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COMMENTARY

A PERSPECTIVE ON THE ROLE OF CHEMICALLY REACTIVE METABOLITES OF FOREIGN COMPOUNDS IN TOXICITY—I CORRELATION OF CHANGES IN COVALENT BINDING OF REACTIVITY METABOLITES WITH CHANGES IN THE INCIDENCE AND SEVERITY OF TOXICITY

JAMES R. GILLETTE
Laboratory of Chemical Pharmacology, National Heart and Lung Institute, National Institutes of Health, Bethesda, Md. 20014, U.S.A.

DURING the past several years it has become increasingly evident that chemically inert foreign compounds can be converted in the body to chemically reactive metabolites that combine with tissue macromolecules and thereby cause carcinogenesis[1–4] and mutagenesis.[5,6] In addition, Brodie[7] and others[8–11] have raised the possibility that drugs as well as environmental toxicants may evoke other serious kinds of toxicities including cellular necrosis, hypersensitivity reactions, blood dyscrasias and fetotoxicities through the formation of reactive metabolites. Because of the seriousness of these toxicities, it has become important to develop rapid test systems to elucidate which kinds of reactive metabolites mediate toxicities and which are innocuous. Our laboratory has approached the problem by identifying toxicities caused by various drugs and other foreign compounds and determining whether the toxicities are mediated by reactive metabolites. The present commentary describes the assumptions on which this approach is based.

Correlation of changes in covalent binding with changes in the incidence and severity of toxicities

In previous studies of the mechanisms of toxicities mediated by chemically reactive metabolites, various laboratories have assumed that the toxicity results from the covalent binding of the metabolite with a single kind of target substance. They have, therefore, expended considerable effort in attempts to identify the target substance that mediates the toxicity. However, in the development of a general test system that would be useful in studies of different kinds of toxicity, this approach seemed fruitless for a number of reasons. It seemed obvious to us that the target substance would depend on the toxicity being studied; some toxicities may be mediated by the covalent binding of the reactive metabolite to nuclear DNA, others may be mediated by the covalent binding to lipids or to certain enzymes. Moreover, in some kinds of toxicity, such as tissue necrosis and hypersensitivity reactions, the target substance may be any one of a number of different intracellular components and indeed may differ

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with the reactive metabolite. For example, many investigators believe that the chemically reactive metabolite of carbon tetrachloride causes liver necrosis by combining with phospholipids in the endoplasmic reticulum and thereby promoting lipid peroxidation.[9–11] But many other substances that also cause centrilobular liver necrosis, such as bromobenzene, do not promote lipid peroxidation[12] and therefore cannot cause liver necrosis by this mechanism. Moreover, we were also cognizant that the specificity of covalent binding of reactive metabolites to macromolecules can vary markedly with the reactive metabolite being studied. At one extreme of the spectrum, some reactive metabolites, particularly those having relatively low chemical reactivities, may become preferentially bound to certain macromolecules in tissues by first combining reversibly with active centers on a specific macromolecule to form a complex that rearranges to form a covalently bound conjugate. Indeed, this mechanism is the basis of affinity labeling of receptor sites by chemically reactive analogues of endogenous chemical mediators[13] and of the preferential inhibition of choline esterases by organophosphate insecticides and their precursors.[14] In these situations, where relatively few macromolecules are covalently bound to the metabolite or where the toxicity mimics well characterized pharmacologic actions, the identification of the target substance is relatively easy. At the other extreme, however, highly reactive metabolites of foreign compounds combine indiscriminately with many different kinds of intracellular components including protein, lipids, glycogen, DNA and RNA.[1–4] In most instances, however, the relative rates of covalent binding to different kinds of macromolecules vary with the reactive metabolite and the tissue. Thus, the identification of the target substance can be very difficult. Indeed, when a reactive metabolite interacts with a number of biochemical systems simultaneously, it is difficult to determine whether changes in cell function result from a sequence of changes originating from a single biochemical alteration or from the concerted action of a number of different initial biochemical alterations.

