1979), administration of BHT to 13 different mouse strains resulted in lung injury, whether this was measured morphologically or by increased [^{3}H]thymidine incorporation into pulmonary DNA. It appears that BHT produces lung damage in mice only (Witschi et al., 1989). The reason for this species specificity is unknown.

XI. Bleomycin-Associated Lung Injury

The bleomycin (BLM) used clinically in the United States and Europe to treat human malignancies is not a single chemical entity, but rather a mixture of 55 to 70% BLM A_2 and smaller amounts of a variety of other BLMs [see Sebti and Lazo (1988) for structures]. The BLMs are a family of glycopeptide antitumor antibiotics first isolated from cultures of Streptomyces verticillus. Initial reports on the use of this material to treat human ma-

lignancies began to appear in the late 1970s, and in this paper, BLM will refer to the clinical mixture, whereas specific isomers will be designated BLM A_2, etc. BLM is a highly effective antineoplastic drug that is widely used in the treatment of lymphomas and testicular and squamous cell tumors. However, its use as an antineoplastic agent is limited by a dose-dependent pneumonitis, which often progresses to interstitial pulmonary fibrosis in humans and animals.

A. Histological and Fine Structural Changes Produced in Lung by Bleomycin

One of the earliest reports on this subject conducted in experimental animals was that of Adamson and Bowden (1974), who treated mice with 0.5 mg BLM (per animal) twice weekly for 4 weeks and observed pulmonary fibrosis as an end-stage lesion. Seven of ten mice survived the 20-week experiment. The initial site of injury was the intima of pulmonary arteries and veins, where endothelial cells became edematous and separated from the underlying basement membrane. These lesions developed after 2 weeks. Capillary endothelial blebbing and interstitial edema were observed after 4 weeks when multifocal necrosis of type I alveolar epithelial cells was accompanied by fibrinous exudate into the alveoli. The process of cellular repair was characterized by proliferation and metaplasia of type II epithelial cells, fibroblastic organization of alveolar fibrin, and interstitial fibrosis within 8 to 12 weeks. The multifocal proliferation of type II epithelial cells that followed necrosis of type I epithelium is reminiscent of the effects of hyperbaric oxygen. Later work in mice (Sikic et al., 1978) examined pulmonary effects of 0, 1, and 20 mg/kg BLM administered twice weekly for 6 weeks. Lung hydroxyproline, an index of collagen, increased approximately 50% above controls at 6 and 8 weeks after BLM (20 mg/kg). Areas of consolidation were observed microscopically after 20 mg/kg at 4 and 6 weeks. Areas of fibrosis ranged from focal lesions, frequently subpleural, to nearly total loss of recognizable architecture at high doses. Alveolar wall thickness was increased at the most distant time point, 8 weeks.

B. The Chemical/Biochemical Mechanism by which Bleomycin Attacks DNA

The cytostatic activity of BLM is due to single and double strand cleavage of DNA, which correlates with the reduction of cell proliferation (Scheulen et al., 1981). The ultimate DNA-attacking molecular species has not been revealed as yet but it is thought to be produced by a ternary BLM-Fe(II)-oxygen complex. A three-dimensional depiction of this active complex between BLM, O_2, and Fe(II) has been published (Sebti and Lazo, 1988, 1994). Physiochemical studies indicate a BLM-metal coordination compound maintaining a square-pyramidal structure with a basal plane that contains the secondary amine nitrogen, the N-4 pyrimidine ring nitrogen, which is the deprotonated peptide bond nitrogen of the histidine imidazole nitrogen (see Sebti and Lazo, 1988). The fifth axial donor has been assigned to the α-amino nitrogen of the β-aminoalanine moiety of BLM and the sixth axial coordination site is thought to be occupied by exogenous oxygen. Cell-free studies in vitro suggest a BLM-Fe(II)-oxygen complex is biologically active. In this rigid square-pyramidal coordination complex, oxygen binding is efficient, and oxygen reduction is possible. Both hydroxyl and superoxide radicals can be formed based on spin-trapping data. DNA strand scission produced by the 'activated' iron BLM complex is thought to be responsible for cell destruction, but at least three metallobleomycins have been shown to mediate oxidative DNA strand scission in vitro (Sebti and Lazo, 1994).

