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COMMENTARY

A PERSPECTIVE ON THE ROLE OF CHEMICALLY REACTIVE METABOLITES OF FOREIGN COMPOUNDS IN TOXICITY—II. ALTERATIONS IN THE KINETICS OF COVALENT BINDING

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IN THE FIRST part of this Commentary,[1] some of the basic concepts of the relationships between covalent binding of reactive metabolites of foreign compounds to tissue macromolecules and the toxicity caused by these metabolites were discussed. It was pointed out that covalent binding studies by themselves do not predict whether a given compound would cause toxicities. But studies on the relationships between changes in the covalent binding of reactive metabolites and changes in the incidence and severity of the toxicity known to be caused by the foreign compound can serve as a rapid test to determine whether the toxicity was mediated by a chemically reactive metabolite or the inert foreign compound or one of its inert metabolites. The validity of the test is based on several fundamental differences in the pharmacokinetics of reversibly and irreversibly acting foreign compounds. In the second part of this Commentary, I shall discuss some of these differences and shall illustrate how they have led to an understanding of seemingly dichotomous results and of the mechanisms of dose thresholds in the toxicity of certain drugs.

Theoretical pharmacokinetics of covalent binding

In our approach, we have pretreated animals with various inducers and inhibitors of drug-metabolizing enzymes and determined their effects on the covalent binding of reactive metabolites to tissue macromolecules. Some of the effects, however, may seem unusual to those who are familiar only with the pharmacokinetics of reversibly acting drugs. In order to gain a perspective of what might be expected after a given treatment, it is perhaps useful to review the basic principles of the kinetics of covalent binding of reactive metabolites to macromolecules in the body.[2] In the derivation of the pharmacokinetic equations shown in Fig. 1, the following assumptions were made: (1) All processes, including the elimination of the parent compound, the formation of the reactive metabolite, the covalent binding of the reactive metabolite to various macromolecules and the conversion of the reactive metabolite to inactive secondary metabolites, follow first-order kinetics. (2) The rate of elimination of the parent compound approaches that predicted by a one-compartment model; that is the rate constant of elimination is very small compared with the rate constants for tissue distribution. (3) The metabolism of the macromolecules with which the reactive metabolite combines is negligible compared with the rate of metabolism of the

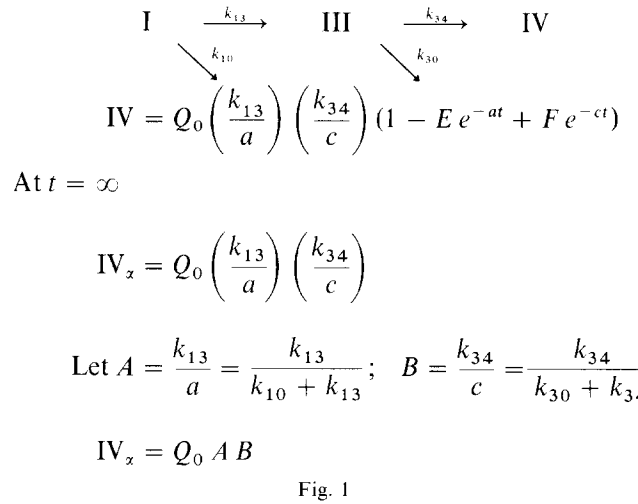


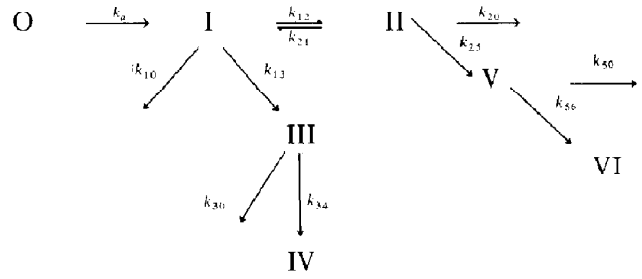
Fig. 1

FIG. 1. One-compartment model for covalent binding of reactive metabolites to macromolecules. Definitions: Q_0 = dose of foreign compound; I = amount of unchanged foreign compound at any given time; III = amount of reactive metabolite at any given time; IV = total amount of covalently bound reactive metabolite, including that bound to target macromolecules, at any given time; k_{13} = rate constant for the formation of the reactive metabolite; k_{10} = sum of the rate constants for the reactions by which the foreign compound is converted to nonreactive metabolites and for its excretion by the lungs and kidneys; k_{34} = sum of the rate constants for the reactions by which the reactive metabolite covalently combines with the different kinds of macromolecules in a given tissue; k_{30} = sum of the rate constants for the reactions by which the reactive metabolite is converted to inactive metabolites. When the reactive metabolite is so stable that it is excreted into urine, the rate constant of the metabolite excreted is also included. $a = k_{10} + k_{13}$; $c = k_{30} + k_{34}$; $E = c/(c - a)$; $F = a/(c - a)$. A = the proportion of the dose that is converted to the reactive metabolite; B = the proportion of the reactive metabolite that becomes covalently bound by the different kinds of macromolecules.

