

# Glutathione: A Vehicle for the Transport of Chemically Reactive Metabolites in Vivo

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The contribution of chemistry to toxicological research has increased dramatically over the past 15-20 years. This development has been due in large measure to the growing awareness that many, if not most, of the serious adverse effects of drugs, pesticides, and other xenobiotics in biological systems are mediated not by the parent compounds themselves but by chemically reactive metabolites thereof.<sup>1,2</sup> These short-lived intermediates, usually generated by the action of enzymes located in the liver, may be either free-radical or even-electron electrophiles that react directly with structural or functional components of the host cell, resulting in the covalent modification of critical macromolecules or the peroxidation of membrane lipids.<sup>3</sup> One of the most important endogenous compounds that protects cells against these highly deleterious processes is the tripeptide glutathione ( $\gamma$ -L-glutamyl-L-cysteinylglycine; GSH), which is the major non-protein thiol in both plants and animals, where it is present at concentrations as high as 10 mM.<sup>4,5</sup> Because of its free sulfhydryl group, GSH may serve as both a nucleophile and a reducing agent, two functions that are facilitated by the action of GSH-dependent enzymes such as the glutathione transferases<sup>6</sup> and glutathione peroxidase.<sup>4</sup> Thus, hazardous electrophilic metabolites may be converted to GSH conjugates through nucleophilic addition or substitution reactions, whereas reactive oxygen species and organic hydroperoxides are reduced to stable products with the concomitant formation of oxidized glutathione (GSSG).

Mechanistic studies of foreign-compound-induced toxicities frequently entail the isolation and structural characterization of GSH conjugates because a knowledge of the structure of a GSH conjugate provides valuable insight into the identity of the reactive intermediate from which it was derived. More recently, however, interest in the structure of GSH adducts has been heightened by the finding that certain classes of GSH conjugate are *themselves* toxic as a consequence of the operation of one or more of the following mechanisms: (i) The GSH conjugate undergoes spontaneous

conversion to a more reactive electrophile by virtue of the adventitious proximity of the nucleophilic glutathionyl sulfur atom to a residual leaving group in the foreign compound. For example, the mutagenic fumigant 1,2-dibromoethane is transformed, via the half sulfur mustard *S*-(2-bromoethyl)glutathione, to a reactive episulfonium ion, which alkylates DNA.<sup>7</sup> (ii) A relatively stable GSH conjugate may undergo auto-oxidation to a chemically reactive product. For example, GSH-derived conjugates of 2-bromohydroquinone are concentrated in kidney cells, where they are oxidized to the corresponding quinone thioethers; the latter species are believed to be responsible for 2-bromohydroquinone-induced nephrotoxicity.<sup>8</sup> (iii) The GSH conjugate is metabolized further to a chemically reactive thiol. For example, the adduct from tetrachloroethene undergoes multistep biotransformation in kidney cells to yield a 1,2,2-trichlorovinyl thiol that may tautomerize to thionoacyl chloride, a potent acylating agent.<sup>9</sup> (iv) The GSH conjugate, although less reactive than the intermediate from which it was formed, nevertheless is electrophilic and reacts covalently with cellular constituents. For example, *S*-(2-chloroacetyl)glutathione, a reactive GSH thiol ester and a putative metabolite of 1,1-dichloroethene, behaves as a selective acylating agent toward biological nucleophiles.<sup>10</sup> (v) The GSH conjugate is formed via an addition reaction that is readily reversible, such that the back-reaction liberates the original electrophilic metabolite. For example, the GSH conjugates of allyl and benzyl isothiocyanate revert to the parent isothiocyanates under physiological conditions.<sup>11</sup>

As a consequence of the above processes, it is now recognized that conjugation with GSH does not always lead to the detoxification of electrophilic foreign com-

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(1) Monks, T. J.; Lau, S. S. *Toxicology* 1988, 52, 1-53.

(2) Utrecht, J. P. *Pharm. Res.* 1989, 6, 265-273.

(3) Nelson, S. D.; Pearson, P. G. *Annu. Rev. Pharmacol. Toxicol.* 1990, 30, 169-195.

(4) Larsson, A.; Orrenius, S.; Mannervik, B., Eds. *Functions of Glutathione: Biochemical, Physiological, Toxicological and Clinical Aspects*; Raven Press: New York, 1983.

(5) Reed, D. J. In *Bioactivation of Foreign Compounds*; Anders, M. W., Ed.; Academic Press: Orlando, 1985; pp 71-108.

(6) Armstrong, R. N. *Chem. Res. Toxicol.* 1991, 4, 131-143.

(7) Peterson, L. A.; Harris, T. M.; Guengerich, F. P. *J. Am. Chem. Soc.* 1988, 110, 3284-3291.

(8) Lau, S. S.; Hill, B. A.; Highet, R. J.; Monks, T. J. *Mol. Pharmacol.* 1988, 34, 829-836.

(9) Dekant, W.; Berthold, K.; Vamvakas, S.; Henschler, D.; Anders, M. W. *Chem. Res. Toxicol.* 1988, 1, 175-178.

(10) Liebler, D. C.; Latwesen, D. G.; Reeder, T. C. *Biochemistry* 1988, 27, 3652-3657.

(11) Bruggeman, I. M.; Temmink, J. H. M.; van Bladeren, P. J. *Toxicol. Appl. Pharmacol.* 1986, 83, 349-359.

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pounds (in contrast to the classical view of the function of GSH), but instead actually may play an important role in mediating their adverse effects. Processes i-iv are relatively well understood, and the underlying mechanisms have been discussed recently in several excellent reviews.<sup>12-16</sup> In contrast, foreign-compound-mediated toxicities that may occur as a result of reversible GSH conjugate formation (class v) have been encountered only in recent years. These are of particular interest because they could involve GSH in the delivery of reactive intermediates from their site of formation (usually the liver) to distant target sites where adverse effects occur. This Account will deal with the emerging view of GSH as a potential vehicle for the in vivo transport of certain classes of reactive metabolites. Particular attention will focus on conjugates of isocyanates (RN=C=O), a group of highly reactive and often very toxic industrial compounds. One member of this group (methyl isocyanate; MIC) gained notoriety for its role in the industrial catastrophe at Bhopal, India, in 1984.