NADPH-dependent enhancement of DNA chain breakage by BLM in microsomes has been reported. Scheulen et al. (1981) studied chain breakage of [^{3}H]thymidine-labeled DNA by release of measureable acid-soluble radioactivity. The following substances were incubated: NADPH, [^{3}H]thymidine-labeled DNA, Fe(III), BLM, and NADPH cytochrome P450 reductase. NADPH oxidation was stimulated approximately ten-fold in the presence of BLM, suggesting that NADPH cytochrome P450 has NADPH-Fe(III)-BLM reductase activity. Omission of any component of the incubation mixture blocked DNA chain breakage. The dependence of DNA chain breakage on oxygen indicated that the formation of oxygen-Fe(II)-BLM is a prerequisite for the production of DNA strand breaks. A nitrogen atmosphere blocked DNA chain breakage and mannitol, GSH, boiled but not native, SOD, catalase, or boiled catalase restored DNA cleavage activity. These findings clearly implicate a role for reactive forms of oxygen in BLM damage to DNA.

At pH 5.5, binding of BLM relaxed supercoiled Co1E1 DNA without breaking it (Povirk et al., 1979). Binding of tripeptide S, a fragment of the drug containing the bithiazole rings, also relaxed and then recoiled supercoiled DNA at pH 5.5 and at pH 8.0, where BLM is normally active. These findings suggested that BLM binding to DNA involves intercalation of the bithiazole rings. In 0.1 M NaCl (pH 8.0), supercoiled Co1E1 DNA was broken at a rate 50% greater than that of relaxed closed circular Co1E1 DNA. The results were interpreted to suggest that intercalative binding is involved in BLM-induced breakage of DNA.

Ferrous BLM is known to cleave DNA in vitro in the presence of oxygen giving rise to oligonucleotides, bases, and compounds resembling MDA in their chromogenic reaction with 2-TBA (Burger et al., 1980). Chromatography of radiolabeled DNA reaction mixture resolves three kinds of MDA-like products, related by sequential conversions. The first chromogenic product is linked to DNA, and its formation does not require base release. It decomposes readily to a second product, a compound containing the base and deoxyribose carbons 1′ to 3′.

Hydrolysis of either product yields a third, which is indistinguishable from authentic MDA. Thus, the oxygen-dependent cleavage of DNA by Fe(II)-BLM can begin with rupture of the deoxyribose 3′-4′-carbon bond, and this is concurrent with another mode of DNA degradation involving release of free base alone in a yield similar to that of TBA chromogen. DNA strand scission catalyzed by BLM in the presence of Fe(II) and oxygen is accompanied by concomitant release of free bases and four low molecular weight compounds that react with TBA to form chromophores that absorb maximally at 532 nm (Giloni et al., 1981). These products, upon hydrolysis, released thymine, cytosine, adenine, and guanine. Reduction of the adenine compound with borohydride followed by silylation and high resolution MS revealed silylated 3-(adenin-9′-yl)-2-propen-1-ol consistent with the original compound being 3-(adenin-9′-yl)-2-propen-1-al. The reaction product containing thymine was similarly shown to be 3-(thymin-1′-yl)-2-propenol. Analogous structures, 3-(guanidin-9′-yl) propenal and 3-(cytosin-1′-yl) propenal, were inferred but not actually detected. It is noteworthy that free MDA was not formed in this system consisting of DNA, BLM, Fe(II), and oxygen. Loss of TBA reactivity was observed after reduction of the aldehydes with $NaBH_4$. It may thus be concluded that it is the base-propenals that form the chromophore after reaction with TBA and not MDA.