foreign compound. Under these conditions, the amount of covalently bound metabolite in any given tissue will accumulate until all the foreign compound is metabolized. After both of the exponential terms shown in Fig. 1 reach zero, i.e. time approaches infinity, the total amount of foreign compound that eventually becomes covalently bound thus depends on the product of the two ratios, A and B. Ratio A is the proportion of the dose of foreign compound that is converted to the reactive metabolite, and ratio B is the proportion of the reactive metabolite that becomes covalently bound to tissue macromolecules.

It is noteworthy that changes in the rate of absorption of a foreign compound or the rate of its distribution to tissues could affect the rate of covalent binding to tissue macromolecules, but would not be expected to affect the maximum amount of covalently bound metabolite in rapidly perfused tissues, except when the rate of metabolism of the drug in poorly perfused tissues is significant (Fig. 2). Notice that after all of the foreign compound is metabolized ($t = \infty$), the equation in Fig. 2 for covalent binding does not contain the rate constant for absorption (k_a) and that when there is negligible metabolism in the deep compartment (compartment II), the equation for covalent binding is identical to that shown in Fig. 1.

From these equations, it is evident that treatments that change the activities of drug-metabolizing enzymes will alter the ultimate amount of covalent binding of



$$IV = Q_0\left(\frac{k_{13}\,b}{\alpha\beta}\right)\left(\frac{k_{34}}{c}\right)[1 - Re^{-kat} + Se^{-\alpha t} - Te^{-\beta t} + Ue^{-ct}]$$

$$VI = Q_0\left(\frac{k_{12}\,k_{25}}{\alpha\beta}\right)\left(\frac{k_{56}}{f}\right)[1 - We^{-kat} + Xe^{-\alpha t} - Ye^{-\beta t} + Ze^{-ft}]$$

At $t = \infty$

$$IV_\infty = Q_0\left(\frac{k_{13}\,b}{\alpha\beta}\right)\left(\frac{k_{34}}{c}\right)$$

$$VI_\infty = Q_0\left(\frac{k_{12}\,k_{25}}{\alpha\beta}\right)\left(\frac{k_{56}}{f}\right)$$

When k_{20} and k_{25} are 0, i.e. negligible amounts of the foreign compound are metabolized in the deep compartment.

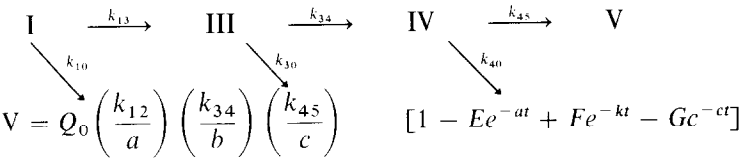
$$IV'_\infty = Q_0\left(\frac{k_{13}}{k_{10} + k_{13}}\right)\left(\frac{k_{34}}{c}\right)$$