### Chemical Considerations in the Reversible Formation of GSH Conjugates

The chemistry that underlies the reversible conjugation of GSH with electrophilic drug metabolites is best rationalized in terms of "hard" and "soft" reactivity as applied to nucleophile/electrophile pairs.<sup>17</sup> This concept, where "softness" may be correlated with the polarizability and size of the positive or negative center, is based on the generalization that the potential energy barrier associated with the reaction of a nucleophile with an electrophile will be minimized when "soft" nucleophiles react with "soft" electrophiles, or "hard" with "hard". In the case of GSH, a "soft" nucleophile, the high polarizability and accessibility of the low-energy d orbitals of the cysteinyl sulfur atom favor the reaction of GSH with "soft" electrophiles.<sup>18,19</sup> A case in point is MIC, which is a "soft" electrophile by virtue of the delocalization of electrons over the N=C=O  $\pi$  bond system. The reaction between MIC and GSH does not require catalysis by glutathione transferase enzymes (although such catalysis may assist in the process), in contrast to reactions of GSH with "hard" electrophiles such as epoxides or nucleic acids where the process is largely enzyme mediated.<sup>19</sup> Therefore, "soft" electrophiles in biological systems may react spontaneously with GSH (leading to depletion of cellular stores of this important tripeptide) and with free sulfhydryl groups on proteins (resulting in covalent modification of macromolecules). Both of these events are believed to initiate a variety of toxic sequelae. It should also be noted that, in addition to the "hardness" of a given nucleophile, basicity is an important criterion of the rate of its reaction with an electrophilic center. For example, basic amino groups on proteins and pep-

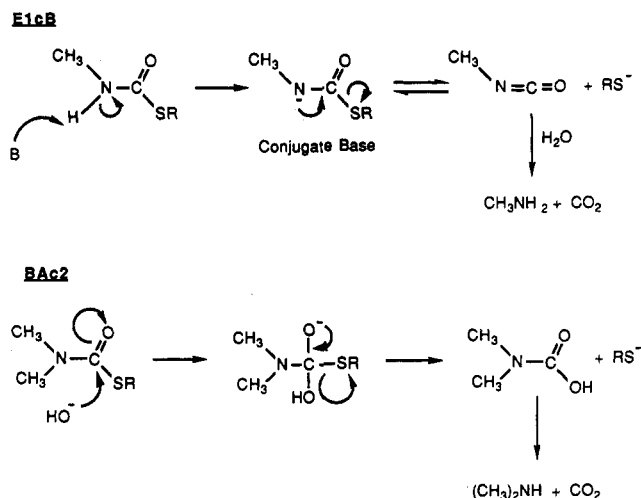


Figure 1. E1cB and BAC2 mechanisms for the hydrolysis of secondary and tertiary carbamate thioesters, respectively.

tides are largely protonated under physiological conditions and, therefore, exhibit relatively low nucleophilicity. However, the thiol moiety of GSH ( $pK_a = 8.66$ ), although not extensively ionized at pH 7.4, nevertheless is reactive toward "soft" electrophiles. Of course, this reactivity may be enhanced in those reactions catalyzed by glutathione transferase enzymes.

With respect to the issue of reversibility in GSH conjugation, the reactions of thiols, amines, and alcohols with cyanates or isocyanates to yield carbamate thioesters, ureas, and carbamates, respectively, are chemically reversible processes whose kinetics have been studied in detail.<sup>20-24</sup> In the 1960s, Stark<sup>21,24</sup> showed that proteins, GSH, and cysteinyl thiols reacted reversibly with cyanate at pH 7. In these studies, the "soft" SH group was shown to add to cyanate at rates that were 22, 190, and 2000 times higher than those observed with the "hard" NH nucleophilic centers of imidazole, glycine, and aminocaproic acid, respectively.<sup>21</sup> The resulting carbamate thioester adducts decomposed rapidly in bicarbonate solution, but were stable at pH values of 5 or less. Kinetic studies demonstrated that the decomposition was not facilitated by neighboring amino or carboxyl groups, but was accelerated by oxidation of the sulfur atom with performic acid. This observation may be rationalized in terms of the increase in polarization of the carbon-sulfur bond which results from sulfoxidation, leading to more facile elimination of the isocyanate.<sup>24</sup> It has been shown that S-linked conjugates can undergo metabolic sulfoxidation,<sup>25,26</sup> so this reaction may play a role in facilitating the release of toxic isocyanates from carbamate thioester adducts in vivo.

Studies on the chemistry of carbamate pesticides have provided mechanistic information that is relevant to the reversible GSH conjugation processes discussed

(12) van Bladeren, P. J. *Trends Pharmacol. Sci.* 1988, 9, 295-299.

(13) Dekant, W.; Vamvakas, S.; Anders, M. W. *Drug Metab. Rev.* 1989, 20, 43-83.

(14) Monks, T. J.; Lau, S. S. In *Reviews in Biochemical Toxicology*; Hodgson, E., Bend, J. R., Philpot, R. M., Eds.; Elsevier: Amsterdam, 1989; Vol. 10, pp 41-90.

(15) Monks, T. J.; Anders, M. W.; Dekant, W.; Stevens, J. L.; Lau, S. S.; van Bladeren, P. J. *Toxicol. Appl. Pharmacol.* 1990, 106, 1-19.

(16) Koob, M.; Dekant, W. *Chem.-Biol. Interact.* 1991, 77, 107-136.

(17) Pearson, R. G.; Songstad, J. J. *Am. Chem. Soc.* 1967, 89, 1827-1836.

(18) Ketterer, B. *Xenobiotica* 1986, 16, 957-973.

(19) Coles, B. *Drug Metab. Rev.* 1984/5, 15, 1307-1334.

(20) Bourne, N.; Williams, A.; Douglas, K. T.; Penkava, T. R. *J. Chem. Soc., Perkin Trans. 2* 1984, 1827-1832.

(21) Stark, G. R. *Methods Enzymol.* 1967, 11, 590-594.

(22) Williams, A.; Jencks, W. P. *J. Chem. Soc., Perkin Trans. 2* 1974, 1753-1759.

(23) Brånstad, J.-O.; Ekberg, G.; Nilsson, I. *Acta Pharm. Suec.* 1973, 10, 1-12.

