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ROLE OF COVALENT AND NONCOVALENT INTERACTIONS IN CELL TOXICITY: Effects on Proteins

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INTRODUCTION

Xenobiotics (foreign chemicals) interact with proteins in a variety of ways. The interactions of drugs with receptors is a fundamental premise for our understanding of the function of many drugs in pharmacology. These interactions are usually reversible in nature. In the field of toxicology one of the most frequently studied interactions between xenobiotics and proteins is that of enzyme-catalyzed metabolism of substrates. These interactions usually lead to more water-soluble products that are excreted. However, certain chemicals are metabolized to electrophilic derivatives (reactive metabolites) that may covalently bind with nucleophilic sites within the cell to produce a toxic effect. A number of amino acids in proteins are nucleophilic and electrophiles may react with these nucleophilic sites to produce altered proteins. These interactions are believed to be the mechanism by which many chemicals produce their toxic effects to cells.

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This review concentrates on the interaction of electrophilic metabolites with proteins. The main focus is on chemicals that interact with the amino acids of the primary structure of proteins to produce alterations believed to be important in toxicities. In most cases these interactions occur in the target cell, which is the site of formation; however, some electrophilic metabolites leave the site of formation and react with proteins in other cells or tissues. These latter interactions are also discussed as background to our general understanding of how toxic metabolites react with proteins.

Chemically induced toxicities may also occur via noncovalent interactions. The best example is oxidative stress. By this mechanism the reactive oxygen species superoxide anion, hydroxyl radicals, and hydrogen peroxide may adversely affect a number of normal cellular functions and thus result in cell death. These species may decrease the reducing equivalents in the cell (i.e. NADPH, NADH, glutathione, ascorbate, vitamin E), and produce lipid peroxidation, and alterations of proteins. For toxic compounds such as quinones and related compounds that produce these reactive oxygen species in concert with covalent binding to protein, it has proven difficult to differentiate between toxic mechanisms that may occur via covalent binding or oxidative stress.

General Concepts of Metabolic Activation

Many chemical induced toxicities are believed to be mediated by metabolism to electrophilic derivatives. Metabolism is usually by the drug-metabolizing enzyme system and reactive metabolites may covalently bind, not only to nucleophilic sites in proteins, but to other nucleophilic sites such as those on DNA, RNA, or react with smaller molecular weight endogenous compounds such as glutathione. Two general concepts have been advanced to explain the mechanisms by which covalent binding may produce toxicity. One mechanism is (*a*) by covalently interacting or altering the protein(s) and/or macromolecules such that normal functioning cannot be maintained, e. g. by covalently binding to a critical enzyme necessary for energy production in the cell or to enzymes involved in ion homeostasis. Another mechanism is (*b*) by binding to protein(s) and thus causing the chemical protein adduct to serve as an immunogen. Subsequent exposure causes an immune reaction that results in a cytotoxic response (see Refs. 1, 2 for recent reviews).

The concept of metabolic activation and its importance in chemical toxicity was derived from an analysis of the formation of protein adducts from toxic chemicals (3). A report published in 1947 from the Millers' laboratory indicated that the livers of rats fed the carcinogen N,N dimethyl 4 aminoazobenzene contained aminoazo dye firmly bound to the protein and that these protein adducts formed before the hepatic tumors (3). It was sub-

sequently determined that formation of the protein adducts correlated with the carcinogenicity of the aminoazodyes under a variety of conditions. It was further shown that a variety of toxic chemicals lead to covalent adducts to numerous nucleophilic sites on proteins as well as DNA, and that the effects of many carcinogens and toxins are mediated via initial metabolism to an electrophilic derivative that covalently binds to nucleophilic sites in the cells (4).

Electrophilic metabolites and chemicals react with many different nucleophilic sites in cells. The role of DNA binding in initiating the carcinogenic process has been extensively investigated (4–7). However, electrophilic metabolites may not only react with nucleophilic sites on DNA, but may also bind to proteins, RNA, and with derivatives of low molecular weight, such as glutathione. Thus, our understanding of the role of electrophilic metabolites binding to nucleophilic sites in DNA is integrally tied both historically and experimentally to binding of electrophilic metabolites to proteins.

The complexity of the reaction of electrophilic metabolites with the various nucleophilic sites within cells and the reasons why different electrophilic reagents react at different sites have been interpreted based upon concepts of hard and soft electrophiles/nucleophiles (hard and soft acids/bases) (8–10). These terms have been qualitatively defined to mean: the donor atom of soft nucleophile is of high polarizability and low electronegativity, is easily oxidized and associated with empty, low-lying orbitals; the donor atom of hard nucleophile is of low polarizability and high electronegativity, is hard to oxidize, and associated with empty orbitals of high energy and hence inaccessible; the acceptor atom of a soft electrophile is of low positive charge, large size, and has several easily excited outer electrons; the acceptor atom of a hard electrophile is of high positive charge, small size, and does not have easily excited outer electrons (8). Soft electrophiles react predominantly with soft nucleophiles, and hard electrophiles with hard nucleophiles. Thus, a soft electrophile such as an α,β-unsaturated carbonyl compound (diethylmaleate or p benzoquinone) reacts predominantly with a soft nucleophile such as the sulfhydryl group of cysteine or glutathione. Similarly, a hard electrophile such as the methyl carbonium ion produced from the carcinogen dimethylnitrosamine reacts with hard nucleophiles such as the O6 of guanine in DNA (Table 1; 9).

COVALENT INTERACTIONS OF CARCINOGENS

The interaction of carcinogens with proteins has been well documented (see Ref. 4). Early studies generally described binding to protein as the basic mechanism of carcinogenesis, but subsequent research has focused on bind-

Table 1 Relative softness and hardness of nucleophiles and electrophiles

Soft				Hard
Nucleophiles				
Sulfhydryl of cysteine or GSH	> Sulfur of methionine	> Primary or secondary amino of lysine, arginine or histidine,	> Amino groups of purine bases in RNA & DNA	> Oxygen of purines & pyrimidines in DNA & RNA
Electrophiles				
α, B-Unsaturated carbonyl compounds, quinones & quinone imines	> Epoxides, alkyl sulfates & halides	> Aryl carbonium & nitrenium ions	> Benzylic carbonium ions	> Alkyl carbonium ions

ing of metabolites to DNA as the critical initiating event. Interest in carcinogen-protein adducts may be classified into: (*a*) basic mechanisms of carcinogen interactions (11); (*b*) molecular dosimetry or biomonitoring (12, 13). In the latter studies it is assumed that the amount of carcinogen protein adducts formed in an accessible tissue such as blood is proportional to exposure as well as to the amount of specific DNA adducts formed.

Binding of Carcinogens to Proteins in Target Tissues

A substantial amount of work has been performed on the binding of the aromatic aminoazo dye carcinogen, N,N-dimethyl-4-aminoazobenzene (DAB) to protein. Analyses of protein-bound residues indicated that binding of DAB and its demethylated N-methyl-4-aminoazobenzene (MAB) to methionine residues on protein as 3-(methion-S-yl) MAB and tyrosine residues on protein as N-(3-tyrosyl)-MAB and 3-(3-tyrosyl)-MAB (4, 14, 16–18).

Specific proteins that covalently bind to aminoazo dye have been purified and identified from rat hepatic cytosol (18–21). One such protein containing azo dye from rats treated with the 3'-methyl analogue of DAB was liver alcohol dehydrogenase (21). This protein (subunit M_r 45,000) contained a minimum 45% of the azo dye and its binding site was identified at methionine 306 as 3-(homocystein-S-yl)-4-methylamino-3-methylazobenzene. It was postulated that the MAB-protein adduct was formed from DAB by an initial demethylation to MAB, followed by N-hydroxylation and sulfation to form the electrophilic metabolite N-sulfoxy-4-methylamino-3'-methylazobenzene, which reacts with the methionine residue of alcohol dehydrogenase. Only minor alterations in alcohol dehyrogenase activity were observed and the reaction was regarded as a major detoxification of activated MAB (21). In

addition, MAB binds to fatty acid binding protein (M_r 14,000) through a methionyl residue and to a minor extent to a glutathione transferase (ligandin), possibly through a cysteinyl residue (15, 18). The presence of lipophilic binding sites may be related to the covalent binding of azo dye residues to these specific proteins (18). The toxicological importance of this binding is unknown, although Sorof and coworkers (19, 22, 23) have suggested that the fatty acid binding protein may be important in cell regulation.