FIG. 2. Two-compartment model for covalent binding of reactive metabolites to macromolecules with first-order absorption. Definitions: Q_0 = dose of foreign compound; O = compartment into which the foreign compound is introduced (e.g. intestine); I = rapidly perfused compartment, which usually includes liver, heart, lung, kidneys and other vital organs; II = deep or poorly perfused compartment, which usually includes muscle and fat; III = amount of reactive metabolite in compartment I at any given time; IV = total amount of covalently bound reactive metabolite in compartment I at any given time, including that bound to target macromolecules; V = amount of reactive metabolite in compartment II at any given time; VI = total amount of covalently bound reactive metabolite in compartment II at any given time, including that bound to target macromolecules; k_a = rate constant for absorption of the foreign compound; k_{13} = rate constant for the formation of the reactive metabolite in compartment I; k_{10} = sum of the rate constants for the reactions by which the foreign compound is converted to inert metabolites in compartment I and for its excretion by lungs and kidneys; k_{34} = sum of the rate constants for the reactions by which the reactive metabolite covalently combines with the different kinds of macromolecules in compartment I; k_{30} = sum of the rate constants for the reactions by which the reactive metabolite is converted to inert metabolites in compartment 1; k_{12} = rate constant for the passage of the foreign compound from compartment I to compartment II; k_{21} = rate constant for the passage of the foreign compound from compartment II to compartment I; k_{25} = rate constant for the formation of the reactive metabolite in compartment II; k_{20} = sum of the rate constants for the reactions by which the foreign compound is converted to inert metabolites in compartment II; k_{56} = sum of the rate constants for the reactions by which the reactive metabolite covalently combines with the different kinds of macromolecules in compartment II; k_{50} = sum of the rate constants for the reactions by which the reactive metabolite is converted to inert metabolites in compartment II. $a = k_{10} + k_{13} + k_{12}$; $b = k_{20} + k_{25} + k_{21}$; $c = k_{30} + k_{34}$; $f = k_{50} + k_{56}$; $\alpha = 1/2\,[a + b + \sqrt{\{(a+b)^2 - 4[(k_{10} + k_{13})\,b + k_{12}\,(k_{20} + k_{24})]\}}]$; $\beta = 1/2\,[a + b - \sqrt{\{(a+b)^2 - 4\,[k_{10} + k_{13})\,b + k_{12}\,(k_{20} + k_{25})]\}}]$; $\alpha\beta = (k_{10} + k_{13})\,b + k_{12}\,(k_{20} + k_{25})$; $R = \alpha\beta c\,(b - k_a)/b\,(\alpha - k_a)\,(\beta - k_a)\,(c - k_a)$; $S = k_a\beta c\,(b - \alpha)/b\,(\alpha - k_a)\,(\beta - \alpha)\,(c - \alpha)$; $T = k_a\,\alpha c\,(b - \beta)/b\,(\beta - k_a)\,(\beta - \alpha)\,(c - \beta)$; $U = k_a\,\alpha\beta\,(b - c)/b\,(c - k_a)\,(c - \alpha)\,(c - \beta)$; $W = \alpha\beta f/(\alpha - k_a)\,(\beta - k_a)\,(f - k_a)$; $X = k_a\beta f/(\alpha - k_a)\,(\beta - \alpha)\,(f - \alpha)$; $Y = k_a\alpha f/(\beta - k_a)\,(\beta - \alpha)\,(f - \beta)$; $Z = k_a\,\alpha\beta/(f - k_a)\,(f - \alpha)\,(f - \beta)$.

highly reactive metabolites to macromolecules in any given tissue only when the treatments alter either the ratio *A* or the ratio *B*, or both, in that tissue. Since ratio *A* may be altered by changing either its numerator or its denominator, the magnitude of the alteration depends on the relative importance of the activation reaction and the other reactions that contribute to the elimination of the foreign compound. When nearly all of the parent compound is converted to its reactive metabolite, increasing or decreasing the activity of the enzyme system that catalyzes the formation of the reactive metabolite would not be expected to cause a marked change in the proportion of the dose converted to the reactive metabolite because changes in the numerator would parallel changes in the denominator of *A*. Even when a relatively small proportion of the parent compound is converted to its reactive metabolite, inducers and inhibitors of the enzyme that catalyze the formation of the reactive metabolite may not markedly change the magnitude of the covalent binding when they also cause parallel changes in the rates of elimination of the foreign compound by the major pathways of elimination. Indeed, inducers and inhibitors of drug-metabolizing enzymes would be expected to have their greatest effect on *A* by changing the activity of an enzyme that catalyzes the formation of the reactive metabolite along a relatively minor pathway without affecting the rate of formation of metabolites along the major pathways of elimination. Hence, inducers and inhibitors may greatly affect *A* without markedly affecting the biological half-life of the parent compound, whereas inducers and inhibitors that greatly change the biological half-life of the parent compound may not always cause marked changes in *A*.

If the covalent binding of the reactive metabolite to macromolecules in tissues occurs nonenzymatically, it seems unlikely that the ratio, *B*, can be markedly altered by changing its numerator unless the treatment caused marked changes in the concentrations of the macromolecules. It seems likely, therefore, that most treatments that alter *B* act by changing the denominator of *B*, that is, they change the rate of elimination of the reactive metabolite. If relatively little of the reactive metabolite were covalently bound to macromolecules and if the rest of the reactive metabolite were inactivated by only one reaction, then an inducer that specifically increased the activity of the enzyme which catalyzed that reaction would be expected to decrease the covalent binding of the reactive metabolite without markedly changing either the biological half-life of the parent compound or the pattern of its urinary metabolites. In most instances, however, the reactive metabolite is inactivated by several reactions, and alterations in the activity of an enzyme that catalyzes the inactivation of the reactive metabolite may usually be detected by measuring changes in the relative amounts of the inactive metabolites excreted into urine. Nevertheless, these considerations illustrate how a foreign compound may be toxic in one animal species and not in another, even though the metabolism of the foreign compound may appear to be virtually identical in the two species.

