Biochemical Pharmacology, Vol. 28, pp. 2941–2946
Pergamon Press Ltd. 1979. Printed in Great Britain.

0006-2952/79/1001-2941 $02.00/0

VARYING EFFECTS OF SULFHYDRYL NUCLEOPHILES ON ACETAMINOPHEN OXIDATION AND SULFHYDRYL ADDUCT FORMATION

ALAN R. BUCKPITT*, DOUGLAS E. ROLLINS†‡ and JERRY R. MITCHELL§

Section on Clinical Pharmacology and Metabolism, Office of the Director of Intramural Research, National Heart, Lung and Blood Institute, Bethesda, MD 20014, U.S.A.

(*Received* 21 *October* 1978; *accepted* 23 *March* 1979)

Abstract—The effects of glutathione, cysteine, *N*-acetylcysteine, cysteamine, α-mercaptopropionylglycine and methionine on the NADPH-dependent metabolism and covalent binding of acetaminophen have been examined in mouse liver microsomal incubations. With the exception of methionine, all of the nucleophiles decreased covalent binding by forming adducts with the electrophilic metabolite of acetaminophen. The adducts were measured quantitatively by high pressure liquid chromatography. In contrast to glutathione, *N*-acetylcysteine and α-mercaptopropionylglycine, both cysteamine and cysteine in high concentrations also decreased covalent binding of acetaminophen through another mechanism, inhibition of the formation of the reactive acetaminophen metabolite. These results indicate that both inhibition of metabolite formation and detoxification of metabolite by sulfhydryl adduct formation are mechanisms that can be important in reducing acetaminophen toxicity in overdosed patients treated with these nucleophiles.

Following the initial demonstrations that glutathione and cysteine decrease the covalent binding of the reactive metabolite formed from acetaminophen, and that cysteine, cysteamine and dimercaprol effectively protect against acetaminophen-induced hepatic injury *in vivo* [1–4], several nucleophiles were shown to effectively reduce hepatocellular necrosis after acetaminophen overdose [5–7]. The mechanisms that are of importance in thiol protection of the hepatocyte from acetaminophen-induced injury are not well-defined; such studies have been hampered by the lack of an adequate quantitative procedure for measuring the thiol conjugates of acetaminophen. The recent development of a high pressure liquid chromatographic method has enabled us to monitor the production of acetaminophen-thiol conjugates, as well as to measure NADPH-dependent disappearance of acetaminophen from *in vitro* microsomal incubations [8]. We now report that, for six sulfhydryl nucleophiles tested, only methionine does not form an acetaminophen-thiol conjugate, and that cysteine and cysteamine are potent inhibitors of the microsomal activation of acetaminophen.

* Supported by a National Heart, Lung and Blood Institute Fellowship 1F32-HL05236. Present address: Clinical Pharmacology Branch, National Cancer Institute, Building 10, Room 6N119, Bethesda, MD 20014.

† Staff Fellow, Pharmacology Research Associate Program, National Institutes of General Medical Sciences. Present address: Division of Clinical Pharmacology, University of Kansas Medical Centre, Kansas City, KS 66103, and Veterans Administration Medical Centre, Kansas City, MO 64128.

‡ Send reprint requests to: Douglas E. Rollins, M.D., Ph.D., Division of Clinical Pharmacology, Department of Medicine, University of Kansas Medical Centre, Rainbow Boulevard at 39th., Kansas City, KS 66103, U.S.A.

§ Present address: Department of Medicine, Baylor College of Medicine, Houston, TX 77030.

MATERIALS AND METHODS

Radiochemical. [^{14}C]-(ring)-4-Hydroxyacetanilide (acetaminophen) (sp. act. 1.33 mCi/m-mole) and [^{14}C-methyl]-L-methionine (sp. act. 12.0 mCi/m-mole) were obtained from New England Nuclear, Boston, MA. Radiolabeled acetaminophen was shown to be greater than 99.9 per cent pure by thin-layer radiochromatography and by high pressure liquid chromatography (h.p.l.c.) as described previously [8]. [^{14}C]Methionine was 80 per cent pure as determined by radiochromatography on Avicel F using a solvent system of ethanol–water (70:30) and was used without further purification. [^{35}S]Cysteamine (β-mercaptoethylamine) (sp. act. 4.6 mCi/m-mole) was at least 90 per cent pure when purchased from Amersham/Searle, Arlington Heights, IL, and was used without further purification.