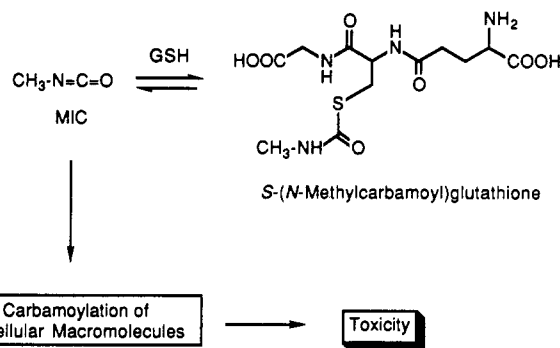
(24) Stark, G. R. *J. Biol. Chem.* 1964, 239, 1411-1414.

(25) Lee, C. W.; Lewis, R. A.; Tauber, A. I.; Mehrotra, M.; Corey, E. J.; Austen, K. F. *J. Biol. Chem.* 1983, 258, 15004-15010.

(26) Sausen, P. J.; Elfarra, A. A. *J. Biol. Chem.* 1990, 265, 6139-6145.

below. In 1964, Christenson<sup>27</sup> suggested that the hydrolysis of monoalkyl carbamates was initiated by the elimination of an isocyanate, and some years later, Krishna and Casida<sup>28</sup> proposed that certain methyl carbamate insecticides were able to modify proteins *in vivo* by means of a "transmethylcarbamoylation" reaction, presumably mediated by MIC liberated from the parent compound. Subsequently, by means of infrared spectrometry, Woodcock<sup>29</sup> was able to detect an intermediate isocyanate in the hydrolysis of a secondary carbamate. From these early studies, it was concluded that the hydrolysis of monoalkyl carbamates proceeded via elimination of an isocyanate, whereas hydrolysis of *N,N*-dialkylcarbamates involved addition of HO<sup>-</sup> to the carbamoyl carbon, followed by elimination of a thiolate leaving group from the tetrahedral intermediate and spontaneous decarboxylation of the resulting carbamic acid (Figure 1). More recent work has supported this view and has provided kinetic evidence for the operation of an E1cB mechanism (unimolecular elimination via the conjugate base) for the hydrolysis of monoalkyl carbamates.<sup>20,23</sup> Interestingly, the rate constant for hydrolysis of an *N*-arylcabamate thioester was found to be 4900 times that of the corresponding carbamate ester. This difference was attributed to the higher acidity of the N-H proton in the thioester and to the role of sulfur in stabilizing the developing negative charge in the transition state leading to the reaction intermediate.<sup>20</sup> Although neighboring-group participation in cyclic concerted eliminations has been ruled out as a mechanism for the release of isocyanates from secondary carbamates,<sup>30</sup> an E2 mechanism (with N-H proton abstraction and thiolate anion release occurring simultaneously) cannot be distinguished readily from the E1cB reaction.<sup>20,30</sup> Both the E1cB and E2 reactions are second-order processes that are dependent upon the nature of the leaving group. However, the available literature favors the E1cB (reversible) reaction for the hydrolysis of carbamates<sup>30</sup> and carbamate thioesters.<sup>20,23</sup> Replacement of the N-H proton in secondary carbamates with an alkyl substituent alters this reaction chemistry significantly, consistent with the expectation that *N,N*-dialkyl (tertiary) carbamate thioesters undergo hydrolysis by a BAc2 (base-catalyzed bimolecular) mechanism (Figure 1).<sup>31</sup> In the case of the *N*-arylcabamate thioester cited above, hydrolysis occurred 60 200 times faster than with an analogous tertiary carbamate thioester.<sup>23</sup>

On the basis of the above considerations, it may be expected that the non-enzyme-mediated reaction between isocyanates (or isothiocyanates) and GSH to yield carbamate thioesters (or thiocarbamate thioesters) would be both facile and reversible in nature. Indeed, the preference of isocyanates<sup>32</sup> and isothiocyanates ("soft" electrophiles) for reaction with GSH (a "soft" nucleophile), as opposed to hydrolysis (which involves attack by water, a "harder" nucleophile), accounts satisfactorily for the high yields of the respective GSH adducts formed in aqueous media.<sup>33</sup> Thus, in a recent



**Figure 2.** Proposed mechanism for MIC-induced toxicity, illustrating the reversible nature of the reaction between MIC and GSH.

*in vitro* study wherein a 1 mM aqueous solution of S-(*N*-methylcarbamoyl)glutathione (the GSH conjugate of MIC; Figure 2) was allowed to react with cysteine (5 mM), an equilibrium was established between the GSH and cysteine conjugates of MIC, with only 32% of the reactants being lost to hydrolysis over 3 h.<sup>34</sup>

### Classes of Compounds That Undergo Reversible Conjugation with GSH

**Isothiocyanates.** Isothiocyanates (RN=C=S) occur in a number of vegetables in the form of their glucosinulates, sugar derivatives that are cleaved readily by the enzyme thioglucosidase when the plant is damaged.<sup>35</sup> As a result, humans may be exposed through the diet to free isothiocyanates, a class of compounds with diverse toxic effects.<sup>11</sup> Studies on the fate of alkyl isothiocyanates in rats and human volunteers indicated that conjugation with GSH represents a major pathway of metabolism.<sup>36-38</sup> Significantly, the investigators commented on the thermal instability of these thiocarbamate thioesters (a property that hampered attempts to accurately quantify their excretion by gas chromatography) and on the tendency of these compounds to undergo base-catalyzed hydrolysis to regenerate the parent isothiocyanates. On the premise that the GSH conjugates themselves might behave similarly and exist under physiological conditions in equilibrium with the toxic isothiocyanates, van Bladeren and co-workers examined the chemical properties of the allyl and benzyl isothiocyanate GSH adducts and determined their cytotoxicity toward isolated rat liver cells.<sup>11</sup> It was found that these conjugates were indeed cytotoxic in this *in vitro* system, where they appeared to act by release of the parent isothiocyanate at cell membranes.<sup>39</sup> Interestingly, toxicity could be abolished by the addition to the medium of an excess of free GSH, consistent with the displacement of an equilibrium between the isothiocyanate and its GSH adduct toward the intact conjugate.<sup>11</sup> On the basis of these findings,

(33) Han, D.-H.; Pearson, P. G.; Baillie, T. A. *J. Labelled Compd. Radiopharm.* **1989**, *27*, 1371-1382.