In some instances, a primary metabolite of a foreign compound is not sufficiently reactive to combine rapidly with tissue macromolecules but is converted in the body to even more chemically reactive metabolites. For example, 2-fluorenyl acetamide is first converted to its *N*-hydroxylamine derivative, a portion of which is converted to its highly reactive sulfate ester and another portion is converted to its relatively stable glucuronide, which is rapidly excreted into urine.[3,4] Thus, the pharmacokinetic equation for the covalent binding of this substance and perhaps certain other ace-



At $t = \infty$

$$V = Q_0\left(\frac{k_{13}}{a}\right)\left(\frac{k_{34}}{b}\right)\left(\frac{k_{45}}{c}\right)$$

$$A = \frac{k_{13}}{a} = \frac{k_{13}}{k_{10} + k_{13}}; \quad B = \frac{k_{34}}{b} = \frac{k_{34}}{k_{30} + k_{34}}$$

$$C = \frac{k_{45}}{c} = \frac{k_{45}}{k_{40} + k_{45}}$$

FIG. 3. One-compartment of model for covalent binding when an inert metabolite is converted to a reactive metabolite. Definitions: Q_0 = dose of foreign compound; I = amount of unchanged foreign compound at any given time; III = amount of the intermediate reactive metabolite at any given time; IV = amount of reactive metabolite at any given time; V = total amount of covalently bound reactive metabolite, including that bound to target macromolecules at any given time; k_{13} = rate constant for the formation of the intermediate reactive metabolite; k_{10} = sum of the rate constants for the reactions by which the foreign compound is converted to inert metabolites and for its excretion by the lung and kidneys; k_{34} = rate constant for the formation of the reactive metabolite; k_{30} = sum of the rate constants for the reactions by which the intermediate reactive metabolite is converted to inert metabolites and for the excretion of the intermediate metabolite by the lungs and kidneys; k_{45} = sum of the rate constants for the reactions by which the reactive metabolite covalently combines with the different kinds of macromolecules; k_{40} = sum of the rate constants for the reactions by which the reactive metabolite is converted to inert metabolites. $a = k_{10} + k_{13}$; $b = k_{30} + k_{34}$; $c = k_{40} + k_{45}$; $E = bc/(b - a)(c - a)$; $F = ac/(b - a)(c - b)$; $G = ab/(c - a)(c - b)$.

tylated aromatic amines can be quite complex (Fig. 3). However, the proportion of the dose that ultimately becomes covalently bound depends on the product of three ratios, i.e., the proportion of the dose that is converted to *N*-hydroxylamine derivative (ratio *A*), the proportion of the *N*-hydroxylamine derivative that is converted to its sulfate ester (ratio *B*) and the proportion of the sulfate ester that becomes covalently bound to tissue macromolecules (ratio *C*). With the increase in complexity of the pharmacokinetics, it becomes even more difficult to predict changes in the magnitude of covalent binding from changes in the biologic half-life of the drug and changes in the pattern of urinary metabolites. For example, suppose that a treatment increased the proportion of the dose that was converted to the hydroxylamine but also either increased the activity of UDPGA transferase or decreased the activity of the sulfotransferase. The treatment then would have decreased the covalent binding even though it increased the proportion of the dose that was excreted as the glucuronide of the acetylated *N*-hydroxylamine metabolite. Thus, the effects of treatments on the magnitude of covalent binding of such secondary metabolites can be very difficult to predict with accuracy.

In the derivations of the equations shown in Figs. 1–3, it was assumed that the metabolite was so chemically reactive that it never left the tissue in which it was

formed. Since foreign compounds may be converted to chemically reactive metabolites in a number of different tissues, however, the degree of covalent binding in the various tissues would depend not only on the rates of formation and inactivation of the reactive metabolite in the given tissue but also on the elimination rate constant of the unchanged foreign compound. Thus, the ratios A and B may differ markedly from one tissue to another but the denominator of all the ratio A's for the various tissues would be identical. In this situation, a treatment that specifically altered the activity of the enzyme that catalyzed the formation of the major metabolite in the tissue that metabolized most of the drug in the body would alter the denominator of the ratio A's in all the other tissues, even though the treatment did not affect the enzyme activities in the other tissues. For example, when the major pathway of elimination of a foreign compound is through the formation of its reactive metabolite in the liver, a substance that induces only the liver enzyme may tend to increase the covalent binding of the reactive metabolite in the liver and decrease it in other tissues, whereas a substance that inhibits the enzyme only in the liver would tend to decrease the covalent binding in the liver but increase it in the other tissues. For this reason, inhibitors that decrease toxicities in one organ may enhance them in others.