C. Biological Inactivation of Bleomycin via Bleomycin Hydrolase

Sebti and Lazo (1994) reported that BLM A_2 was 100-fold more potent than desamido-bleomycin (dBLM) A_2 in killing murine L1210 cells in culture, so the conversion of an amide linkage to a carboxylic acid, catalyzed by BLM hydrolase, must be considered an effective detoxication pathway. Using ion-paired HPLC, Lazo et al. (1982) were able to investigate the metabolism of BLM B_2 in freshly isolated human tumor material in vitro. All 14 tumors examined were capable of metabolizing BLM B_2 to desamido B_2. Metabolites other than desamido B_2 were not detected. Later work revealed that a relative lack of BLM metabolism, by BLM hydrolase, was a critical factor in the biochemical basis of BLM pulmonary toxicity (Lazo and Humphreys, 1983). The in vitro metabolism of BLM A_2 to dBLM A_2 in tissue homogenates from a sensitive species (mice) and its lack in a species relatively resistant (rabbits) to the pulmonary fibrogenic action of BLM can account for this profound difference in toxicity. Lung cytosol fractions from mice lacked detectable BLM hydrolase activity, whereas rabbit lung cytosol had high levels of the enzyme. Injection of [^{3}H]BLM A_2 into mice revealed constant drug levels in lung but extensive metabolism in liver, kidney, and spleen. Mice injected with BLM A_2 twice weekly for 6 weeks developed pulmonary fibrosis, whereas studies of animals treated with equivalent doses of dBLM A_2 did not indicate that the metabolite is less pneumotoxic than the parent. BLM hydrolase was purified 6,000-fold from rabbit lung to homogeneity by the sequential use of various column and liquid chromatographies. The enzyme had a molecular mass of 250,000 Da; however, subjecting it to SDS-PAGE revealed a single band at 50,000 Da, consistent with a pentameric structure. The purified enzyme was stabilized by dithiothreitol and inhibited by divalent cations such as Cu^{++}, Cd^{++}, and Zn^{++}, suggesting a sulfhydryl at or near the active site. Three isoforms of BLM hydrolase (apparent pIs of 5.3, 4.5, and 4.3) were found in rabbit lung cytosol (Sebti et al., 1987, 1989; Sebti and Lazo, 1988).

At some time during the course of chemotherapy, human and animal tumors become resistant to oncolytic drugs and radiation. Often, the mechanism of this resistance is not known, but enhanced metabolism of the drug is one possibility. Burkitt's lymphoma and human head and neck squamous cell carcinoma (A-253) grown subcutaneously in nude mice were found to be resistant and sensitive to BLM treatment, respectively. Within 1 h of subcutaneous injection of [^{3}H]BLM, A_2 Burkitt's xenografts accumulated less BLM and metabolized the drug to a much greater extent than did A-253 xenografts. The BLM-resistant Burkitt's xenografts metabolized BLM A_2 to at least six metabolites, and only a small portion of the drug remained as unchanged BLM A_2. In the BLM-sensitive A-253 xenografts, however, BLM A_2 remained the major component. Incubation of BLM A_2 with Burkitt's xenografts resulted in a complex mixture of metabolites similar to that formed by Burkitt's in nude mice. This BLM metabolite mixture was biologically inactive in plasmid DNA degradation assays. Treatment of mice with Burkitt's xenografts without an inhibitor of BLM hydrolase (E-64) before [^{3}H]BLM A_2 treatment inhibited BLM A_2 metabolism in xenografts without changing tissue uptake. Thus, Burkitt's lymphoma tumor cells resist BLM by metabolically inactivating it, and the inhibition in vivo enhances the antitumor activity of BLM and hence overcomes resistance (Sebti et al., 1991).

D. Strain Differences in Murine Bleomycin Effects

BLM is inactivated in vivo by BLM hydrolase, an enzyme purified to homogeneity from rabbit lung by Sebti et al. (1987). The enzyme is strongly inhibited by N-ethylmaleimide, suggesting it is a thiol enzyme. Resistance to BLM toxicity may be attributable to BLM hydrolase, as the metabolite dBLM is approximately 1% as active as the parent in producing DNA strand scission, tumor cytotoxicity, oxygen radicals, and pulmonary fibrosis in animals. BLM hydrolase activity is high in liver, kidney, and bone marrow, which are resistant to BLM toxicity but low in lung and skin, target sites for BLM toxicity. An interesting bit of serendipity occurred when it was learned that strain differences occurred in mice. For example, BALB/c mice were resistant to pulmonary fibrosis produced by BLM, whereas C57B1/6