Chemicals. Unlabeled acetaminophen was purchased from Eastham Kodak, Rochester, NY. Reduced glutathione, *N*-acetyl-L-cysteine, cysteine HCl, L-methionine, NADP, glucose-6-phosphate and $MgCl_2$ were obtained from the Sigma Chemical Co., St. Louis, MO. Glucose-6-phosphate dehydrogenase was obtained from Boeringer Mannheim, Indianapolis, IN. α-Mercaptopropionylglycine was purchased from Pfaltz & Bauer, Stamford, CT. Spectro quality solvents for use in the h.p.l.c. were purchased from Matheson, Coleman & Bell, Norwood, OH. All other reagents were the best commercially available.

Microsomal incubations. Hepatic microsomes were prepared from male, National Institutes of Health, general purpose mice (23–28 g) by previously described methods [1].

The incubation mixture contained the following in a final volume of 3 ml: 75 mM KCl, 20 mM sodium phosphate, 15 mM $MgCl_2$, 6 mg of microsomal protein and an NADPH-generating system consisting of 0.83 mM NADP, 20 mM glucose-6-phosphate and 4.0 I.U. glucose-6-phosphate dehydrogenase.

[^{14}C]Acetaminophen (final sp. act. 400–600 dis./min./nmole), [^{14}C]methionine (final sp. act. 1450 dis./min./nmole), [^{35}S]cysteamine (final sp. act. 800 dis./min./nmole), unlabeled acetaminophen and unlabeled nucleophiles were added as indicated. The incubation mixture was prepared on ice, the NADPH-generating system was added last and the incubations were transferred to a Dubnoff metabolic shaker. The mixture was incubated at 37° for 10 min during which time the production of the relative metabolite of acetaminophen (as measured by covalent binding) [1] and glutathione conjugate production [8,9] were constant. At 10 min, the incubation vessels were transferred to an ice bath and 3 ml of ice-cold spectro-quality methanol was added to each vessel. The contents of the incubation vessels were centrifuged for 20 min at 1000 g. The supernatant fraction was filtered through a 0.5 μ Millipore filter and an aliquot of either 500 or 1000 μl was reduced to dryness under a stream of nitrogen. Each sample was reconstituted in 200 μl of glass distilled in water prior to high pressure liquid chromatographic analysis.

Covalent binding assay. After the supernatant fraction was removed for high pressure liquid chromatographic analysis, the remaining protein precipitate was analyzed for the amount of covalently bound acetaminophen metabolite by procedures described previously [1]. The extracted protein was dissolved in 1 N NaOH (60°for 15 min), an aliquot was taken for protein determination [10], and an aliquot was taken for liquid scintillation counting. Each sample was counted in 15 ml ACS (Amersham/Searle) for 10 min in a Searle Mark III liquid scintillation counter using an automatic external standard to correct for quenching. As reported in this paper, the covalent binding of the acetaminophen metabolite is presented as the amount of cofactor-dependent binding minus the amount of non-specific binding (less than 1 per cent of the level of the cofactor samples) that occurs in those incubations containing no cofactor.

High pressure liquid chromatography. The high pressure liquid chromatographic analysis of acetaminophen and its glutathione, cysteine and *N*-acetylcysteine conjugates has been described previously [8]. A Waters Associates (Milford, MA) M6000A pump, U6K loop injector, M440 UV detector (254 nm filter), a C_{18} μBondapak reverse phase column (30 × 0.39 cm) and a mobile phase of 12.5% methanol/1% acetic acid/86.5% water (by vol.) at a flow rate of 1.0 ml/min were used for the quantitation of acetaminophen and its glutathione, cysteine, *N*-acetylcysteine and α-mercaptopropionylglycine conjugates. In incubations containing cysteamine, acetaminophen was quantitated using a mobile phase of 20% methanol/1% glacial acetic acid/79% water containing 0.005 M 1-heptane sulfonic acid (PIC B-7, Waters Associates) at a flow rate of 1.5 ml/min.

One hundred μl of the reconstituted sample was injected onto the h.p.l.c. column. As acetaminophen and its conjugates eluted from the column (monitored with the UV detector), they were collected in scintillation vials, ACS counting fluid was added, and each sample was counted as described previously. The amount of acetaminophen or acetaminophen conjugate in each incubation was calculated on the basis of the radioactivity eluting from the h.p.l.c. column. Acetaminophen metabolism was determined by subtracting the amount of acetaminophen remaining in an incubation which contained cofactor from the amount in a corresponding non-cofactor incubation. Thus, four incubations were required for each experimentally determined value. One incubation contained cofactor and nucleophile, one contained cofactor with no nucleophile (nucleophile control), a third contained nucleophile and no cofactor, and a fourth did not contain nucleophile or cofactor (cofactor control).