By contrast, when the reactive metabolite is sufficiently stable to leave the organ in which it is formed, the rate of covalent binding of the reactive metabolite will depend on a host of interrelated factors: (1) the chemical reactivity of the metabolite, (2) the diffusivity of the metabolite, (3) the blood flow rate to the various tissues, and (4) the activities of the enzymes that catalyze the formation and inactivation of the reactive metabolites in the various tissues. Obviously, pharmacokinetic equations that include these interrelationships would be very complex. But even in these situations, the concept of the ratios A and B is useful in elucidating the dominant factors that determine the covalent binding of reactive metabolites in various tissues.

The kinetics of covalent binding can be even more complex when the reactive metabolite inactivates the enzyme that catalyzes its formation. For example, CCl_4[5–7] and dimethylnitrosamine[5] cause a rapid destruction of cytochrome P-450 in liver microsomes. The covalent binding under these conditions would be expected to be self-limiting. Indeed, Glende[8] has reported that the administration of sublethal doses of CCl_4 to animals protects them from the lethal effects of high doses of the toxicant. The kinetics described in Figs. 1–3 will not be valid in these situations, although the general concept of the ratios A and B is still useful in elucidating mechanisms of covalent binding.

There are other situations in which the kinetics of covalent binding of reactive metabolites to tissue macromolecules *in vivo* do not fit the idealized models described in Figs. 1–3, but studies to determine why the kinetics do not fit them have led to a better understanding of the biochemistry of the reactive metabolites.

Effect of drug interactions on threshold doses in covalent binding and toxicity

Many of the pharmacokinetic principles discussed above may be illustrated by our studies on liver necrosis caused by reactive metabolites of bromobenzene, acetaminophen and furosemide. Although the urinary metabolites of bromobenzene were identified many years ago,[9–11] a recent series of experiments *in vivo* and *in vitro* revealed that nearly all of the bromobenzene administered to animals was converted to its chemically reactive arene oxide, 3,4-bromobenzene epoxide,[12,13] by a cytoch-

rome P-450 enzyme system localized mainly in liver microsomes.[13,14] A portion of the epoxide rearranges nonenzymatically to form *p*-bromophenol. A portion is converted to a dihydrol-diol, presumably by an epoxide hydrase in liver microsomes; the dihydro-diol in turn is dehydrogenated to form 4-bromo catechol, presumably by an enzyme in the soluble fraction of liver. About 70 per cent of the epoxide formed in rats receiving a nontoxic dose of bromobenzene is converted to a glutathione conjugate by a glutathione-transferase in the liver soluble fraction; the conjugate is then hydrolyzed to form the cysteinyl derivative, which is then acetylated to the mercapturic acid and excreted into urine.[12] The steady state concentration of the epoxide in liver thus depends on the relative rates at which it is formed and inactivated by the various enzymatic and nonenzymatic reactions.

In light of the theoretical pharmacokinetics of covalent binding, some of the results of studies on covalent binding of bromobenzene metabolites to liver macromolecules *in vivo* were puzzling. For example, studies on the covalent binding of radiolabeled bromobenzene after different dosages revealed that the proportion of the dose that became covalently bound remained low until a critical dose between 1·2 and 2·15 m-moles/kg was used.[15,16] Above this critical dose, the proportion of the dose that became covalently bound was nearly doubled and liver necrosis was manifested. Moreover, prior administration of SKF 525-A or the pretreatment of rats with phenobarbital markedly increased the covalent binding and the toxicity of bromobenzene. But the pharmacokinetic equations predicted that neither of these treatments should have affected the covalent binding of bromobenzene epoxide for the following reasons: Since virtually all of the bromobenzene administered to rats is converted to its epoxide before it is eliminated from the body, the possibility that SKF 525-A could markedly alter the proportion of the dose that was converted to the epoxide seemed remote. Furthermore, pretreatment of rats with phenobarbital cannot increase the proportion of the dose that is converted to the epoxide and therefore should not have increased the covalent binding of bromobenzene epoxide to liver protein, even though the pretreatment markedly increases the rate of bromobenzene metabolism. Instead, the pretreatment with phenobarbital should have caused a small decrease in covalent binding because it increases the activity of the epoxide hydrase.[17] Indeed, the phenobarbital treatment decreased the covalent binding of bromobenzene when low, nontoxic doses of the toxicant were administered.