(34) Pearson, P. G.; Slatter, J. G.; Rashed, M. R.; Han, D.-H.; Grillo, M. P.; Baillie, T. A. *Biochem. Biophys. Res. Commun.* **1990**, *166*, 245-250.

(35) Fenwick, G. R.; Heaney, R. K.; Mullin, W. J. *Crit. Rev. Food Sci. Nutr.* **1982**, *18*, 123-201.

(36) Mennicke, W. H.; Görler, K.; Krumbiegel, G. *Xenobiotica* **1983**, *13*, 203-207.

(37) Mennicke, W. H.; Görler, K.; Krumbiegel, G.; Lorenz, D.; Rittmann, N. *Xenobiotica* **1988**, *18*, 441-447.

(38) Mennicke, W. H.; Kral, T.; Krumbiegel, G.; Rittmann, N. *J. Chromatogr.* **1987**, *414*, 19-24.

(39) Bruggeman, I. M.; Spengelink, A.; Temmink, J. H. M.; van Bladeren, P. J. *Toxicol. in Vitro* **1988**, *2*, 31-35.

(27) Christenson, I. *Acta Chem. Scand.* **1964**, *18*, 904-922.

(28) Krishna, J. G.; Casida, J. E. *J. Agric. Food Chem.* **1966**, *14*, 98-105.

(29) Woodcock, D. J. *J. Chem. Soc., Chem. Commun.* **1968**, 267-268.

(30) Menger, F. M.; Glass, L. E. *J. Org. Chem.* **1974**, *39*, 2469-2470.

(31) Dittert, L. W.; Higuchi, T. *J. Pharm. Sci.* **1963**, *52*, 852-857.

(32) Benson, W. R.; Kagan, B.; Lustig, E.; Chen, J. T.; Shulman, J. J. *Org. Chem.* **1967**, *32*, 3635-3640.

van Bladeren et al.<sup>11</sup> speculated, "Glutathione and cysteine can be regarded as transporting agents for the isothiocyanates through the body. Initial detoxification can be followed by release of the reactive compound at some other site."

**Isocyanates.** Despite the widespread industrial usage of isocyanates in the manufacture of pesticides and plastics, little was known about the biological effects of these reactive compounds prior to the catastrophe in Bhopal, India, which involved the release of some 30–40 tons of MIC vapor into the atmosphere during a 3-h interval.<sup>40</sup> It has been estimated that some 3500 inhabitants of Bhopal died as a result of this acute exposure to MIC vapor, a severe pulmonary toxin. However, a 1988 follow-up study of 1109 survivors of the incident revealed that the majority of patients exhibited symptoms pertaining not only to the respiratory system but also to the cardiovascular, gastrointestinal, musculoskeletal, reproductive, and immunological systems.<sup>41</sup> It would appear, therefore, that inhalational exposure to MIC can lead to long-term effects involving organs other than the lung. Although the molecular basis for these toxicities remains unknown, two studies in rats have reported that radioactivity from inhaled [<sup>14</sup>C]MIC was distributed rapidly and extensively throughout the bloodstream, with the appearance of MIC-derived radioactivity in urine, bile, and tissue proteins shortly after exposure.<sup>42,43</sup> On the basis of these observations, it was speculated that MIC may be converted in vivo to a "transport" form, possibly by reaction with the sulfhydryl groups of either hemoglobin or GSH to yield carbamate thioester adducts, which could revert spontaneously to free MIC at distant sites.<sup>43,44</sup>

Recent work in our laboratories has provided support for this hypothesis. Rats injected intraperitoneally with MIC were found to excrete appreciable quantities of the expected GSH conjugate, *S*-(*N*-methylcarbamoyl)glutathione, in bile<sup>34</sup> and the corresponding *N*-acetylcysteine adduct in urine.<sup>45</sup> Indeed, the latter urinary conjugate accounted for some 25% of the administered dose. Moreover, these carbamate thioesters, together with the cysteine conjugate *S*-(*N*-methylcarbamoyl)-cysteine, proved to be active carbamoylating agents in vitro; under simulated physiological conditions they donated the elements of MIC to sulfhydryl acceptors such as cysteine,<sup>34</sup> GSH,<sup>34</sup> and the reduced form of oxytocin.<sup>46,47</sup> It should be noted that free MIC, whose half-life in aqueous media has been estimated at 2–5 min,<sup>32,44</sup> was not identified in these studies. However, it appears likely that the carbamoylating properties of S-linked adducts of MIC are mediated by the free iso-

cyanate, formed by reversal of the initial conjugation event through an E1cB reaction (Figure 2).<sup>47</sup> This conclusion is supported by recent work on the structurally related carbamate thioester, *S*-[*N*-(1-methyl-3,3-diphenylpropyl)carbamoyl]glutathione, which released the parent isocyanate under mild basic conditions. In this case, the isocyanate was sufficiently stable to be isolated and characterized as such.<sup>48</sup> In light of these observations, it may be concluded that conjugation of reactive, potentially toxic isocyanates with GSH may participate in the transport of these electrophilic species in vivo, in a fashion analogous to that discussed above for the isothiocyanates. In this context, it is noteworthy that lung epithelial lining fluid is known to contain large amounts of GSH,<sup>49</sup> and it is tempting to speculate that *inhaled* MIC may gain access to the systemic circulation (and hence to organs beyond the lung) in the form of its labile GSH conjugate.

**Formamides, Alkyl Carbamates, and Ureas.** These three functionalities, which are encountered in the structures of many drugs and agricultural chemicals, do not react directly with GSH. However, compounds belonging to these classes undergo either metabolic transformation or spontaneous chemical decomposition to yield chemically reactive isocyanates.