2-Acetylaminofluorene (2-AAF) has been extensively studied as a model for aromatic amine carcinogens (4, 5). It produces tumors in numerous sites including the liver of experimental animals and, like the aminoazo dyes, forms covalent adducts with specific proteins in target tissues. The binding of 2-AAF residues to hepatic proteins was first described in the early 1950s (4). Investigations revealed that iminoquinone metabolites were reactive and could bind to protein, but did not react with nucleic acids to produce adducts and were not ultimate carcinogenic metabolites. Work in the Millers' laboratory indicated that N-hydroxylation of 2-AAF was important in 2-AAF-induced cancer. Esterified derivatives and specifically the formation of N-O-sulfate esters of the N-hydroxy group resulted in reactive species that bound to methionine groups on protein as well as DNA. Alkaline hydrolysis products of the 2-AAF methionine adducts yielded 1- and 3-methyl-mercapto-2-acetylaminofluorene. These data are consistent with the concept that the sulfate ester of N-hydroxy-2-AAF binds to protein as 1-(methion-S-yl)-2-acetylaminofluorene and 3-(methion-S-yl) 2 acetylaminofluorene adducts (4, 5, 14, 25). It is not yet clear whether 2-AAF binds to other amino acid residues in protein.

Specific proteins to which 2-AAF residues become bound have been investigated. In early work it was reported that administration of radiolabeled N-hydroxy-2-AAF to rat resulted in binding to a serum protein with a molecular weight of albumin (26). Sorof and coworkers reported that, as with DAB, small molecular weight polypeptide fatty acid binding protein (M_r 14,000) bound 2-AAF residues. In addition, in animals fed hepatocarcinogens, 2-AAF became bound to a 55,000 subunit of a protein with a molecular weight 150,000 (27). This polypeptide subunit was relatively basic with a Pi of 8.4 to 8.6 and was unstable. The protein has not been identified (22, 23, 27).

Polycyclic aromatic hydrocarbons (PAH) have also been reported to bind to protein (28). Treatment of the skin of mice with benzo[a]pyrene resulted in protein binding. In Heidelburger's laboratory a basic protein to which many PAH derivatives covalently bound in vivo was isolated from the liver. This protein was present not only in liver but in most cell types that were examined and, based on available evidence, is most likely a glutathione transferase (ligandin) (28–30). The mechanism is primarily via binding of the metabolite

7β,8α-dihydroxy 9α,10α-epoxy-7,8,9,10,tetrahydrobenzo[a]pyrene (diol epoxide) (91). The covalent binding of benzo[a]pyrene and specific metabolites has been examined in microsomal incubation mixtures by analysis of proteins with SDS gel electrophoresis and detection by fluorography. Extensive binding occurred to a few proteins in the molecular weight range from 45–70 kd (32). Other work on PAH derivatives has concentrated on the binding of PAH residues to nuclear proteins. In cell cultures it has been shown that the diol epoxide metabolite binds specifically to histone H2A and H3 in hamster embryo cell nuclei, mouse cells, and human cells (33–35). Furthermore, incubation of the diol epoxide with nuclei from rat hepatocytes or chicken erythrocytes results in binding to the histone H2A; the nature of the binding was different with different isomeric forms of the diol epoxide (36, 37). Binding of the diol epoxide was shown to occur to a C-8 octapeptide of histone H2A and it was suggested that binding may occur on a histidine residue near the C terminus (37). With 7-hydroxymethyl-benz[a]anthracene, sulfation of the benzylic hydroxyl leads to a mutagenic metabolite that binds to cytosolic proteins as well as DNA (38).

Other chemical carcinogens also bind to specific proteins in target tissue. Aflatoxin, a potent hepato-carcinogen produced by the mold *Aspergillus flavus*, covalently binds to hepatic proteins and as much as 1–3% of the administered dose has been shown to bind to a specific lysine residue on albumin (39, 40). The importance of binding to hepatic protein in cell toxicity is not well defined. Likewise, nitrosamines are metabolically activated to metabolites that alkylate proteins in target tissue. Using the hepatocarcinogen dimethylnitrosamine as a model, Magee and coworkers reported the occurrence of methylation of protein in the liver (41). Evidence was presented that binding occurred to histidinyl residues on protein as 1- and 3-methylhistidine. It was subsequently shown that dimethylnitrosamine methylated nuclear proteins in liver and kidney. Methylation was determined to occur on histidinyl, lysinyl, and cysteinyl residues of protein (42). The colon carcinogen 1,2-dimethylhydrazine methylated lysine, arginine, and histidine of histone proteins and other DNA-binding proteins in colonic epithelial cells of rats (43). With the direct alkylating agents dimethyl sulfate and methyl methanesulphonate, both weak carcinogens and relatively soft electrophiles, methylation was shown to occur primarily on the sulfhydryl group of cysteine and the 3-position of histidine when tested in cultured hepatocytes and V79 Chinese hamster cells. With the harder electrophiles and more potent carcinogens N-methyl-N-nitrosourea and N-methyl-N'-nitro-N-nitrosoguanosine, methylation in these cells occurred predominantly on lysine and arginine residues (44). Other reports have indicated that N-methyl-N'-nitro-N-nitrosoguanidine also binds to cysteine groups on protein in cultured hepatocytes (46) and this binding is important in cytotoxicity. Comparison of methylation of dimethyl

sulfate and methyl methane sulphonate to N-methyl-N-nitrosourea and N-methyl-N'-nitro-N-nitrosoguanosine on the binding to histone proteins indicated different patterns (44). Support for the concept of the site of alkylation being related to relative electrophilicity was shown in subsequent work using the ethylating agents N-ethylnitrosourea, a very weak electrophile, and ethyl methanesulfonate, an alkylating agent of intermediate electrophilicity (47). Incubations in vitro of the alkylating agents methyl methane sulfonate and 1-methyl-1-nitrosourea with horse-heart cytochrome *c* resulted in methylation of lysine and other unidentified amino acids (48).

Carcinogen Protein Adducts in Molecular Dosimetry Studies

A major area of research into carcinogen-protein adducts is molecular dosimetry. Carcinogen-protein adducts in a readily obtainable protein such as hemoglobin or albumin have been used as a biomarker of exposure (12, 13).

In the 1970s, Ehrenburger and coworkers showed that treatment of mice with the direct-acting mutagen and alkylating agent ethylene oxide resulted in covalent binding to hemoglobin and the amount of adducted hemoglobin decreased after exposure at a rate proportional to the life span of the erythrocyte (49, 50). Hydroxyethyl-histidine accounted for approximately 40% of the adducted hemoglobin (49). This group subsequently showed that administration of ethene, a plant-growth regulator that is metabolized by epoxidation to ethylene oxide, resulted in ethene becoming bound to hemoglobin as S-(2-hydroxyethyl)cysteinyl residues and a mixture of N-1- and N-3-(2-hydroxyethyl)histidinyl residues (50). The same three adducts were observed in hemoglobin following administration of vinyl chloride to mice. These data indicated that chloroethylene oxide, and not chloroacetaldehyde, was the main reactive metabolite of vinyl chloride (51). With propene or propylene oxide, N-(2-hydroxypropyl)histidinyl residues on hemoglobin were determined to be the primary adduct. Again the data were consistent with an epoxide as the reactive intermediate (52, 53). Subsequently, it was shown that ethylene oxide not only alkylated cysteine and histidine residues in hemoglobin, but also reacted with the N-terminal valine residues of hemoglobin to produce the adduct N-(2-hydroxyethyl)valine (54, 55). A similar adduct has been reported for propene and propylene oxide (53). A very sensitive assay was developed for these adducts using pentafluorophenyl isothiocyanate to cleave the alkylated N-terminal adduct from the hemoglobin by a modified Edman degradation procedure. The resulting thiohydantoin derivative was extracted and assayed using a gas chromotography–mass spectrometry (GC-MS) procedure (55). This procedure has been used to analyze smokers for the presence N-(2-hydroxyethyl)valine hemoglobin residues in hemoglobin (56, 57). These adducts have also been analyzed in fruit workers exposed to ethene (58) and animals exposed to automobile exhaust (59).