The reason for the threshold dose became clear when it was realized that the levels of glutathione in liver are decreased after the administration of toxic doses of bromobenzene until the rate of formation of the glutathione conjugate was limited by the availability of this co-substrate. Under these conditions the ratio *B* in Fig. 1. would be increased as the dose of bromobenzene was increased. This view was confirmed by Jollow *et al.*,[13] who showed that the rate of covalent binding of bromobenzene metabolites to liver macromolecules was markedly increased after the glutathione levels were depleted. Moreover, the severity of the necrosis and the magnitude of the covalent binding can be markedly increased by the prior administration of diethyl maleate,[15,16] which depletes the liver of glutathione without causing necrosis. Thus, glutathione protects the liver against the toxic effects of bromobenzene epoxide by combining with it to form the glutathione conjugate.

The mechanism of the effects of SKF 525-A administration and phenobarbital pretreatment on the covalent binding of bromobenzene metabolites is more subtle. If

the synthesis of glutathione were negligible during the metabolism of bromobenzene, then the amount of bromobenzene epoxide that would be converted to the mercapturic acid after the administration of bromobenzene should not be changed by accelerating or decelerating epoxide formation. But glutathione is rapidly synthesized in liver and thus a considerable portion of the bromobenzene epoxide is still inactivated by its conjugation with glutathione (about 50 per cent in rats), even after the liver levels of glutathione have been decreased to very low levels.[12] However, the rate of synthesis of the glutathione conjugate is now limited by the rate of synthesis of glutathione and thus the proportion of the bromobenzene epoxide that is inactivated by formation of the glutathione conjugate can be altered by changing the rate of formation of the epoxide. Hence, phenobarbital pretreatment decreases the proportion of the epoxide that is inactivated through the formation of the glutathione conjugate by accelerating the formation of the epoxide.

In contrast to the effects of phenobarbital, pretreatment of rats with 3-methylcholanthrene decreases both the covalent binding of bromobenzene to liver protein and the severity of liver necrosis[12,18] by a number of interdependent mechanisms. Since the pretreatment does not alter the biological half-life of bromobenzene in rats and actually increases the rate of bromobenzene metabolism by liver microsomes, the protective effect must have occurred by a marked alteration in the pattern of metabolism of bromobenzene. Studies on the pattern of urinary metabolites of rats receiving a toxic dose of bromobenzene revealed that the 3-methylcholanthrene treatment resulted in a decrease in the mercapturic acid and 4-bromophenol but an increase in the bromocatechol, bromophenyldihydrodiol and 2-bromophenol.[12] Since 2-bromophenol cannot be formed from the nonenzymatic rearrangement of 3,4-bromobenzene epoxide, it seems likely that 3-methylcholanthrene induces the formation of a different epoxide, presumably 2,3-bromobenzene epoxide. Moreover, the increase in the bromocatechol and the bromophenyldihydrodiol fractions at the expense of mercapturic acids in the urine of 3-methylcholanthrene-treated rats implies that the epoxide hydrase, as well as the cytochrome P-450 enzyme, was induced.[12,17] Increasing the formation of the 2,3-bromobenzene epoxide thus decreases the proportion of the dose of bromobenzene that is converted to 3,4-bromobenzene epoxide, which may be the more reactive arylating intermediate. At the same time, increasing the activity of epoxide hydrase decreases the dependence of hepatic cells on the glutathione transferase in inactivating the epoxides and hence decreases the rate of utilization of glutathione. By decreasing the rate of formation of glutathione conjugates, the concentration of glutathione in liver cells is more easily maintained at relatively high levels by the synthesis of glutathione and the mobilization of nucleophilic substances, such as cysteine, from body stores. The net effect is thus a decrease in both ratio *A* and ratio *B* in the pharmacokinetic equation shown in Fig. 1.

Recent studies have shown that the liver necrosis caused by large doses of acetaminophen is probably mediated through the formation of a chemically reactive metabolite.[19,20] Studies on the covalent binding of radiolabeled acetaminophen to liver protein in mice revealed that little covalent binding and no necrosis occurred until the dose was greater than 300 mg/kg.[21] As with bromobenzene, the requirement of a threshold dose for toxicity is apparently caused by the depletion of endogenous co-substrates required for the conjugation reactions. In accord with this view, there was a delay in the covalent binding after the administration of a toxic dose of aceta-

minophen; indeed, there was little covalent binding until most of the acetaminophen in the body had been eliminated.[20]