Because highly reactive metabolites can react with so many different kinds of macromolecules in tissues, and because we know so little about the mechanisms by which a given metabolite–macromolecular conjugate might lead to diverse toxicities and much less about how combinations of metabolite–macromolecular conjugates might evoke toxicities, there seemed to be little reason for selecting any given type of macromolecule as the basis of a general test system for determining whether a toxicity caused by a given foreign compound is mediated by a reactive metabolite. It also seemed apparent that the finding of covalently bound radiolabel to tissue macromolecules after the administration of a radiolabeled foreign compound would not be sufficient proof that a reactive metabolite mediated the toxicity under investigation or any other toxicity.

Nevertheless, it seemed likely that the incidence and severity of any toxicity mediated by a chemically reactive metabolite would be roughly proportional to the number of target macromolecule–metabolite conjugates formed in the tissue after the administration of the foreign compound. It also seemed likely that changes in the concentration of the reactive metabolite within a given tissue would alter not only the rate of covalent binding of the reactive metabolite to the target macromolecule but also its rate of covalent binding to other macromolecules. Thus, treatments of animals that alter the pattern of metabolism of the toxicant should cause parallel changes in the amount of covalent binding to both the target macromolecules and

other macromolecules and in the incidence and severity of the toxicity. According to this view, it would not be necessary to identify either the reactive metabolite or the target macromolecule in order to determine whether the toxicity was mediated by a chemically reactive metabolite, an inert drug or inert metabolites. Indeed, with highly reactive metabolites it may not even be necessary that the target substance be present in the sample being assayed for covalent binding.

When target macromolecule–metabolite conjugates are not rapidly replaced by new target macromolecule, or new subunits of the macromolecules, the maximum amount of the conjugate formed in any given tissue will occur after the parent compound and its reactive metabolite have left the body. Thus, the time at which the covalent binding is determined becomes important in attempting to relate changes in the amount of macromolecule–metabolite conjugate with changes in the incidence and severity of the toxicity. Suppose, for example, that virtually all of a substance is converted to a reactive metabolite by a cytochrome P-450 enzyme in liver microsomes and virtually all of the reactive metabolite becomes covalently bound to a target macromolecular substance and that it cannot be eliminated in any other way, and suppose that the formation of the conjugate results in a toxicity. Measuring the amount of covalent binding of the substance to the macromolecule shortly after administration of the substance might suggest to the investigator that pretreatment of the animals with phenobarbital, which induces cytochrome P-450 enzymes, should increase the severity of the toxicity because the covalent binding at that time would be increased. In a similar manner, he might be led to believe that β-diethylaminoethyl diphenylpropylacetate (SKF 525-A) would decrease the toxicity, because the covalent binding at that time would be decreased. In this hypothetical situation, however, neither of the treatments would have altered the severity of the toxicity, as would have been evident had the investigator measured the covalent binding after it had reached its maximum value.

In another hypothetical situation, suppose that the entire doses of two different substances became covalently bound to the target macromolecule but that one was metabolized much more rapidly than the other. Measuring the covalent binding of their reactive metabolites soon after their administration might lead the investigator to the mistaken impression that there was no relationship between covalent binding of the substances and their toxicity, because at that time the covalent binding of the rapidly metabolized substance would be greater than that of slowly metabolized substance.

On the other hand, waiting too long before measuring the covalent binding may also lead to erroneous conclusions, because some macromolecular–metabolite conjugates in tissues may be either lost from the tissue after necrosis or replaced by newly synthesized macromolecules in other kinds of toxicity. Thus, in these kinds of correlation studies, covalent binding should be determined when the covalent binding is maximal, a precaution whose importance has not been realized in some studies reported in the literature. For this reason, time-course studies are invaluable in assessing relationships between covalent binding of reactive metabolites to macromolecules and toxicities.

In order to test the validity of correlating changes in the incidence and severity of toxicities with changes in the maximum amount of covalent binding, studies were carried out on the mechanism of liver necrosis induced by large doses of bromoben-