Simple *N*-monoalkylformamides, such as *N*-methylformamide (NMF), have been shown to possess antitumor activity in rodent models, and NMF has been investigated as a potential anticancer drug in human trials. However, liver toxicity has been a serious problem with NMF<sup>50,51</sup> and has restricted the tolerated dose to the point where antitumor response is minimal. Although both the origin of the antitumor activity of NMF and the mechanism by which the drug causes hepatic injury remain to be firmly established, a number of lines of evidence point to the involvement of MIC as a key mediator of both types of toxic event. Thus, administration of NMF to rodents depletes stores of GSH in liver tissue<sup>52</sup> and results in the appearance in bile of *S*-(*N*-methylcarbamoyl)glutathione, the GSH conjugate of MIC (Figure 2).<sup>53</sup> Although details of the metabolic pathway by which formamides undergo net two-electron oxidation to yield isocyanates remain to be elucidated, it appears from the limited available structure-toxicity data that only those formamides that are substrates for this pathway of biotransformation cause liver injury.<sup>54</sup> Therefore, it has been proposed that MIC, generated by metabolic oxidation of NMF in liver tissue, is the reactive intermediate responsible for the hepatotoxic effects of the parent formamide.<sup>54</sup> It is also possible that S-linked conjugates of MIC contribute to this liver injury (by acting as latent forms of MIC, as discussed above), and it has been shown that such carbamate thioesters are indeed cytotoxic to iso-

(40) Heylin, M. *Chem. Eng. News* 1985, 63, 14–15.

(41) Gupta, B. N.; Rastogi, S. K.; Chandra, H.; Mathur, A. K.; Mathur, N.; Mahendra, P. N.; Pangtey, B. S.; Kumar, S.; Kumar, P.; Seth, R. K.; Dwivedi, R. S.; Ray, P. K. *Ind. J. Exp. Biol.* 1988, 26, 149–160.

(42) Bhattacharya, B. K.; Sharma, S. K.; Jaiswal, D. K. *Biochem. Pharmacol.* 1988, 37, 2489–2493.

(43) Ferguson, J. S.; Kennedy, A. L.; Stock, M. F.; Brown, W. E.; Alarie, Y. *Toxicol. Appl. Pharmacol.* 1988, 94, 104–117.

(44) Brown, W. E.; Green, A. H.; Cedel, T. E.; Cairns, J. *Environ. Health Perspect.* 1987, 72, 5–11.

(45) Slatter, J. G.; Rashed, M. R.; Pearson, P. G.; Han, D.-H.; Baillie, T. A. *Chem. Res. Toxicol.* 1991, 4, 157–161.

(46) Baillie, T. A.; Pearson, P. G.; Rashed, M. S.; Slatter, J. G.; Howald, W. N. In *Biological Mass Spectrometry*; Burlingame, A. L., McCloskey, J. A., Eds.; Elsevier: Amsterdam, 1990; pp 567–578.

(47) Pearson, P. G.; Slatter, J. G.; Rashed, M. S.; Han, D.-H.; Baillie, T. A. *Chem. Res. Toxicol.* 1991, 4, 436–444.

(48) Mutlib, A. E.; Talaat, R. E.; Slatter, J. G.; Abbott, F. S. *Drug Metab. Dispos.* 1990, 18, 1038–1045.

(49) Cantin, A. M.; North, S. L.; Hubbard, S. C.; Crystal, R. G. *J. Appl. Physiol.* 1987, 63, 152–157.

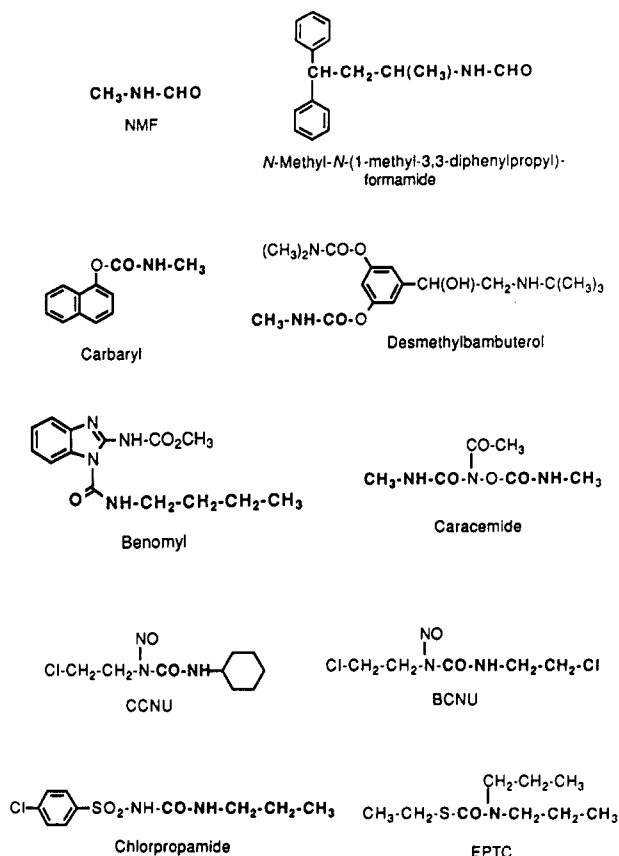
(50) McVie, J. G.; ten bokkel Huinink, W. W.; Somonetti, G.; Dubbelman, R. *Cancer Treat. Rep.* 1984, 68, 607–610.

(51) Ettlinger, D. S.; Orr, D. W.; Rice, A. P.; Donehower, R. C. *Cancer Treat. Rep.* 1985, 69, 489–493.

(52) Pearson, P. G.; Gescher, A.; Harpur, E. S. *Biochem. Pharmacol.* 1987, 36, 381–384.

(53) Threadgill, M. D.; Axworthy, D. B.; Baillie, T. A.; Farmer, P. B.; Farrow, K. C.; Gescher, A.; Kestell, P.; Pearson, P. G.; Shaw, A. J. *J. Pharmacol. Exp. Ther.* 1987, 242, 312–319.

(54) Kestell, P.; Threadgill, M. D.; Gescher, A.; Gledhill, A. P.; Shaw, A. J.; Farmer, P. B. *J. Pharmacol. Exp. Ther.* 1987, 240, 265–270.