The major routes of acetaminophen metabolism in man and experimental animals are through the formation of its glucuronide and sulfate conjugates. Thus, it might seem plausible that high doses of acetaminophen could result in the depletion of PAPS, the co-substrate required for sulfate conjugation, and thereby increase ratio *A*. Indeed, by decreasing the total body clearance of acetaminophen, Büch *et al.*[22] have reported that the amount of acetaminophen excreted into urine as its sulfate conjugate in rats is not increased as the dose is increased from 300 to 600 mg/kg, but that the amount of the sulfate conjugate is increased by the prior administration of sodium sulfate. Moreover, Jollow *et al.*[23] compared the pattern of urinary metabolites after the administration of a nontoxic (25 mg/kg) and a toxic dose (400 mg/kg) of acetaminophen in hamsters and found that the proportion of the dose excreted as sulfate conjugates was about 40 per cent after the nontoxic dose and about 18 per cent after the toxic dose. From these findings, however, it can be calculated that the decrease in clearance of acetaminophen through sulfate conjugation after the administration of the toxic dose of acetaminophen should account for only about a 28 per cent increase in the proportion of the dose that would be converted into the reactive metabolite. It therefore seems unlikely that sulfate depletion was the major mechanism by which the covalent binding per dose was increased as the dose was increased.

In addition to its conversion to glucuronide and sulfate conjugates, acetaminophen is also metabolized by a minor pathway to a mercapturic acid[24] (< 15 per cent in untreated hamsters).[23] Since it seemed unlikely that acetaminophen itself would react with glutathione, the presence of the mercapturic acid in urine suggested the possibility that a small proportion of the acetaminophen was converted to a reactive metabolite which reacts with glutathione. It also seemed possible that as the dose of acetaminophen was increased the liver levels of glutathione would decrease, until the rate of inactivation of the reactive metabolite would be limited by the rate of glutathione synthesis or the rate of mobilization of cysteine from proteins in the body tissues. Under these conditions, ratio *B* would be increased because the concentration of the reactive metabolite in liver would increase and thereby result in an increase in its covalent binding to liver macromolecules. In accord with this view, the proportion of the acetaminophen excreted as the mercapturic acid in hamsters was smaller after the administration of a toxic dose than it was after the administration of a nontoxic dose,[23] presumably because the reactive metabolite now was channelled not only toward covalent binding but also to an unknown metabolite. Moreover, in mice the level of glutatione in liver decreased as the dose of the drug was increased.[21] In addition, the severity of the liver necrosis paralleled not only the magnitude of the covalent binding of radiolabeled acetaminophen to liver protein but also the decrease in liver glutathione levels. For example, little covalent binding and no liver necrosis occurred at doses of acetaminophen that depleted liver glutathione less than 85 per cent.[21] Furthermore, the delay that occurs in the covalent binding of acetaminophen metabolites to liver macromolecules after the administration of a toxic dose of the drug was also related to the time required to decrease the liver levels of glutathione to less than 15 per cent of its original level.

Although it might seem possible that the decrease in liver levels of glutathione by

itself might result in the liver necrosis caused by acetaminophen, other substances, such as diethyl maleate, decrease the glutathione levels in liver[25] to about the same extent as does acetaminophen but do not result in liver necrosis. However, the administration of diethyl maleate immediately before the injection of acetaminophen increases both the covalent binding of the reactive metabolite of acetaminophen to liver protein and the severity of the liver necrosis.[21] On the other hand, the administration of cysteine,[21] a precursor of glutathione, or of cysteamine or dimercaptrol,[26,*] which presumably react chemically with the reactive metabolite, partially prevents the decrease in liver glutathione, and decreases both the covalent binding of the reactive metabolite to liver protein and the severity of the liver necrosis.[21,*]

Although the formation of the reactive metabolite of acetaminophen is catalyzed by cytochrome P-450 enzymes in liver microsomes,[27] the formation of the glucuronide and sulfate conjugates is not catalyzed by these enzymes. Thus, inducers and inhibitors of cytochrome P-450 enzymes might be expected to increase the formation of the reactive metabolite but would not necessarily alter the biological half-life of acetaminophen. In fact, pretreatments of mice with either phenobarbital or cobaltous chloride did not appreciably alter the biological half-life of acetaminophen, even though they markedly changed the covalent binding of acetaminophen to liver macromolecules and the severity of the liver necrosis.[19,20] These findings thus illustrate that treatments can markedly change the toxicity of drugs even though they do not markedly change the total body clearance of the drugs. They also illustrate the problem of finding an animal that would mimic humans in the metabolism and toxicity of drugs when the toxicity is mediated by metabolites formed along minor pathways.