zene. Although this compound is chemically inert, it is converted to its mercapturic acid and its dihydro-diol derivatives, presumably through the formation of its chemically reactive arene oxide. Initial studies revealed that pretreatment of rats with phenobarbital, which hastened the metabolism of bromobenzene, increased the severity of centrilobular necrosis caused by large doses of the toxicant.[15,16] In contrast, SKF 525-A, which slowed the metabolism of bromobenzene, decreased the severity of the necrosis. Moreover, the incidence of necrosis caused by bromobenzene in various animal species paralleled the rates of metabolism of the toxicant in these species.[17] Although these initial studies indicated that the toxicity was not mediated by bromobenzene but by one of its metabolites, they did not indicate whether the active metabolite was chemically reactive or inert. Nevertheless, radioautographic studies after the administration of radiolabeled bromobenzene revealed that a reactive metabolite combined preferentially with macromolecules in the centrilobular necrotic areas of liver.[15] Moreover, subsequent studies showed that pretreatment of rats with phenobarbital increased the rate and the maximum amount of covalent binding of radiolabeled bromobenzene to liver macromolecules, whereas the prior administration of SKF 525-A decreased it.[16–20] In addition, the prior treatment of rats with 3-methylcholanthrene decreased both the covalent binding of radiolabeled bromobenzene and the severity of the necrosis.[19,21]

Apparently, several other halogenated benzenes cause liver necrosis by mechanisms similar to that of bromobenzene.[18,22] At doses of 1·0 m-mole/kg administered to rats, radiolabeled chlorobenzene, iodobenzene and *o*-dichlorobenzene as well as bromobenzene become covalently bound to liver macromolecules and cause liver necrosis, whereas fluorobenzene and *p*-dichlorobenzene are not appreciably bound and do not cause liver necrosis. Moreover, the pretreatment of rats with phenobarbital increases the covalent binding and toxicity of all of the toxic halogenated benzenes but does not markedly affect the covalent binding or toxicity of the nontoxic halogenated benzenes.

In another series of studies, the centrilobular necrosis caused by large overdoses of the commonly used analgesic, acetaminophen, was also shown to be mediated by a chemically reactive metabolite.[23–26] As with the halobenzenes, the severity of the necrosis parallels the magnitude of the covalent binding of radiolabeled acetaminophen to liver macromolecules. For example, pretreatment of mice with phenobarbital increases both the severity of the centrilobular necrosis and the covalent binding to liver macromolecules, whereas pretreatment of mice with cobaltous chloride, piperonyl butoxide[23,24] or α-naphthylisocyanate (ANIT)* decreases both of them. Dose–response studies revealed that the amount of covalent binding per dose was negligible until a critical threshold dose was reached (about 300 mg/kg in mice). Below this critical threshold dose, there is not only negligible covalent binding to protein but also no necrosis, which presumably accounts for the remarkable safety of the drug when used at the usual therapeutic dosage. (The reason for the dose threshold will be discussed in Part II of this Commentary.)

Large doses of another commonly used drug, furosemide, cause midzonal and centrilobular necrosis in male mice, apparently through the formation of a chemically reactive metabolite.[27,28] In support of this conclusion, the administration of piperonyl butoxide, cobaltous chloride or ANIT prevented the necrosis caused by furose-

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mide and markedly decreased the covalent binding of radiolabeled furosemide metabolites to liver protein. As with that caused by acetaminophen, the necrosis caused by furosemide occurs only at high doses. Below a critical dose of 150 mg/kg there is little covalent binding and no necrosis in mice.[29]

As a result of these studies on the correlation between changes in the severity of liver damage and changes in the covalent binding of metabolites to liver macromolecules *in vivo*, there is now little doubt that the toxicities caused by halobenzenes, acetaminophen and furosemide are mediated through the formation of chemically reactive metabolites. This laboratory is now engaged in studies to identify the reactive metabolites and to elucidate the mechanisms by which they cause liver necrosis.

Another aspect of the approach was demonstrated with studies on liver necrosis caused by carbon tetrachloride. In the past, various investigators have shown that the chemically reactive metabolite of carbon tetrachloride becomes covalently bound to both phospholipid and protein in liver,[30–32] even though the majority of evidence now indicates that the target substance is probably phospholipid in the endoplasmic reticulum.[9–11] Recent studies have revealed, however, that various treatments that change the severity of the liver necrosis caused by carbon tetrachloride also cause parallel changes in the maximal covalent binding of radiolabeled carbon tetrachloride to liver protein as well as to phospholipid. For example, pretreatment of rats with isopropanol or acetone, which markedly increases the toxicity of carbon tetrachloride,[33] also increases the covalent binding of carbon tetrachloride to both protein and phospholipid in liver,[34] whereas pretreatment of rats with dibenamine, which decreases the toxicity of carbon tetrachloride, decreases the covalent binding of carbon tetrachloride to both protein and phospholipid.[34] The changes in the covalent binding to protein thus paralleled changes in both the covalent binding to the presumed target substance and the severity of the toxicity. These studies, therefore, illustrate that the identity of the target need not always be known nor that it always be measured directly.