**Figure 3.** Representative formamides, alkyl carbamates, and urea derivatives that give rise to alkyl isocyanates in vivo. These alkyl isocyanates, the sources of which are shown in bold type, may be transported in vivo in the form of labile GSH conjugates.

lated mouse hepatocytes.<sup>55</sup> Furthermore, the glutathione conjugate of MIC proved to be a potent inhibitor of the growth of mouse TLX5 lymphoma cells in vitro.<sup>55</sup> Because this cell type is deficient in GSH, these results were interpreted to indicate that tumor cell killing probably was mediated by the release of free MIC from its "carrier" GSH conjugate. Consistent with this view was the finding that addition of GSH to culture media protected TLX5 cells against the toxic effects of the conjugate, presumably by decreasing the concentration of free MIC at cell membranes.<sup>55</sup> Along similar lines, a series of carbamate thioesters structurally related to the cysteine conjugate of MIC have been reported to exhibit antitumor activity in animals, possibly by acting as delivery systems for toxic isocyanates.<sup>56,57</sup> These studies on NMF metabolism thus have provided insight into the mechanism of both the liver toxicity of formamides and their antitumor activity. Central to both are the carbamoylating properties of MIC and the reversible nature of its reaction with GSH.

A second class of compounds that represent potential sources of reactive isocyanates in vivo are secondary alkyl carbamates, exemplified by metabolites of the novel bronchodilator prodrug bambuterol. Although the parent drug itself is a relatively unreactive tertiary

carbamate, it undergoes metabolic oxidation to yield monomethyl (secondary) carbamates (e.g., desmethylbambuterol; Figure 3) which have been shown to undergo facile elimination of MIC. Thus, when preformed monomethyl metabolites were incubated in vitro in the presence of an excess of either GSH or cysteine, MIC was trapped as the corresponding S-linked conjugate.<sup>58</sup> Whether such a process occurs in vivo when bambuterol is administered has yet to be determined.

Historically, the first indication that certain foreign compounds could release an isocyanate in vivo resulted from the identification of GSH and cysteine conjugates of *n*-butyl isocyanate in animals dosed with the pesticide benomyl (Figure 3).<sup>59</sup> This urea derivative liberates *n*-butyl isocyanate in a reaction that has been proposed to involve a zwitterionic intermediate arising from abstraction of the N-H proton.<sup>22</sup> The *N*-carbamoyl moiety of the experimental antitumor drug caracemide [*N,O*-bis(*N*-methylcarbamoyl)acetohydroxamic acid; Figure 3] has been shown to be a source of MIC, as is the *O*-carbamate group.<sup>60,61</sup> Thus, 2 equiv of MIC can result from the spontaneous breakdown of caracemide in vivo, and this may account for some of the side effects of this compound (mucosal burning and neurotoxicity), which limit both the dose and the therapeutic utility of caracemide.<sup>62</sup> Another group of urea derivatives that serve as latent forms of isocyanates are the antitumor nitrosoureas, such as BCNU and CCNU (Figure 3). These compounds decompose spontaneously in aqueous solution with the formation of an alkylating agent (believed to be the chloroethyl carbonium ion) and an alkyl isocyanate.<sup>63</sup> Although the cytotoxic properties of the nitrosoureas usually are attributed to their ability to alkylate and cross-link DNA, there is evidence that at least some tumor cell types are more sensitive to the carbamoylating properties of the isocyanate.<sup>64</sup> Not unexpectedly, nitrosourea-derived isocyanates form conjugates with GSH,<sup>65</sup> and it has been shown that the carbamate thioester adduct generated from BCNU, S-[(2-chloroethyl)carbamoyl]glutathione, is a potent mutagen that induces single-strand breaks in DNA.<sup>66</sup> In light of this finding, and in view of the reversible nature of the reaction between isocyanates and GSH, isocyanate-GSH conjugates may play a significant, albeit unexplored, role in mediating the antitumor effects of the parent nitrosoureas.

Sulfonylureas comprise an important class of hypoglycemic drugs employed clinically in the treatment of diabetes. One member of this family, chlorpropamide (Figure 3), is known to elicit a flushing reaction when

(58) Rashed, M. S.; Pearson, P. G.; Han, D.-H.; Baillie, T. A. *Rapid Commun. Mass Spectrom.* 1989, 3, 360-363.

(59) Axness, M. E.; Fleeker, J. R. *Pestic. Biochem. Physiol.* 1979, 11, 1-12.

(60) Newman, R. A.; Farquhar, D. *Invest. New Drugs* 1987, 5, 267-271.

(61) Slatter, J. G.; Davis, M. R.; Han, D.-H.; Pearson, P. G.; Baillie, T. A. *Proceedings of the 38th ASMS Conference on Mass Spectrometry and Allied Topics*, Tucson, AZ, May 1990; American Society for Mass Spectrometry: East Lansing, MI, 1990; pp 802-803.

(62) Pazdur, R.; Chabot, G. G.; Baker, L. H. *Invest. New Drugs* 1987, 5, 365-371.

(63) Reed, D. J. In *Oxidative Stress*; Sies, H., Ed.; Academic Press: New York, 1985; pp 115-130.

(64) Gibson, N. W.; Hickman, J. A. *Biochem. Pharmacol.* 1982, 31, 2795-2800.

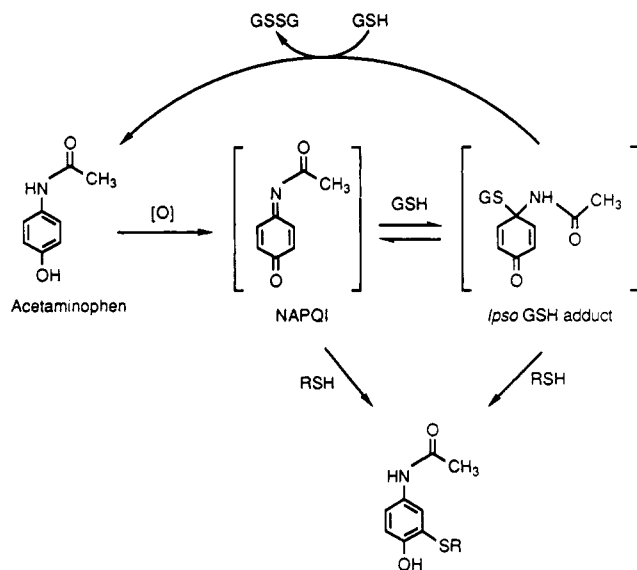
(65) Babson, J. R.; Abell, N. S.; Reed, D. J. *Biochem. Pharmacol.* 1981, 30, 2299-2304.