Raney-Nickel hydrolysis. Approximately 25 nmoles of the acetaminophen–cysteamine conjugate was isolated from incubation mixtures containing 0.5 mM [^{14}C]acetaminophen, 0.5 mM cysteamine and cofactor by h.p.l.c. using 20% methanol/80% water containing PIC B-7 as the mobile phase. The sample was lyophilized, and the conjugate was dissolved in a small amount of ethanol and refluxed for 30 min in the presence of 200 mg Raney-Ni (ICN/K&K). Acetaminophen was isolated by extraction with ethyl acetate. The ethyl acetate extract was reduced to dryness under a stream of nitrogen and the residue was redissolved in water. The presence of [^{14}C]acetaminophen was shown by h.p.l.c. in two solvent systems as described previously.

RESULTS

Formation of thiol–acetaminophen conjugates. The microsomal formation of the glutathione, cysteine and N-acetylcysteine conjugates of acetaminophen along with their identification and analysis by h.p.l.c. has been described previously [8].

A u.v. peak, containing ^{14}C radioactivity and eluting at 13.5 min (Fig. 1A) in 20% methanol/80% water containing PIC B-7, was obtained from incubations containing [^{14}C]acetaminophen (0.5 mM), cysteamine (0.5 mM) and cofactor. This peak was identified as the cysteamine conjugate of acetaminophen by the following criteria: (1) neither the u.v. nor the radioactive peaks were present in identical incubations containing no cysteamine (Fig. 1B); (2) incubations of unlabeled acetaminophen (0.5 mM) with [^{35}S]cysteamine (0.5 mM) and cofactor resulted in the elution of a small radioactive peak at 13.5 min (data not shown) which was not present in a microsomal incubation containing only [^{35}S]cysteamine and cofactor; and (3) Raney–Nickel hydrolysis (see Materials and Methods) of the [^{14}C]acetaminophen–cysteamine conjugate isolated by h.p.l.c. yielded [^{14}C]acetaminophen as determined by h.p.l.c. in two mobile phases.

High pressure liquid chromatography of an aliquot from an incubation containing [^{14}C]acetaminophen (0.05 mM), liver microsomes, an NADPH-generating system and α-mercaptopropionylglycine (0.1 mM) in 12.5% methanol/1% glacial acetic acid/86.5% water yielded both a u.v. and a radioactive peak (Fig. 2A), eluting at 21.7 min, that was not present (Fig. 2B) in identical incubations which did not contain α-mercaptopropionylglycine.

No conjugate could be detected in incubations of acetaminophen and methionine. Panels A-D of Fig. 3 show the elution of radioactivity from incubations of cofactor and (A) [^{14}C]acetaminophen plus methionine,

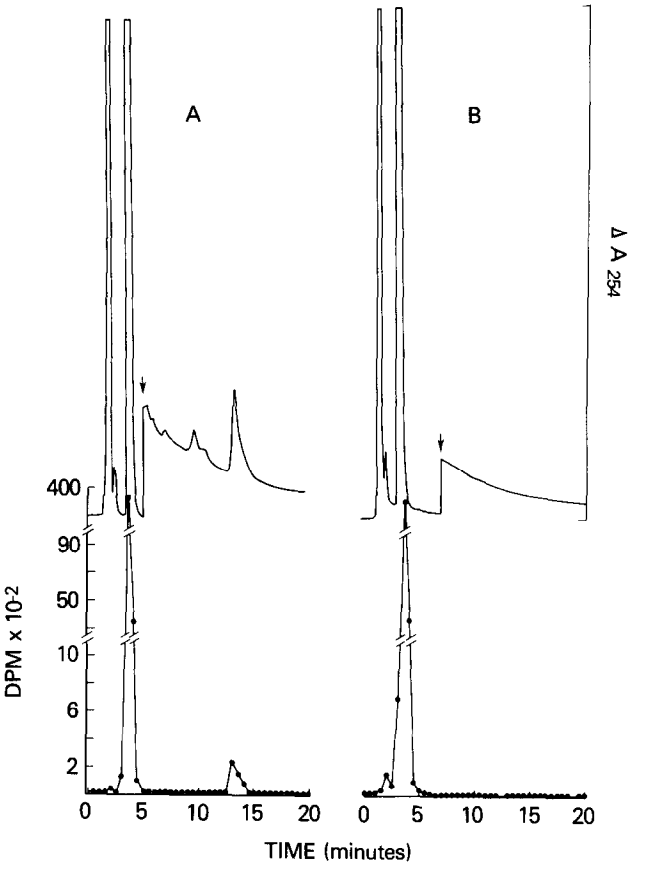

Fig. 1. High pressure liquid chromatography and radioactive elution profile of a [^{14}]acetaminophen–cysteamine conjugate. One hundred μl of a sample prepared from a microsomal incubation containing [^{14}C]acetaminophen (0.5 mM), an NADPH-generating system and either (A) 0.5 mM cysteamine or (B) no cysteamine was injected onto a C_{18} μ Bondapak column using 20% methanol/0.005 M PIC B-7/80% water as the mobile phase at a flow rate of 1.5 ml/min. Ultraviolet absorbance was measured at 254 nm; the arrow indicates a 5-fold increase in detector sensitivity. Radioactivity was measured by collecting fractions of the column eluate every 30 sec for liquid scintillation counting.