Methylated adducts of hemoglobin have been reported to occur following both a direct alkylating agent and the nitrosamine dimethylnitrosamine, which is metabolized to an alkylating agent. Administration of dimethylnitrosamine resulted in 3-methylhistidine adducts on hemoglobin at a level of approximately 10–15% of the adducted protein (49). Administration of the direct alkylating agent methyl methanesulfonate to mice resulted in the formation of S-methylcysteinyl and 3-methylhistidinyl-hemoglobin adducts (60). The same adducts have been reported in serum proteins after treatment of rats with dimethyl nitrosamine (61). With the tobacco-specific nitrosamines 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone or N'nitrosonornicotine, Hecht and coworkers (62–64) showed that hemoglobin adducts were formed. Base treatment of the adducts from hemoglobin treated with both compounds yielded 4 hydroxy-1-(3-pyridyl)-1-butanone that could be derivatized and the product quantitated by a GC–MS procedure. The derivatized amino acid residue in hemoglobin was not definitively identified; however, the susceptibility of the adduct to base hydrolysis and not acid hydrolysis suggested that cysteine was not involved and the adduct may be an ester of aspartate or glutamate (64). A polyclonal antibody assay has recently been developed to study the interaction of tobacco-derived nitrosamines with protein (65).

Hemoglobin adducts have been used as biomarkers of aromatic amine exposure with a high degree of success (13). Weisburger and coworkers (66) initially noted that administration of the N-hydroxy derivative of 2-AAF as well as 2-AAF caused a significant fraction of the administered dose to bind to hemoglobin. In the mid-1980s two other groups (67–69) showed that the aromatic amines aniline (or nitrobenzene) (68) and 4-aminobiphenyl (69) covalently bind to hemoglobin. It was postulated that the aromatic amine was N-hydroxylated, presumably in the liver, and that the N-hydroxy metabolite subsequently entered the erythrocyte where it was oxidized by hemoglobin (with methemoglobin formation) to produce the corresponding aromatic nitroso derivative. This nitroso compound, a soft electrophile, reacts preferentially with cysteine groups of hemoglobin to yield sulfinic acid amides. Treatment of the hemoglobin with alkali hydrolyzed the adduct to the corresponding aromatic amine, which could be extracted into organic solvents and quantified by GC (68) or GC-MS analyses (69). As much as 5% of a dose of 4-aminobiphenyl, administered at 5 mg/kg to the rat, became bound to hemoglobin; chronic administration caused adducts to accumulate; adduct levels decreased at a rate directly proportional to the life span of the erythrocyte (69).

Hemoglobin adducts of aromatic amines have been successfully used to monitor exposure of human populations to both carcinogenic and noncarcinogenic aromatic amines. Cigarette smokers were shown to have hemoglobin adducts of 15 different aromatic amines and significant adduct levels of

the human carcinogens 4-aminobiphenyl and 2-naphthylamine (70, 71). The binding of 4-aminobiphenyl has been shown to be at the β-93 cysteine residue on human hemoglobin (72). Other aromatic amines have also been shown to produce hemoglobin adducts, including various methyl and ethylaniline derivatives (71), chlorinated aniline derivatives (73–75), benzidine derivatives (76), and the food pyrolysis products 2-amino-3,8-dimethylimidazo[4,5-*f*] quinoxaline (77).

Serum albumin adducts as well as hemoglobin adducts have been reported in rats administered 4-aminobiphenyl. Skipper and coworkers (78) found 3-tryptophanyl-4-acetylamino-biphenyl adducts at position 214 in serum albumin. It was postulated that the metabolite N-sulfonyloxy-N-acetylaminobiphenyl reacted with the single tryptophan moiety of albumin, which is near the fatty acid binding site of albumin (78).

Molecular dosimetry studies also have been performed with polycyclic aromatic hydrocarbons. Shugart (79, 80) used acid to hydrolyze hemoglobin from mice treated with benzo[a]pyrene and by using HPLC separation methods coupled with fluorescent detection showed that tetrols of benzo[a]pyrene become bound to hemoglobin. These data indicated that the benzo[a]pyrene diol epoxide bound to hemoglobin. Santella and coworkers (81, 82) developed an immunoassay to analyze for the presence of benzo[a]pyrene adducts and tetrols released from macromolecules under acidic conditions. Their data indicated that in mice adducts occurred in both serum and hemoglobin as well as DNA. With fluoranthene it was shown that a diol epoxide bound specifically to a sulfhydryl in the β-125 cysteine residue (83), a highly reactive nucleophilic residue peculiar to rodents and not present in human hemoglobin (84–86). Binding to cysteine β-93, the nucleophilic sulfhydryl amino acid present in human hemoglobin, was not detected (83). Skipper and coworkers obtained oxygen-18 hydrolysis data which indicated that the benzo[a]pyrene diol epoxide binds to human hemoglobin as a carboxylic acid ester (binding to a carboxylic acid residue) (86, 87). Further evidence for binding of benzo[a] pyrene diol epoxide to a carboxylic acid residue was obtained by fluorescence line-narrowing spectral analysis (88) and recently α chain aspartate 47 has been implicated as a site of binding (89). Other work using metabolites from five polycyclic aromatic hydrocarbons and styrene indicated that binding to carboxylic acid residues on hemoglobin as the corresponding esters occurred with many PAHs (90).

Aflatoxin B1 binds to a lysine residue in serum albumin of rats. This adduct is believed to occur via initial condensation of the ring-open form of aflatoxin B1-8,9-dihydro-8,9-diol with a lysine residue to form a Schiff base. The same intermediate could also be formed via reaction of the 8,9-epoxide with the lysine residue. The product subsequently undergoes a series of rearrangements to yield a stable ring-closed product. As much as 1–3% of a single dose

may bind to serum proteins (39). The adduct has been used in molecular dosimetry studies (91, 92).

COVALENT INTERACTIONS OF DRUGS AND OTHER CHEMICALS

The covalent binding of reactive metabolites of drugs to proteins may be a key event in the development of many drug-induced toxicities. Many toxicities may be mediated by a reactive metabolite covalently binding to critical proteins that are important in maintaining cellular function(s) (1, 93). Certain other toxicities may be mediated via immunological mechanisms and a critical component may be the development of hypersensitivity against drug-protein adducts (2).

Although covalent binding of reactive metabolites to protein correlates with the development of many drug-induced toxicities, the actual sequence of events leading to the development of the toxicities is unknown. Moreover, with many drugs the reactive metabolite that may covalently bind to protein may also produce active oxygen metabolites that may produce the toxicity. Thus, additional investigations are needed for many drugs to determine the role of covalent binding to protein in the toxicity.

Covalent Binding of Model Toxins: Bromobenzene, Acetaminophen, and Halothane

Brodie initially proposed a role of covalent binding of reactive metabolites to protein as the mechanism of drug-induced cytotoxicities (94). Using bromobenzene, a model compound for aromatic drugs that produces centrilobular hepatic necrosis, he and coworkers showed that covalent binding of radiolabeled compound to hepatic protein correlated with the hepatotoxicity of the chemical. It was postulated that bromobenzene was converted by cytochrome P-450 to a reactive metabolite, bromobenzene-3,4-oxide, which bound to protein. Evidence was presented that the metabolite either spontaneously rearranged to 4-bromophenol, was detoxified by glutathione to form a conjugate or by epoxide hydrolase to form a dihydrodiol, or the detoxification pathways were overwhelmed following a large dose and residues covalently bound to protein (95, 96).