Depletion of glutathione from tissues, however, is not the only mechanism by which the "threshold" phenomenon can occur. Although the dose–response curves with furosemide revealed that negligible covalent binding and no necrosis occur below a critical dose of about 150 mg/kg, furosemide,[26,28,29] unlike bromobenzene and acetaminophen, does not deplete the liver of glutathione.[29] Moreover, it seems unlikely that the threshold is due to the depletion of other nucleophilic substances or co-substrates in liver, because there is no delay in the covalent binding to liver macromolecules after the administration of a toxic dose of furosemide. Thus, as the dose of furosemide is increased there must be an increase in ratio A rather than in ratio B of the equation shown in Fig. 1, that is, the clearance of the drug by the enzyme that catalyzes the formation of the reactive metabolite relative to the total body clearance of the drug increases as the dose is increased. In accord with this view, at subtoxic doses of furosemide, most of the drug is excreted unchanged into the urine, but as the dose is increased to above the threshold amount, the proportion of the dose that is metabolized increases.[29]

Since it seems unlikely that the drug activates the enzyme that catalyzes the formation of the reactive metabolite, it seems probable that the increase in ratio A is due to a decrease in the clearance of the drug by the kidney or perhaps by biliary excretion. A possible mechanism by which this might occur was suggested by the finding that threshold toxic doses result in plasma levels of the drug at which reversible binding sites of the plasma proteins become saturated, and that higher doses result in disproportionate increases in the plasma levels of the unbound drug and in the liver

* J. R. Mitchell, S. S. Thorgeirsson, W. Z. Potter, D. J. Jollow and H. Keiser, *Clin. Pharmac. Ther.*, in press.

levels of the drug.[29] Although increasing the proportion of drug in plasma existing in its unbound form should not decrease its clearance (rate of excretion/concentration of unbound drug) by glomerular filtration, increasing the plasma concentration of unbound drug could decrease the urinary or biliary clearance by active transport systems in kidney or liver. For example, when the clearance of highly bound drugs by active transport systems in kidney is calculated from the concentration of unbound drug, its value may exceed the blood flow rate through the kidney, because the reversible drug–protein complexes dissociate as the concentration of unbound drug in plasma is decreased by the transport system. Thus, the clearance of drug would tend to decrease as the per cent of bound drug in plasma decreases. Alternatively, the transport systems in kidney or liver may become saturated as the concentration of unbound drug is increased and thereby cause a decrease in the clearance into urine or bile.

As pointed out above, changes in the rate of absorption of the drug from the gastrointestinal tract ordinarily would not be expected to change the proportion of the dose that becomes covalently bound to tissue macromolecules. But when the clearance of the drug is decreased as the concentration of unbound drug is increased, the total body clearance of the drug would depend on the plasma level of the drug and therefore on the rate of absorption of the drug. Thus the rate of absorption would affect the proportion of the dose that is converted to the reactive metabolite and covalently bound to tissue macromolecules. With certain kinds of foreign compounds, therefore, the magnitude of covalent binding may depend on the rates of absorption and excretion of the drug as well as on the activity of enzyme systems that catalyze the formation of reactive metabolites.

GENERAL COMMENTS

The concept of the ratios *A* and *B* in studies of covalent binding of reactive metabolites of foreign compounds to tissue macromolecules have helped to clarify the mechanisms by which chemically reactive metabolites are formed, distributed and eliminated. The concept has also helped to elucidate basic principles by which seemingly contradictory or inconsistent results can be obtained. The finding that a proportion of the dose of foreign compound that becomes covalently bound can increase with increasing doses has led to the concept of dose thresholds in the toxicity of certain compounds. Moreover, such studies in combination with those on the distribution of urinary metabolites have provided estimates of the relative importance of the metabolic pathways that lead to reactive and inert metabolites. These studies in turn have led to the understanding of how certain treatments can cause opposite effects on the covalent binding of reactive metabolites after low and high doses of foreign compounds.

The concept of the ratios *A* and *B* also has provided an insight into the reasons why there may be species differences in toxicity of foreign compounds, even when both the biological half-life and the pattern of urinary metabolites may be similar in the various species. In addition, the concept illustrates how various treatments that seemingly have little effect on the metabolism of a foreign compound may markedly affect its toxicity, whereas those that markedly change the half-life of a foreign compound may have little effect on the toxicity.

A perusal of the pharmacokinetic equations for covalent binding reveals how various treatments may increase toxicities in some tissues but decrease them in others. It further shows how a toxicity in a given tissue may be decreased by either an inducer or an inhibitor of drug-metabolizing enzymes.

From these considerations, it becomes obvious that it is difficult to predict with accuracy whether a given treatment will increase or decrease the covalent binding of reactive metabolites to macromolecules in any given tissue. As was pointed out in Part I of this Commentary,[1] however, studies of covalent binding can be used to determine whether a toxicity caused by a foreign compound is mediated through the formation of a reactive metabolite. When a treatment alters the incidence or severity of the toxicity by changing the metabolism of the toxicant, changes in the magnitude of the covalent binding should parallel changes in the toxicity, regardless of the effect of the treatment on the covalent binding of the reactive metabolite.

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