Covalent binding to macromolecules in extrahepatic tissues

Although chemically reactive metabolites of foreign compounds that covalently bind to liver macromolecules are probably formed in the liver, the source of reactive metabolites that become covalently bound to macromolecules in extrahepatic tissue is not easily discerned and varies with the compound. At one extreme of the spectrum of chemically reactive metabolites, the metabolite may be so chemically reactive and polar that it never leaves the tissue in which it is formed. In this situation, the covalent binding would be restricted to those tissues that contain the enzymes that catalyze the formation of the reactive metabolite. At the other extreme, the metabolite may be carried by the blood to virtually every tissue in the body. In this situation, the tissue specificity in its covalent binding would depend on whether the various tissues contained macromolecules having high affinity binding sites for the reactive metabolite. In many instances, however, the reactive metabolite can not only be formed in extrahepatic tissues but also be carried to the tissues from the liver. In these instances, it is difficult to determine solely on the basis of studies *in vitro* or *in vivo* whether most of the covalently bound metabolite was formed mainly in the liver or in the extrahepatic tissue.

By comparing the effects of inducers and inhibitors on the covalent binding of reactive metabolites to macromolecules in various tissues both *in vivo* and *in vitro*, investigators can sometimes determine the major source of the reactive metabolite and thereby obtain an indirect measure of the reactivity of the metabolite. As will be explained in Part II of this Commentary, treatments that decrease the biological half-life of certain foreign compounds by increasing the activity of enzymes in liver may decrease the covalent binding of highly reactive metabolites in extrahepatic tissues, even though the inducer does not affect the drug-metabolizing enzymes in extrahepatic tissues. On the other hand, if the reactive metabolite were formed in the liver and carried to the extrahepatic tissues by the blood, the effects of inhibitors and inducers by the liver drug-metabolizing enzymes on the covalent binding to macromolecules in the extrahepatic tissue should parallel their effect on the covalent binding in liver.

The validity of this approach was confirmed by studies with bromobenzene, which revealed that the reactive metabolites of bromobenzene are bound to macromolecules in blood and a number of other tissues, in addition to liver.[18,22] The finding that bromobenzene metabolites were covalently bound to proteins in blood plasma after the administration of toxic doses of bromobenzene raised the possibility that bromobenzene epoxide was sufficiently stable to leave the liver and be carried by blood to other organs, although it was still possible that covalently bound bromobenzene metabolites could have been released from damaged liver cells or associated with newly synthesized albumin during its passage through the lumen of the endoplasmic reticulum. On the other hand, the finding that bromobenzene metabolites became covalently bound during incubation *in vitro* with tissue preparations such as lung also suggested that reactive metabolites of bromobenzene could be formed in extrahepatic tissues. Since pretreatment of mice or rats with phenobarbital increased the formation of reactive bromobenzene metabolites by liver microsomes but did not alter the rate of formation of the reactive metabolites by lung microsomes, it was possible to resolve the problem by studying the effects of phenobarbital pretreatment on the covalent binding of bromobenzene metabolites in extrahepatic tissues. As shown by Reid *et al.*,[35,36] pretreatment with phenobarbital increased the covalent binding of bromobenzene to macromolecules in both the lung and liver of mice receiving toxic doses of bromobenzene. It was, therefore, apparent that the epoxide of bromobenzene can escape the liver after depletion of glutathione and can be carried to the lung where it becomes covalently bound. These studies thus demonstrated that the finding of an enzyme that catalyzes the formation of a reactive metabolite in an extrahepatic tissue does not necessarily mean that all of the reactive metabolite that becomes covalently bound in that tissue is formed there.