The conceptual aspects of the toxicity of bromobenzene—metabolism to a reactive epoxide metabolite, detoxification by glutathione and covalent binding of the reactive epoxide to critical protein—have been an excellent model for research on many drug- and chemical-induced toxicities; however, the mechanism of metabolism and toxicity of bromobenzene may be more complicated than was originally envisioned. Work on the formation of glutathione conjugates from bromobenzene-3,4-oxide indicated that the epoxide was de-

toxified in vitro only in the presence of glutathione transferase and two isomeric glutathione conjugates, derived from reaction of the sulfhydryl group with the 3- and 4-positions of the epoxide, were formed (97). The necessity for glutathione transferase for the conjugation reaction indicated that the 3,4-epoxide metabolite was not highly reactive toward the sulfhydryl group and suggested that it may not be highly reactive to cysteine groups in protein, the presumed primary site of binding (97). Since bromobenzene is metabolized to multiple metabolites including hydroquinones and catechols, which may be oxided to species that bind to proteins in microsomal incubation mixtures, it has been difficult to determine the reactivity of the 3,4-epoxide to proteins (98–102). Monks and coworkers (103) showed that as much as 35% of the 3,4-epoxide metabolite diffused out of hepatocytes in in vitro incubation mixtures and was stable enough (half-life 13.5 sec) to diffuse into the blood of treated animals (103). However, in hepatocyte incubations, addition of purified glutathione transferase B to the incubation medium resulted in the metabolite binding to the protein (104). Thus, the epoxide does not appear to be highly reactive, but rather to covalently bind to selective proteins. Hanzlik and coworkers, using 14C-bromobenzene specifically labeled with tritium, presented evidence that in vitro and in vivo covalent binding of bromobenzene to protein was consistent with the primary amount of adducts being derived from the binding of quinones. They later developed methods for determining binding of specific adducts to sulfur groups in protein. It was shown that bromobenzene epoxides alkylate cysteine groups on protein, but account for only 0.4% of total covalent binding (105). Also, evidence was found for binding of both brominated and debrominated quinone metabolites to sulfur groups on protein (cysteine plus methionine) and four different adducts were detected. The binding of these quinone metabolites to protein accounted for only 6.7% and the majority of the adducts (approximately 92%) were unidentified (106). Thus, the hepatotoxicity of bromobenzene may be dependent upon two factors: glutathione depletion, which is mediated primarily by bromobenzene-3,4-epoxide conjugation with glutathione and covalent binding of various metabolites, and/or oxidative stress mediated via quinone-semiquinone metabolites.

Bromobenzene-induced nephrotoxicity appears to be mediated via different mechanisms from those mediating the hepatotoxicity. Bromobenzene and some of its metabolites produce a necrosis of the proximal convoluted tubules (107–114). Interestingly, the relative order of nephrotoxicity (weakly nephrotoxic to extremely nephrotoxic) has been shown to be: bromobenzene $<$ 2 bromophenol $<$ bromohydroquinone $<$ bromohydroquinone monoglutathione conjugate $<$ bromohydroquinone diglutathione conjugate. Oxidation of the hydroquinone metabolite(s) to the benzoquinone(s), which may bind to protein, appears to be critical in the production of the toxicity; however, the

role of each metabolite in producing the nephrotoxicity is unclear and the toxicity may be mediated by multiple metabolites. Interestingly, the glutathione conjugate(s) is very nephrotoxic (112–115). Administered mono- or diglutathione bromohydroquinone conjugates are metabolized by γ-glutamyl transpeptidase in the proximal tubules, the site of the highest concentration of this enzyme, resulting in accumulation of the adducts at this site (115, 116). The mechanism of oxidation of these conjugates to the benzoquinone derivatives is not known, nor is the specific protein or amino acids to which these derivatives bind. In addition, the benzoquinone cysteine conjugates may produce cyclized products via an intramolecular rearrangement of the quinone derivative to produce 1,4-benzothiazine, which is very insoluble (116, 117). The contribution of each metabolite to the nephrotoxicity is not clearly defined. Redox cycling and production of oxygen radicals is not believed to be important in the toxicity (115, 118, 119).

The hepatotoxicity of the analgesic acetaminophen appears to conform to the conceptual model developed by Brodie and coworkers to explain mechanisms of drug-induced toxicities (94). This commonly used drug produces a centrilobular hepatic necrosis in overdose in both humans and experimental animals (120, 121). In mice the drug is metabolized to a reactive metabolite by the cytochrome P-450 drug-metabolizing system (96, 120, 121). After a therapeutic dose the drug is detoxified by glutathione; after a toxic dose, however, glutathione detoxification is overwhelmed and the reactive metabolite covalently binds to protein. Covalent binding to protein correlates with the incidence and severity of the hepatotoxicity.

Many details of the metabolism of acetaminophen as it relates to the hepatotoxicity have been worked out. The drug is metabolized by cytochrome P-450 directly to the reactive metabolite N-acetyl-*p*-benzoquinone imine (96, 120, 122–124). The cytochrome P-450 species that catalyze this reaction in the human are cytochromes P-450IIE1 (ethanol inducible form) and P-450IA2 (125, 126). The reactive metabolite is a soft electrophile and undergoes a Michael-type addition reaction with the sulfhydryl of glutathione to form 3-(glutathion-S-yl)acetaminophen (127). Even though the nonenzymatic reaction is very rapid, in vivo the conjugation reaction is believed to be catalyzed by a glutathione transferase (128). Mass spectral analysis of hydrolysates of liver from mice treated with toxic doses of acetaminophen indicate that the major protein adduct was 3-(cystein-S-yl)acetaminophen (129).

The covalent binding of acetaminophen to proteins in liver has been analyzed by the use of immunological approaches. Hinson, Roberts and coworkers developed a quantitative immunoassay that specifically recognized 3-(cystein-S-yl)acetaminophen derivatives (130, 131). The dose and time relationships of acetaminophen binding to liver S-9 proteins were determined in mice (132). Of specific interest was the finding that lysis of hepatocytes

following toxic doses of acetaminophen to mice resulted in the release of acetaminophen protein adducts into the serum. The presence of these adducts in serum correlated with the increase in hepatic specific transaminase levels in serum (132). 3-(Cystein S-yl)acetaminophen protein adducts were also detected in the serum of patients who overdosed on acetaminophen and developed hepatic toxicity (133). The binding of acetaminophen to proteins in specific liver fractions was also analyzed in mice. Interestingly, binding was very high in hepatic plasma membranes (134), a postulated site of toxification (135), as well as in hepatic mitochondria (134), another postulated site of toxification (136, 137). Alteration of plasma membrane-mediated ion flux and mitochondrial function have been postulated to be important events in mediating the toxicity (135, 137, 138). Levels of binding to protein were low in microsomes (132). In addition, Hinson and coworkers and Cohen and coworkers, who have utilized a polyclonal antibody specific for acetaminophen, have investigated the proteins to which acetaminophen binds using immunoblotting techniques (139–142). Both groups determined that covalent binding occurs at high levels to an unidentified cytosolic protein with a molecular weight of approximately 55 kd, as well as to other unidentified proteins. None of the proteins to which acetaminophen binds have thus far been identified. Also, both groups have investigated covalent binding of acetaminophen to individual hepatocytes by using immunohistochemical approaches (143, 144). The data indicated that binding preceded the development of cytotoxicity and most hepatocytes with acetaminophen protein adducts became necrotic, although somc cells containing adducts did apparently survive (143). These data are consistent with a threshold of binding before toxicity develops, or other parameters may be important in the development of the toxicity.

Acetaminophen is also nephrotoxic in approximately 10% of severely poisoned patients (120, 145). Histologically the nephrotoxicity is a proximal tubular necrosis. Protein binding of acetaminophen has presented a confusing picture about the mechanism of the nephrotoxicity in humans. In Fischer rats acetaminophen is a nephrotoxin (146) and evidence indicates that the mechanism may be mediated via deacetylation of acetaminophen to 4-aminophenol, a potent nephrotoxin (147–149). Examination of binding of radiolabeled derivatives of acetaminophen indicated that more binding of ring-labeled acetaminophen occurred than with acetyl-labeled acetaminophen. It was postulated that the mechanism of the toxicity is deacetylation of acetaminophen, followed by oxidation of 4-aminophenol to a quinone imine metabolite that binds to protein (149, 150). However, evidence has been presented that in the mouse kidney the mechanism of metabolic activation is mediated via binding of N-acetyl-p-benzoquinone imine. Using immunoblotting procedures high levels of acetaminophen-protein adducts were detected in the proximal tu-

bules. Thus, the mechanism of nephrotoxicity in the mouse may be similar to that which is believed to occur in the liver (151). At present it is unclear what the mechanism is in humans or whether both mechanisms are important.