mice were highly sensitive and DBA/2 mice were intermediate in sensitivity. BLM hydrolase activity was determined by measuring the rate of formation of dBLM A_2 from the parent BLM A_2 using HPLC techniques in tissue cytosol fractions. Resistant BALB/c mice had the highest lung BLM hydrolase activities, 2.6-fold higher than the sensitive C57B1/6 and 1.6-fold higher than the intermediate strain DBA/2. An identical pattern emerged in skin, another sensitive BLM target (Filderman and Lazo, 1991). Interestingly, BLM hydrolase activities in kidney and liver, organs insensitive to BLM fibrosis, did not differ among the three strains. However, the pattern in lung BLM hydrolase activity in vitro correlated inversely with the pulmonary sensitivity to parenterally administered BLM. BLM was administered to mice by Schrier et al. (1983) by intratracheal injection, and pulmonary collagen "synthesis" was estimated by measuring hydroxyproline content. Animals were all females of the C57B1/6, DBA/2, BALB, and Swiss strains. C57B1/6 strain responded to BLM with a nearly two-fold increase in hydroxyproline, DBA was slightly less, and BALB was virtually unresponsive. Values for the outbred strain, Swiss, were nearly as high as the C57Bl/6.

After a single dose of [^{3}H]BLM (80 mg/kg, iv) to sensitive and resistant mouse strains, lung collagen content was increased 35% in C57Bl/6 mice (sensitive) but only approximately 8% in BALB/c mouse (resistant). Plasma $t_{½}$ in the resistant strain was significantly longer than in the sensitive strain. V_D values did not differ. Thirty minutes after iv injection, plasma radioactivity was 50% higher in the resistant strain than the sensitive. Similarly, tissue levels in the resistant strain were 50% higher than the sensitive strain 30 minutes after injection of [^{3}H]BLM into saline-perfused mouse lungs, and the activity 30 minutes after injection of [^{3}H]BLM into intact animals was 50% higher in the resistant strain. Thus, it was concluded that strain differences in pharmacokinetics cannot explain the resistance of BALB/c mice to BLM pneumotoxicity.

Hoyt and Lazo (1992) measured lactate dehydrogenase (LDH) release and nuclear poly (ADP-ribose) polymerase (PAP), which is stimulated by DNA breakage using lung slices from BLM sensitive (C57B1/6) and resistant (BALB/c) mice. LDH release from sensitive lung slices increased two-fold by 8.5 h after treatment with BLM. In contrast, BLM failed to increase LDH release from resistant lungs. Coincubation of sensitive lung slices with 3-aminobenzamide (3-AB), a PAP inhibitor, prevented BLM-induced LDH release. Nuclear PAP was activated 3- to 4-fold, 75 minutes after exposure of sensitive lung slices to BLM but returned to control levels by 225 minutes. Nuclear PAP was only marginally affected at these times in resistant lung slices. Thus, murine strain sensitivity to acute cell injury and early PAP activation by BLM in lung slices parallels the in vivo sensitivity of lungs. In addition, 3-AB suppresses PAP activation and acute cell injury in lung slices. Differential activation of PAP appears to govern mouse strain differences to BLM and supports the view that activation of PAP participates in acute pneumocyte injury, initiating BLM-induced fibrosis. BLM cleaves DNA and causes pulmonary fibrosis. 3-AB, a PAP inhibitor, prevents enzyme activation and cell injury. The potential role of adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide (NAD) depletion in injury of BLM-sensitive and -resistant murine lung slices treated with BLM or deprived of glucose, the major metabolic substrate of lung was examined. Lung slices were either treated with various concentrations of BLM or were incubated with glucose in the presence or absence of 3-AB. No significant alterations occurred in ATP concentrations. In contrast, glucose deprivation caused 50 to 70% ATP depletion in slices from both strains. BLM alone caused a sustained 30 to 70% NAD depletion from 75 through 400 minutes in sensitive mouse lung slices. In the resistant (BALB/c) lung slices, NAD depletion by BLM appeared only at 400 minutes. 3-AB almost completely blocked NAD depletion in slices from both strains. In contrast to BLM, glucose deprivation did not decrease NAD levels unless 3-AB was present in sensitive slices. Thus, ATP depletion may play a role in the injurious effects of glucose deprivation but does not appear to be a major factor in pneumocyte injury caused by BLM. NAD depletion or other effects of PAP activation appear to account for the strain-specific toxic effect of BLM on lung tissue.