(66) Stahl, W.; Denkel, E.; Eisenbrand, G. *Mutat. Res.* 1988, 206, 459-465.

(55) Han, D.-H.; Pearson, P. G.; Baillie, T. A.; Dayal, R.; Tsang, L. H.; Gescher, A. *Chem. Res. Toxicol.* 1990, 3, 118-124.

(56) Németh, L.; Somfai-Relle, S.; Kellner, B.; Sugár, J.; Farkas, J.; Bálint, J.; Pályi, I.; Tóth, K.; Szentirmay, Z.; Somosy, Z.; Pokorny, E. *Arzneim.-Forsch.* 1978, 28, 1119-1123.

(57) Jayaram, H. N.; Lui, M. S.; Plowman, J.; Pillwein, K.; Reardon, M. A.; Elliott, W. L.; Weber, G. *Cancer Chemother. Pharmacol.* 1990, 26, 88-92.



**Figure 4.** Proposed role of reversible ipso adduct formation in the reactions of NAPQI, the electrophilic metabolite of acetaminophen. RSH denotes GSH or protein sulfhydryl groups.

ingested together with alcohol. The basis for this drug-drug interaction appears to be that chlorpropamide acts as an inhibitor of aldehyde dehydrogenase and the adverse effects of the chlorpropamide-alcohol combination result from the accumulation of ethanol-derived acetaldehyde. Recent studies have suggested that chlorpropamide and some of its congeners release *n*-propyl isocyanate in vivo, which carbamoylates, and thereby inhibits, aldehyde dehydrogenase.<sup>67,68</sup> Interestingly, administration of *S*-(*N*-*n*-propylcarbamoyl)-glutathione or *S*-(*N*-*n*-propylcarbamoyl)cysteine to rats gave rise to an even more pronounced inhibition of aldehyde dehydrogenase than chlorpropamide itself, consistent with the hypothesis that these carbamate thioesters serve as latent forms of *n*-propyl isocyanate.<sup>68</sup> Therefore, as in the case of simple formamides, the reversible reaction of a metabolically generated isocyanate with GSH appears to play an important role in modulating the adverse effects of the parent drug. Finally, the *N,N*-dialkylcarbamate thioester herbicide EPTC (Figure 3) has been found to give rise to a monoalkyl carbamate thioester conjugate with GSH through a series of metabolic steps.<sup>69</sup> This adduct would be expected to act as a carbamoylating agent and thus may mediate some of the toxic effects of EPTC in vivo.

**Hydroxyacetanilides and Quinone Imines.** Acetaminophen (4-hydroxyacetanilide; Figure 4) is a widely used nonprescription analgesic and antipyretic drug that is known to cause serious liver and kidney injury in both animals and human subjects when administered in very high doses.<sup>70</sup> Extensive research over the past 20 years into the mechanism of acetaminophen-induced hepatic injury has indicated that the toxicity is mediated by a highly reactive metabolite, *N*-acetyl-*p*-

benzoquinone imine (NAPQI), which is formed primarily in liver tissue, where it arylates cellular macromolecules (Figure 4).<sup>71</sup> Somewhat unexpectedly, given the high chemical reactivity of NAPQI,<sup>72</sup> acetaminophen was also shown to become attached covalently to hemoglobin in red blood cells following injection into mice.<sup>73</sup> Moreover, the bound residue was deemed to have been generated from NAPQI formed in liver tissue and then transported by some mechanism to red blood cells. On the basis of the known chemistry of the reaction of quinone imines with nucleophiles,<sup>74</sup> it was proposed that GSH might attack NAPQI at the imine carbon to generate a tetrahedral intermediate at C-1, and that the product of this ipso addition<sup>74,75</sup> was sufficiently stable to diffuse away from its site of formation in the liver. Subsequent reaction with cellular nucleophiles (e.g., protein sulfhydryls or a second molecule of GSH) could occur either in liver cells or at distant loci and thus account for the observed arylation of tissue proteins, the formation of the stable GSH conjugate, 3-(glutathion-*S*-yl)acetaminophen, and the generation of oxidized glutathione (GSSG) (Figure 4). Indeed, recent studies have provided mass spectrometric evidence for the existence of the putative ipso GSH adduct of NAPQI,<sup>76</sup> although the role of GSH as a vehicle for the transport of NAPQI in vivo remains speculative. Interestingly, the quinone imine derived from 2,6-dimethylacetaminophen has been shown to form stable ipso addition products in vitro<sup>74</sup> and to give rise to labile protein adducts that can be cleaved by reduction with dithiothreitol.<sup>77</sup> It would appear, therefore, that reversible conjugation with GSH may be important in the disposition of toxic, electrophilic metabolites of acetaminophen and its congeners. The formation of ipso adducts of quinone imines clearly is a reaction that merits further investigation.

**$\alpha,\beta$ -Unsaturated Carbonyl Compounds.** Although the Michael addition of GSH to  $\alpha,\beta$ -unsaturated carbonyl compounds is a reversible process,<sup>78</sup> most adducts formed by this reaction are relatively stable and the rates of the back-reaction tend to be very slow. For this reason, conjugation of agents such as  $\alpha,\beta$ -unsaturated aldehydes (many of which, such as acrolein, are highly toxic in biological systems) appears to represent an effective means for their inactivation. Several cysteine conjugates of  $\alpha,\beta$ -unsaturated aldehydes have been evaluated as potential cancer chemotherapy delivery systems for their cytotoxic parent aldehydes.<sup>79</sup> However, these compounds were only weakly effective against Ehrlich ascites tumors in mice, probably because of their low propensity to undergo retro Michael

(71) Dahlin, D. C.; Miwa, G. T.; Lu, A. Y. H.; Nelson, S. D. *Proc. Natl. Acad. Sci. U.S.A.* 1984, 81, 1327-1331.

(72) Dahlin, D. C.; Nelson, S. D. *J. Med. Chem.* 1982, 25, 885-886.

(73) Axworthy, D. B.; Hoffmann, K.-J.; Streeter, A. J.; Calleman, C. J.; Pascoe, G. A.; Baillie, T. A. *Chem.-Biol. Interact.* 1988, 68, 99-116.

(74) Fernando, C. R.; Calder, I. C.; Ham, K. N. *J. Med. Chem.* 1980, 23, 1153-1158.