The hepatitis observed in some patients treated with the anesthetic halothane is believed to be mediated by covalent binding of a reactive metabolite to proteins during exposure and the subsequent development of antibodies against the protein halothane adducts. Additional exposure(s) causes a hypersensitivity reaction to develop that is manifested as a hepatotoxicity (2). Although halothane may be metabolized by reductive mechanisms (152, 153), oxidative metabolism to a reactive trifluoroacetyl halide metabolite is believed to be important in the toxicity (152, 154). This metabolite may acylate nucleophilic amino acids on protein including lysyl groups to produce trifluoroacetyl-lysine-protein adducts. Pohl and coworkers (155), using an immunological assay specific for trifluoroacetylated proteins, showed that treatment of rats with halothane resulted in trifluoroacetylation of a number of microsomal proteins, including a phenobarbital-inducible form of cytochrome P-450. It was later established that patients with halothane hepatitis had antibodies that recognized the trifluoroacetylated peptides (156–158). In immunoblot analyses, sera from halothane hepatitis patients recognized microsomal proteins covalently modified with trifluoroacetyl groups with the following molecular weights: 100 kd, 80 kd, 63 kd, 59 kd, 58 kd, and 57 kd. Some proteins have been isolated, sequenced, and identified by comparing the sequence to that of known proteins. A major protein which is trifluoroacetylated during halothane metabolism is microsomal carboxylesterase (M_r 59 kd) (159). In addition, the 57-kd microsomal protein, protein disulfide isomerase, is also trifluoroacetylated (160). The mechanism governing the exposure of these and other microsomal proteins to the immune system is not understood; however, luminal transport out of the endoplasmic reticulum to the outer surface of the hepatocyte may occur (158). Immunologically active proteins have been detected on the outer surface of the hepatocyte (2, 161).

Covalent Binding of α,β-Unsaturated Carbonyl Compounds

α,β-Unsaturated ketones and aldehydes are some of the more ubiquitously occurring reactive compounds that covalently bind to protein. These compounds are soft electrophiles (Table 1) and may react at the β carbon with soft nucleophiles such as the sulfhydryl groups on cysteine. The reaction is a Michael-type addition. The simplest member of this series is acrolein (2-propenal). Its toxicity and a number of precursors that are metabolized to acrolein have been attributed to its reactive properties (162). These compounds include allyl alcohol, which produces a periportal hepatic necrosis and allylamine, a cardiac toxicant, allyl formate, and the chemotherapeutic agent

cyclophosphamide (163–166). In vitro, acrolein has been reported to bind to, or inhibit the following enzymes: DNA methylase (167), cytochrome P-450 (168), NADPH-cytochrome P-450 reductase (169), aldehyde dehydrogenase (170), and those involved with arachidonic acid metabolism (217).

The toxicity of a number of furans may be mediated via α,β-unsaturated aldehyde metabolites. In experimental animals large doses of the following furan derivatives are very toxic: the solvent furan, the drug furosemide (172), the moldy sweet potato toxin 4-ipomeanol (173), the environmental toxin 2-methylfuran (174), and menthofuran, a metabolite of pulegone from pennyroyal oil (175, 176). The sites of toxicities include liver, lung, and kidney, and protein binding correlates with the site of toxicity and presumed metabolism. Metabolism studies with 2-methylfuran indicated that the metabolite that covalently bound to protein was 4-oxo-2-pentenal and was given the trivial name acetyl-acrolein. The mechanism of formation was postulated to be rearrangement of a metabolite or intermediate formed by the cytochrome P-450 mixed function oxidase (presumably a hydroxy or epoxide metabolite) (177, 178). Another furan derivative that covalently binds to proteins in microsomal incubation mixtures in vitro is 8-methoxypsoralen. Cysteine was effective in inhibiting covalent binding and SDS gel electrophoresis coupled with a fluorographic procedure indicated that most binding was to proteins in the 50–56-kd range (179). Additional work will be required to determine if the metabolic intermediates that bind to protein of all furans are mediated by mechanisms similar to 2-methylfuran.

Quinones are also α,β-unsaturated keto derivatives and many of these derivatives may become covalently bound to protein. These metabolites are formed by two-electron oxidation of the corresponding dihydroxylated aromatic derivatives. The 1,4-dihydroxylated aromatic compounds are called hydroquinones and are oxidized to the corresponding p-benzoquinones, whereas 1,2-dihydroxylated aromatic derivatives are called catechols, and may be similarly oxidized to o-quinones. Quinones have been postulated to be the toxic metabolites of various compounds (180). Peroxidases are known to catalyze these oxidation reactions (181, 182) in vitro, although their role in the metabolism of these compounds in vivo is not clearly understood. With some derivatives evidence has been presented that quinone formation is via oxidation by other free radicals; however, a thorough understanding of the general importance of these mechanisms awaits further investigation (183).

As discussed above, bromobenzene is metabolized to 2-bromo-p-benzoquinone and apparently p-benzoquinone, which covalently bind to cysteine groups in proteins (106). By a similar mechanism the reactive metabolite of acetaminophen, N-acetyl-p-benzoquinone imine, an α,β-unsaturated imine derivative, reacts with cysteine groups on protein to form 3-(cystein-S-yl)acetaminophen protein adducts (123, 127, 129). Both o- and p-benzo-

quinones may also be formed from benzene and phenol, via oxidation of the catechol and hydroquinone metabolites, respectively. These compounds may react with proteins to produce adducts, and with cysteine and glutathione to form conjugates (184–186). In addition, p-benzoquinone is a hydrolysis product of N-acetyl-p-benzoquinone-imine, the reactive metabolite of acetaminophen, and is metabolized to cysteine- and N-acetyl-cysteine-hydroquinone conjugates and binds to hemoglobin in mice (187, 188). Another quinone metabolite that covalently reacts with protein is 4'4''-diethylstilbestrol quinone, the two-electron oxidation product of diethylstilbestrol, which has been shown to bind in vitro to albumin (189). The quinone metabolite is postulated to be causative in the carcinogenicity of this synthetic estrogen (189, 190). Other toxic quinones that have been reported to arylate protein thiols include: menadione (191, 192), tetrachloro-p-benzoquinone (metabolite of pentachlorophenol) (193, 194), urushiols (toxins from poison ivy/oak) (195), and o-catechol estrogens (196, 197).

Quinone methides are similar to quinones in their reactivities. These derivatives are the two-electron oxidation products of 2- or 4-methylphenols or related derivatives and the products 2-methylene-3,5-cyclohexadienone and 4-methylene-2,5-cyclohexadienone, respectively. One of the most important compounds that is metabolized to a quinone methide is the food additive butylated hydroxytoluene or BHT (2,6-di-*tert*-butyl-4-methylphenol). Although generally regarded as safe, at high doses this compound is a hepatotoxin and a pneumotoxin, and has been found to be a carcinogen in experimental animals (199). Peroxidation of the compound leads to an electrophilic quinone methide that may react with cysteine, glutathione, or covalently bind to protein (200, 201). Furthermore, BHT reportedly functions as a tumor promoter (202). Kensler's laboratory recently reported that in a skin-tumor promotion protocol in mice the quinone methide can elicit the promotion stage of carcinogenesis. It was hypothesized that covalent interaction of the quinone methide with critical sulfhydryl groups or other critical nucleophiles on protein occurred in the keratinocyte. Modification of these proteins, which are postulated to be functionally involved in the control of second-messenger signals (i.e. calcium ions), may provide enhanced growth stimulus to initiated cells (203).

Eugenol is another compound that is metabolized to a quinone methide-type reactive metabolite. This food flavor and fragrance agent has been shown to be toxic to various cell types, including hepatocytes. It may be peroxidized to the quinone methide derivative that reacts with glutathione to form a conjugate or covalently binds to protein, presumably sulfhydryl groups (204–206). No studies to date have examined whether o- or p-cresol is oxidized to the corresponding quinone methides in biological systems or determined whether they can bind to protein. However, toluene is metabolized to both

compounds and has been reported to bind covalently to protein in microsomal incubation mixtures and liver slices. Moreover, the binding is greatly decreased by incubation with either ascorbate, cysteine, or superoxide dismutase but not lysine. These data are consistent with binding via formation of cresol, or possibly hydroquinone or catechol and its further oxidation to a quinone methide or quinone metabolite that binds to protein (207, 208).