Harrison et al. (1989) reported that after the administration of toxic doses of BLM (80 mg/kg) to sensitive (C57BL/6N) and resistant (BALB/c) mouse strains, BLM produced striking differences in nucleoid sedimentation distance in both strains within 15 minutes after injection, indicating extensive DNA scission. Repair of DNA strand breaks was complete in the resistant mice by 5 h, whereas only partial repair (~60%) occurred in the sensitive strain during that time. It should be remembered that the terms "sensitive" and "resistant" were coined to describe the pulmonary fibrotic response to BLM in the two strains of mice. In addition, a seven-fold increase in the type I:type III protocollagen mRNA ratio was seen in both strains 3 to 6 days after BLM injection. Therefore, BLM injection rapidly produces extensive pulmonary DNA damage in vivo, and that persistence of DNA damage rather than the initial level of strand scission is associated with sensitivity to BLM lung disease in these mice.

One last point is that BLM dramatically synergizes pulmonary fibrosis evoked by cyclophosphamide (Muggia et al., 1983) or by hyperoxia (Tryka et al., 1984). For example, Syrian golden hamsters treated intratracheally with BLM and then immediately exposed to 70% oxygen for 72 h displayed a 90% mortality compared with 15% in a control group treated with BLM alone. Histologically, animals receiving the combined treat-

ments revealed only lung lesions characterized by widespread thickening of both alveolar and interstitial walls, type II cell hyperplasia, intraalveolar debris, and fibrosis. It was suggested that simultaneous treatment with BLM and hyperoxia has a synergistic effect on mortality and pulmonary fibrosis.

Several excellent reviews on the toxicology and clinical utility of BLM have appeared (Hay et al., 1991; Lazo et al., 1993; Dorr, 1992). They are all highly recommended to the reader.

XII. Pulmonary Toxicology of Trialkyl Phosphorothioates (Contaminants in Malathion)

The commercially important organophosphorus insecticide, malathion, contains several impurities, one of which, O,O,S-trimethylphosphorothioate (OOSTMP), causes an unusual delayed toxicity in rats. At low oral doses, OOSTMP causes body weight loss, red staining around the nose and mouth, and death from 3 to 28 days after treatment. No typical cholinergic signs of toxicity are produced.

A. Ultrastructural Changes

Morphological studies showed that OOSTMP and another impurity, O,S,S-trimethylphosphorothioate (OSSTMP), cause pulmonary abnormalities in rats consisting of a significant reduction of the number of Clara cells (Gandy et al., 1983). The mean number of Clara cells, estimated by SEM, was reduced from a mean of 69 cells/unit area in controls to approximately 20 by the administration of 20 mg/kg OOSTMP. This reduction in Clara cells was completely blocked by the coadministration of PB (75 mg/kg for 4 days). Later work (Imamura et al., 1983b) confirmed that OOSTMP reduced the number of Clara cells in rat lung but also doubled the LDH activity, a pneumocytic cytoplasmic enzyme in bronchopulmonary lavage fluid from treated rats. Dinsdale et al. (1984) reported that the other contaminant in technical grade malathion, viz OSSTMP, when administered to rats (25 mg/kg, orally) produced a pronounced increase in lung weight, extensive injury to the alveolar epithelium, but no signs of damage to the cells of the bronchiolar epithelium. OSSTMP evoked necrosis of type I pneumocytes within 2 to 4 days after administration, but no ultrastructural signs of cellular injury were found in either the ciliated cells or the Clara cells. Significant symptoms of cholinergic stimulation were observed with this isomer, which may have accounted for the immediate liberation of secretory granules from Clara cells. Returning now to OOSTMP, Gandy et al. (1984) determined its temporal and dose-dependent effects on bronchiolar epithelium in rats using SEM as a rather dubious quantitative tool. Twenty-four h after oral dosing, Clara cell numbers declined by 30%, and after 3 days, by 70%; by 7 days, Clara cells had returned to control values. Five days after OOSTMP adminstration, LDH activity in lung lavage was doubled and at 7 days had returned to control values. Gandy et al. (1984) reported that in addition to Clara cell damage and changes in LDH levels, OOSTMP also caused selective inhibition of pulmonary CYP450-mediated monooxygenases.