By contrast, phenobarbital pretreatment of male mice increases the covalent binding of the reactive metabolite of chloroform to liver macromolecules but decreases the covalent binding of the reactive metabolite to kidney macromolecules. Accordingly, pretreatment with phenobarbital increases the severity of liver necrosis but decreases the severity and incidence of kidney damage.[37] Moreover, the administration of piperonyl butoxide to male mice decreases the covalent binding of the chloroform reactive metabolite and the toxicity in both liver and kidney. These findings thus suggest that the reactive metabolite of chloroform is restricted mainly to the tissue in which it is formed and illustrates that the toxicities, especially

those in extrahepatic tissues, can be decreased by either inducers or inhibitors of drug-metabolizing enzymes. Regardless of the effects of the various treatments on the kinetics of covalent binding, however, it was evident that changes in the covalent binding of the reactive metabolite to macromolecules in the different tissues paralleled changes in the incidence and severity of the tissue damage.

GENERAL COMMENTS

Caution should be used in the interpretation of studies on the covalent binding of reactive metabolites to macromolecules in tissues. The reactive metabolites may be bound to macromolecules that are neither essential to the life of cells nor to their function in the body. Moreover, metabolite–macromolecular conjugates may be rapidly replaced in the cell by repair mechanisms. Furthermore, the mechanism by which a given kind of toxicity may be manifested is usually dependent on a host of other factors in addition to the magnitude of the covalent binding of the reactive metabolite to target macromolecules. Even when the reactive metabolite becomes covalently bound to target macromolecules as well as to nonessential macromolecules, the relative proportion of these metabolite–macromolecular conjugates may vary markedly with the foreign compound and the tissue. Thus, studies of covalent binding to tissue macromolecules by themselves have little predictive value in determining whether a given compound will evoke a given kind of toxicity. Indeed, without correlative studies, no more emphasis should be placed on the finding that a reactive metabolite becomes covalently bound to tissue macromolecules than would be placed on the finding that a reversibly acting drug is localized in a given tissue in drug distribution studies. For example, the finding that a reversibly acting drug is localized in a tissue need not mean that it acts in that tissue, and the finding that a reversibly acting drug metabolite is localized in a tissue need not mean that it was formed there.

In combination with various kinds of toxicity studies, however, studies on the covalent binding of reactive metabolites to tissue macromolecules can be very useful. As pointed out above, studies on the covalent binding of acetaminophen, bromobenzene and furosemide have shown a direct correlation between the changes in the magnitude of the covalent binding of the reactive metabolites to liver macromolecules and changes in the incidence and severity of the liver necrosis caused by these substances. Those treatments that resulted in decreases in the covalent binding of the reactive metabolites also resulted in decreases in the incidence and severity of liver necrosis, whereas those treatments that resulted in increases in covalent binding also resulted in increases in the incidence and severity of liver necrosis.

Covalent binding studies should also be useful in long-term toxicity tests. The proportion of the dose of a potential toxicant that becomes covalently bound can change as the dose is increased.[18,22,24] Studies on covalent binding thus may be useful in determining what doses should be used in long-term toxicity tests and in evaluating whether the toxicities are mediated through chemically reactive metabolites and whether there are dose thresholds for the toxicity. Covalent binding studies should be useful in monitoring changes in the formation and inactivation of reactive metabolites that occur during the course of the toxicity study and thereby should be useful in resolving conflicts in the results of various toxicity tests. Perhaps many of the

strain differences in carcinogenesis may be partially due to differences in the amount of substances that becomes covalently bound, as well as to other factors.

In combination with studies on the metabolism of foreign compounds *in vitro*, studies of covalent binding *in vivo* can provide an indirect insight into the chemical reactivity of various metabolites. For example, the finding that pretreatment of rats or mice with phenobarbital increases the covalent binding of bromobenzene epoxide to lung[35] and kidney[36] macromolecules, even though it does not increase the covalent binding of bromobenzene *in vitro* in these tissues, suggests not only that bromobenzene epoxide is sufficiently stable to leave the liver but also that it is sufficiently stable to reach these tissues during the time required for the blood to flow from the liver to the tissues. Such studies as these may prove invaluable in assessing the plausibility of using intravenous injections of reactive metabolites in toxicity studies. For example, if it were found that the reactive metabolite did not leave the tissue in which it was formed, it seems unlikely that the reactive metabolite would be sufficiently stable to reach the tissue after its intravenous injection; it would also seem unlikely that it could be isolated from biological fluids. Indeed, in these instances, it might be stated, somewhat facetiously, that if a reactive metabolite can be isolated from biological fluids, such as urine and blood, it probably is not the reactive metabolite one is looking for.