The imine methide (methylene imine) formed from 3-methylindole is also believed to be a reactive toxic metabolite. 3-Methylindole, is a degradation product of tryptophan and is present in cattle and human feces. It is a pneumotoxin and believed to be oxidized by the lung microsomal mixed function oxidase system to the imine methide (the two-electron oxidation product), which covalently binds to protein. The glutathione conjugate has been identified, but the nature of the protein-bound adducts is not known (209–212).

Covalent Binding of Halogenated Compounds

Halogenated aromatic hydrocarbons represent a major class of protein-binding compounds. The metabolism and covalent binding of bromobenzene is discussed above. In addition, as stated above, pentachlorophenol, a metabolite of hexa-chlorobenzene, has been recently reported to be metabolized to tetra-chloro-p-benzoquinone, a highly reactive species that may covalently bind to protein. The mechanism was postulated to be loss of a chloride anion from the microsomal 3,4-epoxide metabolite of penta-chlorophenol (193, 194). Other halogenated aromatic derivatives may covalently bind to proteins, e. g. chlorobenzene is hepatotoxic when administered in large doses to rats. The mechanism is believed to be similar to that of bromobenzene (213). A number of polyhalogenated aromatic derivatives are known to be metabolized to derivatives that bind to protein. These include o-dibromobenzene (214) and certain polychlorinated aromatic hydrocarbons (215).

Halogenated aliphatic hydrocarbons are another major class of halogenated compounds that may be metabolized to species that become covalently bound to protein. These include carbon tetrachloride, chloroform, vicinal dihaloalkanes, and haloalkenes. Though structurally related, the metabolism and binding to protein of these derivatives presents a complex array of mechanisms. Carbon tetrachloride has been reported to bind to various hepatic protein fractions, including nuclear proteins (216). Studies using radiolabeled carbon tetrachloride under anaerobic conditions have demonstrated that covalent binding occurs to a number of microsomal proteins. SDS gel electrophoresis coupled with fluorography revealed radiolabeled proteins in the M_r range of 52, 54, and 51–62 kd (217). Other investigators identified a 54-kd cytochrome P-450 as a specific protein to which carbon tetrachloride

became bound (218). Investigation of the adduct indicated that the trichloromethyl free radical metabolite of carbon tetrachloride became bound to the heme moiety. Heme adduction resulted in histidine residue 93 of the cytochrome P-450 becoming covalently attached to the heme moiety. The resulting adducted heme protein can undergo a redox cycling, as evidenced by its facile reduction of molecular oxygen and carbon tetrachloride (219, 220). This action has been termed "suicide activation" (220).

Oxidative metabolism of chloroform is believed to be the mechanism of its conversion to a metabolite that binds to protein. Pohl and coworkers presented evidence that phosgene is an electrophilic metabolite. This metabolite is believed to be formed by cytochrome P-450 catalyzed hydroxylation to form trichloromethanol, followed by a dehydrochlorination (221). This acyl dihalide is extremely reactive toward amino groups and sulfhydryl; however, the nature of the specific adducts that are formed with protein have not been characterized. Fluorography of bound radiolabeled chloroform in rat liver microsomes indicated that binding was primarily to proteins of M_r 52 and 56 kd and secondarily to protein of M_r 62 and 73 kd. Reductive metabolism was also suggested to produce protein adducts (222).

Vicinal dihaloalkanes are activated to reactive metabolites that covalently bind to protein (223). Many of these compounds have been reported to be carcinogens or mutagens, and most are cytotoxic. 1,2-Dichloroethane and 1,2-dibromoethane, may produce a diversity of toxicities: nephrotoxicity, hepatotoxicity, testicular toxicity, mutagenesis, and carcinogenesis. These derivatives may be metabolized by microsomal mixed function oxidase and glutathione conjugation mechanisms to species that covalently bind to protein and DNA (224–226). Whereas the oxidative binding is believed to be mediated by the haloacetaldehyde, glutathione conjugation leads to a glutathione haloethane adduct that may spontaneously rearrange to produce the episulfonium ion, an alkylating agent. This hard electrophile may alkylate DNA and produce a different amino acid binding profile than the one expected from the soft electrophile chloroacetaldehyde (224, 227, 228). Administration of disulfiram to rats to induce hepatic glutathione transferase levels followed by administration of radiolabeled 1,2-dichloroethane increased hepatotoxicity, hepatic DNA binding of radiolabel and hepatic protein binding at 24 hr (229, 230). Whole body autoradiography of mice administered 1,2-dichloroethane revealed binding to liver, kidney, nasal olfactory mucosa, and the tracheobronchial epithelium (231). The importance of glutathione-mediated versus mixed function oxidase catalyzed binding in the nasal mucosa and the tracheo-bronchial epithelium is not known.

The nephrotoxicity of various haloalkenes is believed to be mediated by reactive metabolites formed from the corresponding cysteine conjugates (223, 232–234). The conjugate dichlorovinyl cysteine, a product of the extraction

of soybean meal with trichloroethylene, provided insight into the mechanism of the nephrotoxicity of these derivatives. This conjugate was found to be a potent nephrotoxin in mice (235) and it underwent cleavage by a lyase to produce a reactive thiol, pyruvate, and ammonia (236). Further research has determined that the mechanism is applicable to other nephrotoxic haloalkenes. The generalized scheme involves initial hepatic glutathione conjugation of the haloalkene, conversion to the cysteine conjugate, and metabolism of the cysteine conjugate in the kidney by the enzyme cysteine conjugate β-lyase to produce the corresponding thiol derivative. The thiol derivative is unstable and may spontaneously rearrange to produce a thioacylating metabolite that binds to protein. Various halogenated alkenes may be metabolically activated by this mechanism: tetrafluoroethylene (237), chlorotrifluoroethylene (238), 1,1-dichloro-2,2-difluoroethylene (239), hexafluoropropene (240), trichloroethylene (241), tetrachloroethylene (242), hexachloro-1,3-butadiene (243) as well as dichloroacetylene (244). The nature of the toxicity (cytotoxicity of the proximal tubular cells versus genotoxicity) varies with the compound. With fluoroalkenes the corresponding halogenated alkane conjugates are formed, whereas with the chloroalkenes the corresponding vinylic conjugates are formed. Two reactive thioacylating metabolites have been postulated: the thioacyl halide may be formed from both conjugates, and the corresponding ketene derivative may also be formed with the vinylic conjugates (232–234). With the model compound dichlorovinyl cysteine, administration of the [14C]vinyl compound to mice followed by whole body autoradiography and microautoradiography showed selective binding in the straight portion of the renal proximal tubules. The primary binding site was observed to be the site of toxicity and presumed activation; however, a lesion unrelated to binding was also observed (245–246).

Other Covalent Binding Reactions

Many thiono-sulfur compounds may be activated to reactive metabolites that covalently bind to protein (for review, see Ref. 247). Examples of this class of chemicals that are toxic in animals are thioacetamide, carbon disulfide, thiourea, and α-naphthylthiourea. The drugs propyl-thiouracil and methimidazole are members of this class of chemicals. A broad spectrum of toxicities have been observed: bone marrow depression, liver toxicity, lung toxicity, and cancer. Depending upon the particular derivative, these compounds may be metabolically activated by either cytochrome P-450 and/or microsomal flavin-containing monooxygenase to derivatives that result in covalent binding of sulfur and/or other portions of the molecule to protein (247). Carbon disulfide, a hepatotoxin in rats, has been shown to be metabolized to produce a protein hydrodisulfide-type derivative (sulfur bound to cysteine) (248). However, thioacetamide, another hepatotoxin, covalently binds to protein as

N-ε-acetyllysine. The mechanism is believed to be metabolism of thioacetamide to thioacetamide S-oxide by microsomal flavin-containing monooxygenase and subsequent oxidation to the reactive acylating metabolite (249, 250). Other thiono sulfur compounds such as α-naphthylthiourea are pulmonary toxins and mechanisms of toxicity are also believed to be related to reactive metabolite that may covalently to protein (251).