B. Cytochrome P450 and Biochemical Alterations

The oral 28 day LD_{50} of OOSTMP was 67 mg/kg in rats (Konno et al., 1984). Male rats were dosed with the compound at 40, 100, and 160 mg/kg and killed 24 h later. Neither lung nor liver weight was affected at any dose. In lung, NADPH cytochrome c reductase activity was altered up and down in a non-dose–related manner. BP hydroxylase was generally reduced to ~35% of control, again dose-independently. Lung GSH was reduced 40% by OSSTMP (40 mg/kg). Imamura et al. (1983a) treated rats with OOSTMP at 10, 20, and 40 mg/kg po, and they were killed 3 days after treatment. The oral 28-day LD_{50} under these conditions was 60 mg/kg. Lung microsomal P450 was reduced marginally but significantly by 17%. Relative lung weight nearly doubled at the high dose (40 mg/kg), whereas liver weight declined slightly. 7-Ethoxycoumarin-O-deethylase declined in a dose-related manner to approximately 10% of control at the high dose.

Oral treatment of rats with [^{3}H]OOSTMP resulted in substantial amounts of radiolabeled material being covalently bound in lung. [^{3}H]OOSTMP was administered at 10, 20 and 40 mg/kg and covalent binding was measured as functions of dose and time. Binding was dose-dependent in lung, liver, kidney, ileum, spleen, and thymus, but not in testes, brain, muscle, or blood. Pretreatment of rats with PB for 4 days before [^{3}H]OOSTMP reduced binding in lung, kidney, and other organs but not in liver (Imamura and Hasegawa, 1984). Dose-dependent GSH depletion was observed in kidney (nadir at 55% of control at the highest dose), lung (40%), and kidney (55%) and to lesser but significant degrees in spleen, testes, and brain at higher doses. Pretreatment of rats with piperonyl butoxide reduced binding of [^{3}H]OOSTMP in lung, kidney, and liver by approximately 70% and reduced binding in other organs to some extent. Thus, pretreatment of rats with either PB or piperonyl butoxide reduced the level of radiolabel bound in lung, GSH depletion, and the toxicity of OOSTMP. Dinsdale et al. (1982, 1984) had reported that OOSTMP caused selective destruction of alveolar type I cells and proliferation of type II cells to cover the basal membrane. Gandy and Imamura (1985) found that [^{3}H]OOSTMP bound throughout all regions of the lung. Using light microscopy of plastic sections, the compound was bound to cells of the alveoli as well as bronchiolar epithelial cells, interstitial cells, and connective tissue under the bronchiolar basement membrane. [^{3}H]Thymidine incorporation into DNA was evaluated in control animals and animals that received 40 mg/kg OOSTMP and sacrificed 12 h, and 1, 3, and 7 days later. LIs were time-related. In no case was labeling different from con-

trols at 12 or 24 h. However, 3 days after [^{3}H]Thd, the LI increased ten-fold in type I cells, 20-fold in type II cells, and 17-fold in Clara cells. Seven days after ^{3}H-Thd, the LI was increased 25-fold in type I cells and 15-fold in type II and Clara cells. It is noteworthy that at no time point did the LI in bronchiolar ciliated epithelial cells differ in OOSTMP and control animals.

Verschoyle et al. (1993b) reported that OOSTMP inhibited the dealkylation of pentoxyresorufin by approximately 10-fold in rat lung microsomes. Pentoxyresorufin is a specific substrate for CYP2B1; p-xylene is a specific inhibitor of CYP2B1. Thus, pulmonary CYP2B1 is responsible for the bioactivation and toxicity of OOSTMP in rats. Treatment of rats with p-xylene gave effective protection against OOSTMP pulmonary toxicity.

XIII. Summary

After reading this lengthy review, the reader may consider it foolhardy to attempt to summarize the data contained herein. If so, the author and the reader are in complete accord. I set out to integrate, wherever possible, pathological changes in tissues and cells as determined by light, scanning, and TEM with chemical, biochemical, molecular, biological, and genetic techniques. I am not so naive as to equate correlation with causation, but in experimental biology, one is often compelled to rely on a number correlations to suggest causation. These data may also act as a stepping-off point helping the investigator design experiments to support/confirm or refute/disprove the hypothesis under investigation. Science is rarely black and white but rather gray, and the young investigator must be wary of scientists who argue their points too vociferously, too loudly, or too selectively. The truth in science is often a Gordian knot.

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