Ratioautographic studies of covalently bound reactive metabolites may also be useful in toxicity studies. For example, radioautographic studies reveal that the covalently bound metabolites are evenly distributed throughout a tissue or localized to certain cells within the tissue. In combination with time-course studies, radioautography also provides a way of determining the rates at which the drug–macromolecular conjugates are destroyed and in what cells the removal of these conjugates take place. Thus, the time-course radioautographic studies on acetaminophen-induced necrosis revealed that the covalent binding of the reactive metabolite initially was only slightly greater in the centrilobular hepatocytes than in the periportal hepatocytes. But after the total amount of covalent binding to liver macromolecules reached maximal values, the covalent binding decreased in the periportal regions until the binding was almost entirely restricted to the centrilobular necrotic cells.[24] These findings thus suggest that the decrease in the covalent binding in liver is mainly due to repair mechanisms in the periportal hepatocytes rather than to a loss of macromolecules by necrotic cells.

Because studies on covalent binding *in vivo* provide insights into the relative rates of formation and inactivation of reactive metabolites, into their intercellular and intracellular distribution and into their stability, such studies, in combination with metabolic studies *in vitro* should be especially useful in evaluating the validity of toxicity tests *in vitro*. Many laboratories have developed a number of tests for mutagenesis, including chromosomal aberrations, the dominant-lethal test, the reversion of mutant bacteria and other micro-organisms, cell-culture systems and host-mediated assays.[5] The reliability of these tests in predicting hereditary changes in the progeny of animals and humans has become highly controversial, largely because most laboratories do not determine the disposition of reactive foreign compounds or their reactive metabolites in living animals. Covalent binding studies of potential mutagens would at least demonstrate whether reactive metabolites can enter or be formed in seminiferous tubules in the testes or Graafian follicles in ovaries, which

presumably would have to occur if the substance were to cause inheritable changes in animals.

Many investigators believe that several of the mutagenic tests may be useful in detecting potential carbinogens.[38–40] An obvious deficiency of the mutagenic tests *in vitro*, however, is that the toxicity may not be caused by the parent compound but by a metabolite. When the toxic metabolites are relatively inert chemically, this deficiency may not be serious, because the various metabolites of the foreign compound could be identified, synthesized and then tested in the system. But when the metabolite is very reactive chemically, it may have to be generated *in situ*. In attempting to solve this problem, Legator and Malling[41] developed the host-mediated assay in which mutant micro-organisms are injected into the peritoneal cavity before the administration of the foreign compound. This test requires that the reactive metabolite be stable enough to leave the tissue in which it is formed and enter the peritoneal cavity; thus it would presumably not detect highly reactive metabolites. On the other hand, other investigators[42] have added mutant micro-organisms to liver microsomal systems in order to generate reactive metabolites of the foreign compound. These tests neglect the mechanisms by which tissues can inactivate potent reactive metabolites and hence may give false positive results. In this regard, it is noteworthy that the covalent binding of many drugs, such as diphenylhydantoin, by liver microsomes *in vitro* approaches that of bromobenzene and other toxicants,[43] but these drugs become covalently bound only to a negligible extent *in vivo*, and do not cause necrosis.

Studies with hepatic cell cultures and other tissue culture systems have been suggested in order to solve the problem of reactive metabolite formation. However, the relative activities of the enzymes that catalyze the formation and inactivation of the chemically reactive metabolites or the levels of nucleophilic substances in these systems may differ from those occurring in living animals. It seems possible that many of the discrepancies between the results obtained with the various test systems and those with living animals might become clear if the covalent binding occurring in the test systems were compared with that occurring in living animals.

In conclusion, studies on the covalent binding of reactive metabolites to tissue macromolecules provide a relatively rapid and simple way of monitoring changes in the formation and distribution of reactive metabolites of foreign compounds. In combination with toxicity tests, these studies should prove invaluable in the future as a rapid screen for substances that evoke toxicity through the formation of reactive metabolites and as an aid in the extrapolation of results from test systems *in vitro* to living animals.

The kinetics of covalent binding of reactive metabolites differ in several ways from the pharmacokinetics of reversibly acting drugs. In Part II of this Commentary, I shall discuss several of these differences and how a better understanding of the pharmacokinetics of covalent binding has led to the concept of dose thresholds in toxicity.

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