The lung toxicities produced by a number of chemicals have been attributed to reactive metabolites that covalently bind to protein. The toxicities of 3-methylindole (209), 4-ipomeanol (173), and α-naphthylthiourea (251) have been previously discussed. In addition, the pneumotoxicity, as well hepatotoxicity, of the pyrrolizidine alkaloid monocrotaline, a product derived from plants, is believed to be mediated by hepatic metabolism to a compound that binds to protein and conjugates with glutathione (209). The covalent binding of radiolabeled monocrotaline to various tissues has been examined in the rat and the structure of the glutathione conjugate determined by mass spectral methods (252, 253). A potentially useful assay for covalent binding of pyrrolizidine alkaloids to protein as sulfur conjugates has recently been developed by Mattocks & Jukes (254). These investigators showed that the thioether bond could easily be broken by treatment of the bound adducts with silver nitrate. Subsequently, the pyrrolic derivative could be extracted by solvents and analyzed by chromatographic methods. These methods may be applicable to other protein sulfur bound adducts.

Buckpitt and coworkers have examined the mechanism of the pulmonary toxicity of naphthalene. This work has revealed that mice are very sensitive to this toxicity and metabolized naphthalene stereoselectively to 1R,2S-naphthalene oxide. Other species were not sensitive to this toxicity and metabolized naphthalene to form the 1S,2R-naphthalene oxide, in addition to lesser amounts of the 1R,2S-naphthalene oxide (209). Both metabolites may be detoxified to form glutathione conjugates. It is unclear whether binding of the 1R,2S-naphthalene oxide metabolite to protein in lung (255) is responsible for the toxicity. It is known that 1-naphthol, a rearrangement product of the 1R,2S-naphthalene oxide (255), covalently binds to protein and the mechanism appears to be via quinones (256). However, 1-naphthol is not a lung toxin even though covalent binding to protein of 1 naphthol occurs in vivo at levels similar to those observed following naphthalene administration to mice. These findings have been interpreted to indicate that 1-naphthol is not an obligate intermediate in naphthalene-induced lung toxicity (257).

Many hydrazine derivatives are reportedly metabolized to derivatives that covalently bind to protein. As discussed above, administration of the colon carcinogen 1,2-dimethylhydazine to rats results in methylation of lysine, arginine, and histidine residues of histones and DNA-binding proteins of colonic epithelial cells (43). The hepatic toxicity of antituberculosis drugs

isoniazid and iproniazid has been postulated to be via metabolic activation to reactive metabolites that covalently bind to protein (258).

The neurotoxicity and testicular atrophy produced by the commonly used solvents hexane and methylbutyl ketone have been attributed to their metabolism to derivatives that bind covalently to protein. The proximate toxic metabolite with both solvents is 2,5-hexanedione, which reacts with the ϵ-amino groups of lysine on proteins and cyclizes to form pyrroles. Pyrrole oxidation and protein cross-linking may be involved in the toxicities (259, 260). The importance of binding to tubulin and its altered role in microtubule assembly has been examined as an important parameter in this toxicity (261, 262).

Both alkyl and aryl isocyanates are electrophilic and have been reported to react with various proteins in vitro. The primary nucleophilic sites on proteins that these derivatives may react are: ϵ-amino group of lysine, hydroxyl group of serine, sulfhydryl group of cysteine, and amino terminal groups (263). Acute and chronic toxicities have been reported and both may be related to protein binding. Methyl isocyanate is the chemical responsible for the poisoning disaster in Bhopal, India, in 1984. Like certain other isocyanates, it inhibits choline esterase activity in vitro (263). Administration of radiolabeled compound to rats resulted in binding to amino groups in liver, kidney, lung, and brain protein and specific binding to N-terminal groups in hemoglobin (264, 265). Toluene diisocyanate is an economically important member of the isocyanates because of its use in the plastics industry, specifically in the production of polyurethane foam. A primary human toxicity of toluene diisocyanate, as well as certain isocyanates is a pulmonary sensitivity. This toxicity occurs in previously exposed individuals and is believed to be an immunological response. A protein reportedly derivatized by toluene diisocyanate in the guinea pig is serum albumin (266--268). Recent metabolic evidence suggests that certain formamides such as the hepatotoxin N-methylformamide (268, 269) and N-(1-methyl-3,3-diphenylpropyl)formamide (271) may be metabolized to isocyanates that may covalently bind to protein or react with glutathione.

Other chemicals have been reported to covalently bind to protein. One important example is the drug chloramphenicol, which covalently binds as N-ϵ-chloramphenicol oxamyl lysine to cytochrome P-450 during its in vitro metabolism in a reconstituted system. Binding was associated with inactivation of enzyme activity. It was postulated that the reactive metabolite was the corresponding oxamyl chloride (272). Other examples include the solvent acrylonitrile (273), the tricyclic antidepressant amineptine (274, 275), phenytoin (276), valproic acid (277, 278), and zomepirac glucuronide (279). The covalent binding of zomepirac glucuronide is mechanistically interesting since it is believed to occur by isomerization of the glucuronide and formation

of an imine linkage between the glucuronic acid moiety of the conjugate and a lysine or terminal amino group on the protein.

Other types of protein covalent binding reactions are important in toxicology. One of these, termed "suicide destruction", is inhibition of cytochrome P-450 activity by a reactive metabolite that covalently binds to heme (for reviews see Refs. 280, 281). Coupled with heme binding, porphyria and disruption of heme biosynthesis may occur (282). Briefly, compounds such as olefins, acetylenes, and certain heteroatomatic substrates are metabolized to derivatives that bind to heme of cytochrome P-450. The olefins described to inactivate cytochrome P-450 include vinyl halides, ethylene, propene, fluroxene, 2-isopropyl-4-pentenamide, and secobarbital, to list a few. The acetylenes include acetylene, propyne, ethinyl estradiol, and norethisterone. 1-Aminobenzotriazole and phenylhydrazine are two of the heteroatomic compounds that bind to heme. The species that bind to heme often appear to differ from those that may bind to the primary structure of the protein. Frequently, the binding to heme occurs on the cytochrome P-450 species that metabolized the compound, and thus is a mechanism-based inactivation. Another important type of covalent binding reaction involves the organophosphate esterase inhibitors (see Ref. 283).

Reversible Covalent Adducts

Aldehydes are a major group of chemicals that form reversible covalent adducts with proteins. A major source for exposure of these chemicals is the oxidation of alcohols by the enzyme alcohol dehydrogenase, contained in high concentrations in the liver. Another source of exposure to aldehydes within the body is the cytochrome P-450-catalyzed N-, O-, or S-demethylation or deethylation of drugs and other chemicals. Formaldehyde and certain other aldehydes have commercial applications and, being volatile, may lead to human exposure. The carbonyl group of aldehydes is electrophilic and may react with lysyl amino groups on protein to produce Schiff bases. Schiff bases may have relatively long half-lives; however, reduction (i.e. borohydride) is required to produce a stable derivative. Aldehydes may also react with sulfhydryl groups to produce unstable thiohemiacetals. Thus, formaldehyde has been reported to deplete protein sulfhydryl groups in rat hepatocytes (284). The covalent binding of acetaldehyde to protein has been widely studied. Acetaldehyde is the aldehyde produced by oxidation of ethanol by hepatic alcohol dehydrogenase. In in vitro incubations aceteldehyde has been shown to react with numerous proteins including serum albumin (285) and collagen (286), and to inhibit the activities of glucose-6-phosphate dehydrogenase and RNAase, enzymes that have lysyl residues at their active sites (287). Analysis of liver proteins from ethanol-treated rats using immunoblot procedures and an antiacetaldehyde-specific immunoglobin re-

vealed acetaldehyde bound to cytochrome P-450IIE1 (288). Other investigators have shown that in rats a major protein containing acetaldehyde-protein adducts is an unidentified cytosolic 37-kd protein (289). In humans, immunoassays have revealed high titers of acetaldehyde protein adducts in serum of alcoholics (290) and immunohistochemical assays have shown acetaldehyde-protein adducts in the centrilobular region of the liver (291). Another reversible reaction of compounds with proteins is the reaction of the alkyl isocyanates with cysteine (263).

Immunological Consequences of Protein-bound Adducts

This area has been recently reviewed (2, 292). Briefly, it is well known that certain chemicals react directly with proteins, and that these adducts may serve as immunogens. Also, metabolic activation to form a chemical-protein adduct has more recently been recognized as an important determinant. In both cases these interactions may result in various immunological toxicities: hypersensitivity reactions, cytotoxicities, formation of immune complexes, or delayed hypersensitivities. In addition, chemical protein adducts may be important in the development of some autoimmune diseases (292).