(75) Smith, C. V.; Mitchell, J. R. *Biochem. Biophys. Res. Commun.* 1985, 133, 329-336.

(76) Nelson, S. D.; Tirmenstein, M. A.; Rashed, M. S.; Myers, T. G. In *Biological Reactive Intermediates-IV*; Witmer, C. M., Snyder, R. R., Jollow, D. J., Kalf, G. F., Kocsis, J. J., Sipes, I. G., Eds.; Plenum Press: New York, 1991; pp 579-588.

(77) Rundgren, M.; Porubek, D. J.; Harvison, P. J.; Cotgreave, I. A.; Moldéus, P.; Nelson, S. D. *Mol. Pharmacol.* 1989, 34, 566-572.

(78) Esterbauer, H.; Zollner, H.; Scholz, N. *Z. Naturforsch.* 1975, 30, 466-473.

(79) Tillian, H. M.; Schauenstein, E.; Ertl, A.; Esterbauer, H. *Eur. J. Cancer* 1976, 12, 989-993.

(67) Nagasawa, H. T.; Elberling, J. A.; DeMaster, E. G.; Shirota, F. N. *J. Med. Chem.* 1989, 32, 1335-1340.

(68) Shirota, F. N.; Elberling, J. A.; Nagasawa, H. T.; DeMaster, E. G. *Pharmacologist* 1990, 32, 149.

(69) Hubbell, J. P.; Casida, J. E. *J. Agric. Food Chem.* 1977, 25, 404-413.

(70) Hinson, J. A. In *Reviews in Biochemical Toxicology*; Hodgson, E., Bend, J. R., Philpot, R. M., Eds.; Elsevier: Amsterdam, 1980; Vol. 2, pp 103-129.



cleavage under physiological conditions. However, one example has been reported of a biological retro Michael reaction that involved the GSH conjugate of a substituted acrylonitrile metabolite of furazolidone, a drug used in veterinary medicine.<sup>80</sup> A possible additional example of GSH acting to deliver a toxic compound to its target site involves muconaldehyde, a reactive ring-opened dialdehyde metabolite of benzene that is thought to bind to DNA.<sup>81</sup>

**Simple Aldehydes.** It is well established that GSH reacts spontaneously in biological media with simple aldehydes such as formaldehyde<sup>82</sup> and acetaldehyde<sup>83</sup> to form thiohemiacetals that are in equilibrium with the parent aldehydes. Recently, however, it has been found that aqueous mixtures of formaldehyde and GSH react to form a variety of cyclized adducts in addition to *S*-(hydroxymethyl)glutathione, and that these adducts are in dynamic equilibrium with each other.<sup>84</sup> These observations suggest that GSH not only may function as a cofactor in the metabolic oxidation of formaldehyde to formate and hence to carbon dioxide (the principal metabolite of formaldehyde) but also may play a role in the transport of this aldehyde *in vivo*. Since formaldehyde can react with nucleic acids to yield cross-linked products, the reversibility of its thiohemiacetal formation with GSH could assume toxicological importance.

### Conclusions

The examples cited in this brief Account illustrate the potential role for the endogenous tripeptide GSH to serve as a vehicle for the transport of certain classes of chemically reactive metabolite *in vivo*. Although the contribution of reversible GSH conjugation to the pa-

thogenesis of foreign-compound-induced toxicities remains to be determined, the release of electrophilic metabolites from labile GSH adducts in GSH-depleted cells, or in an environment of altered pH, represents an attractive mechanism for selective cellular injury in tissues which themselves have only weak drug-metabolizing capacity.<sup>85</sup> In the case of direct-acting toxins such as allyl isothiocyanate, reversible GSH conjugation may explain the selective bladder carcinogenicity of this compound because the relatively high pH of nascent urine would favor the release of the free isothiocyanate from its GSH conjugate.<sup>12,86</sup> On the other hand, the factors that determine the selective lung toxicity mediated by sulfur conjugates of the pyrrolizidine alkaloid monocrotaline are not yet understood.<sup>87</sup> Much research needs to be done to better define the scope of these reversible processes in the conjugation of foreign compounds and their metabolites with GSH, and it is encouraging to note the development of novel assay systems by which reactive metabolite formation and disposition can be studied *in vitro*.<sup>88</sup> It is becoming increasingly evident, however, that the conjugation of chemically reactive foreign compounds with GSH does not always result in detoxification of these substances in a classical sense, and that reversible GSH adduct formation represents an intriguing mechanism for the transport of certain short-lived electrophilic agents *in vivo*.

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**Registry No.** Glutathione, 70-18-8.

(80) Vroomen, L. H. M.; Berghmans, M. C. J.; Groten, J. P.; Koeman, J. H.; van Bladeren, P. J. *Toxicol. Appl. Pharmacol.* **1988**, *95*, 53-60.

(81) Witz, G. *Free Radical Biol. Med.* **1989**, *7*, 333-349.

(82) Koivusalo, M.; Baumann, M.; Uotila, L. *FEBS Lett.* **1989**, *257*, 105-109.

(83) Viña, J.; Estrela, J. M.; Guerri, C.; Romero, F. J. *Biochem. J.* **1980**, *188*, 549-552.

(84) Naylor, S.; Mason, R. P.; Sanders, J. K. M.; Williams, D. H. *Biochem. J.* **1989**, *249*, 573-579.

(85) Gillette, J. R. *Drug Metab. Rev.* **1982**, *13*, 941-961.

(86) Dunnick, J. K.; Prejean, J. D.; Haseman, J.; Thompson, R. B.; Giles, H. D.; McConnell, E. E. *Fundam. Appl. Toxicol.* **1982**, *2*, 114-120.

(87) Huxtable, R. J.; Bowers, R.; Mattocks, A. R.; Michnicka, M. In *Biological Reactive Intermediates-IV*; Witmer, C. M., Snyder, R. R., Jollow, D. J., Kalf, G. F., Kocsis, J. J., Sipes, I. G., Eds.; Plenum Press: New York, 1991; pp 605-612.

(88) Riley, R. J.; Roberts, P.; Coleman, M. D.; Kitteringham, N. R.; Park, B. K. *Br. J. Clin. Pharmacol.* **1990**, *30*, 417-426.