Chemicals known to react directly with protein to produce adducts and immunological consequences include: halodinitrobenzenes, penicillin breakdown products, cephalosporin breakdown products, captopril, penicillamine (292), and toluene diisocyanate (266). Specific chemical-protein adducts have been conclusively identified with some chemicals. Administration of fluoro-2,4-dinitrobenzene to rats resulted in metabolites consistent with detoxification via the glutathione pathway and a long-lived metabolite which was identified as an acetylated lysine with the dinitrophenyl group attached to the ϵ-amino group. The latter metabolite was postulated to be derived from 2,4-dinitrobenzene covalently bound to lysine groups in proteins (293). Penicillin is one of the most extensively studied compounds that produces immunological consequences. It has been established that the major antigenic determinant is reaction of the β-lactam carbonyl of penicillin with ϵ-amino groups of lysine in protein and a minor determinant is the formation of disulfide bonds between proteins and a penicillin metabolite (294). Similarly, captopril and penicillamine covalently bind to protein via the formation of disulfide linkages (292).

Other chemicals must be metabolically activated to produce proteins adducts that may function as immunogens. Two major examples have been previously discussed: halothane (155–161), and urushiols (195, 196). Metabolic activation may be important with many aromatic amines, including drugs. Procainamide has been studied extensively and the mechanism, as has been postulated to occur with other aromatic amines (13), may be via N-hydroxylation and conversion to the nitroso derivative that may bind to

protein (295, 296). The hepatotoxicity of the drug tienilic acid may have an immunological mechanism. Patients with hepatitis induced by this drug produced antibodies against a cytochrome P-450 involved in the hydroxylation of this drug (297). Subsequent research has determined that the drug is metabolized by human microsomal enzymes to metabolites that covalently bind to protein (298). Other drugs that promote metabolic activation and protein binding have been postulated to be important in their immunological toxicities: these include hydrazaline, practolol, phenytoin, amodiaquine, ethynylestradiol, and propylthiouracil (292, 299, 300).

EFFECT OF OXIDATIVE STRESS ON PROTEINS

Some toxicities are mediated by mechanisms termed "oxidative stress". By this mechanism reactive oxygen metabolites interact with cellular constituents to produce toxic effects such as glutathione depletion, alteration in redox balance, and lipid peroxidation. Three reactive oxygen species have been implicated in oxidative stress: superoxide anion, hydrogen peroxide, and hydroxyl radical. The formation of these species is interrelated: superoxide anion may dismutate (enzymatically or nonenzymatically) to hydrogen peroxide plus oxygen, and hydroxy radical may be produced by interaction of hydrogen peroxide or superoxide anion with iron ions by a Fenton reaction, or a Haber-Weiss reaction. Each species is an oxidizing agent and may contribute to the oxidative stress reaction; however, hydroxyl radical is extremely reactive and may be the primary toxic metabolite. Reactive oxygen species are formed from certain enzyme processes or may be formed from redox cycling compounds such as paraquat, nitrofurantoin, and adriamycin. The mechanism of redox cycling is reduction of the compound by an endogenous reductant (i.e. NADPH) to an intermediate such as a free radical that is subsequently oxidized by molecular oxygen with concomitant production of superoxide anion and regeneration of the parent compound. Under conditions of glutathione depletion, the toxicities of these compounds are enhanced because detoxification of peroxides by glutathione peroxidase is diminished. Reported adverse effects include lipid peroxidation, mitochondrial damage, alterations in calcium metabolism, and cell death. With certain other compounds it is believed that metabolism may produce a derivative that may undergo redox cycling to produce oxidative stress. This stress reaction may be exacerbated by depletion of glutathione (for reviews see Refs. 301, 302).

Oxidative stress produces mixed disulfides of proteins with low molecular weight thiols such as glutathione (303). Sies et al used thin gel isoelectric focusing to show that a 30-kd protein in rat liver cytosol underwent S-thiolation with glutathione disulfide (304). In hepatocytes it was shown that *t*-butyl hydroperoxide and diamide produced rapid S-thiolation with this

protein and rapid repair; however, the redox cycling compound menadione produced a slow sustained rise in the level of S-thiolation during the course of the incubation (304). It was subsequently determined that the protein was carbonic anhydrase III (305). Isolated cultured heart cells have likewise been shown to undergo S-thiolation, and two different enzymes, creatine kinase and glycogen phosphorylase are modified (306–308).

Many investigators have been pursuing the role of protein oxidation by reactive oxygen species. Stadtman and coworkers have been examining the importance of protein oxidation in age-related changes in microorganisms and have found that certain enzymes such as glutamine synthetase in *E. coli* may be inactivated by reactive oxygen species. The mechanism may be formation of a hydroxyl radical on the enzyme and subsequent oxidation of histidine 269 or of arginine 344, both situated at the metal binding site on the enzyme. The oxidation products are aldehydes and oxidation may increase susceptibility to proteolysis (309, 310).

The importance of oxidative stress on protein oxidation has been examined in young and old rats. It was determined that 26-month old rats had hepatic protein aldehyde residues threefold greater than 3-month old rats. Exposure of the rats to a 100% oxygen atmosphere did not alter hepatic protein aldehyde residues in the 3-month old animals; however, similar treatment of the 26-month old rats resulted in a threefold increase in protein aldehyde residues (311).

Methods for quantitation of protein oxidation have used aldehyde/keto-specific reactions such as reaction with 2,4-dinitrophenylhydrazine or reduction with tritiated sodium borohydride (312, 313). These approaches have been widely employed to study the role of protein oxidation in various pathological conditions such as myelinolysis (314) and muscular dystrophy (315). At present information on the relationship between protein oxidation and the toxicity of chemicals is limited.

CONCLUSIONS

The mechanisms by which covalent and noncovalent interactions may produce cell toxicity are not clearly understood. Chemical protein adducts have been shown to occur with a large number of toxic compounds. Quantitative amounts of adducts have been determined in various organs, subcellular fractions, and in tissues distant from the site of metabolic activation. Specific binding sites on proteins have been determined with many chemicals. Coupled with this binding data, correlative data have been obtained with various biochemical parameters: calcium metabolism, mitchondrial function, and membrane alterations to list some of the more active areas. However, it is still uncertain if reactive metabolites inhibit one critical enzyme, or if inhibition of

multiple enzymes may contribute to the development of some toxicities, or the role of oxidative stress in the toxicities.

An extensive body of data now exists on the specific amino acids to which various toxins bind. These data indicate that known carcinogens are generally observed to bind to the hard nucleophilic amino acids, while the majority of the noncarcinogenic toxins are observed to bind to the sulfhydryl groups of cysteine. As reported in this review, the reactive metabolites of a relatively large number of compounds that produce cell toxicity are α,β-unsaturated aldehydes or ketones, or related derivatives. Moreover, current data suggest that epoxides may not be as important as was once envisioned.

Many excellent analytical procedures have been used to study the binding of reactive metabolites to specific amino acids. Immunological methods have been used with a high degree of success to evaluate covalent binding of drugs to protein. SDS gel electrophoresis coupled with immunostaining has revealed with both acetaminophen and halothane that only a limited number of proteins contain high levels of adducts. Previously it was generally assumed that binding occurred indisciminately; this may not, however, be the case. Also, these methods may be used to study human toxicities. Moreover, these approaches have been coupled with protein purification, sequence analysis, and comparison to previously reported proteins to identify some proteins to which halothane binds. This approach in future studies may be extremely valuable in determining the mechanisms of many chemically induced toxicities.

Protein oxidation may be an important area for future research. Oxidative stress is well characterized as a major mechanism of many chemical toxicities, but its effects on proteins are not known. The pioneering work on protein oxidation in microorganisms in Stadtman's laboratory indicates that hydroxyl radicals oxidize amino groups on proteins to aldehydes. If this occurs in chemically induced oxidative stress, it may be another parameter to evaluate. Moreover, if protein oxidation is applicable, then covalent binding and oxidative stress may be mechanistically related to alteration in critical enzyme activities.

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