(B) [^{14}C] acetaminophen and no methionine, (C) acetaminophen plus [^{14}C]methionine, and (D) [^{14}C]methionine. The mobile phase was 12.5% methanol/1% glacial acetic acid/86.5% water. High pressure liquid chromatography of identical samples in 10% methanol/1% glacial acetic acid/89% water containing PIC B-7 at a flow rate of 1.0 ml/min yielded similar results. Furthermore, thin-layer chromatography of an aliquot from an incubation of [^{14}C]acetaminophen, cofactor and methionine on Avicel F (500 μm, Analtech) in *n*-propanol–water (70 30, v/v), followed by scintillation counting of 0.5 cm sections of the plate, showed that all of the radioactivity chromatographs with acetaminophen (R_f 0.9).

Effects of nucleophiles on the covalent binding, conjugate production and metabolism of acetaminophen. The rates of covalent binding, conjugate production and cofactor-dependent metabolism of acetaminophen were determined in samples prepared by the simultaneous incubation of [^{14}C]acetaminophen (0.05 mM), nucleophile (0.1 mM) and cofactor (Fig. 4). With the exception of methionine, all of the nucleophiles significantly reduced ($P < 0.05$, Student's two-tailed '*t*'-test) the rates of covalent binding of acetaminophen when compared to controls without nucleophile. Cysteine was the least effective inhibitor of convalent binding (52 per cent of control); glutathione, *N*-acetylcysteine and α-mercaptopropionylglycine were approximately equipotent (34 per cent of control) whereas cysteamine was the most potent inhibitor (27 per cent of control).

Detectable amounts of conjugate were formed when [^{14}C]acetaminophen (0.05 mM) was incubated with cofactor and 0.1 mM glutathione, α-mercaptopropionylglycine, *N*-acetylcysteine of cysteine (Fig. 4). Although cysteamine formed a conjugate with acetaminophen when the concentrations of the substrates were equimolar, no conjugate could be detected from these incubations where the concentration of cysteamine was double that of acetaminophen.

Of the nucleophiles tested, only cysteine and cysteamine significantly decreased the rate of metabolism of acetaminophen. In the experiment shown in Fig. 4, cysteine reduced the metabolism of acetaminophen to 62 per cent of the control level (no nucleophile) and cysteamine reduced the metabolism to 46 per cent of the control level. These results were confirmed in a

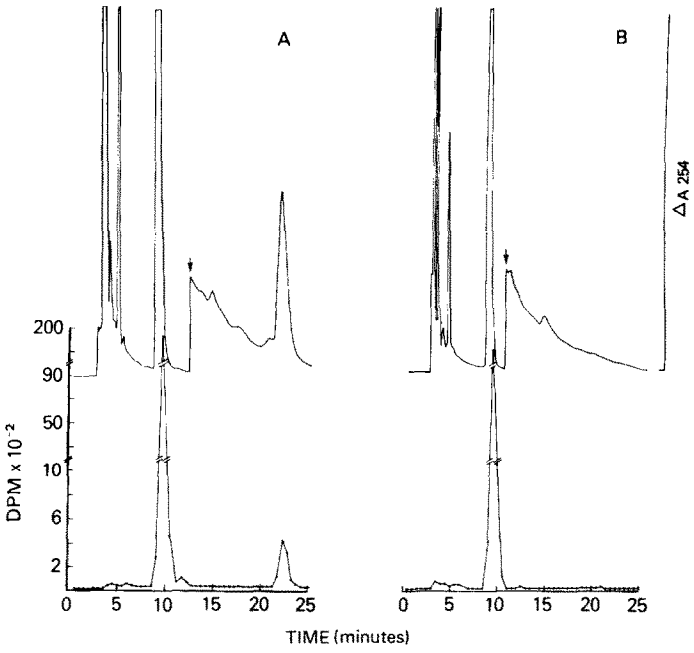

Fig. 2. High pressure liquid chromatography and radioactive elution profile of a [^{14}C]acetaminophen–α-mercaptopropionylglycine conjugate. One hundred μl of a sample prepared from a microsomal incubation containing [^{14}C]acetaminophen (0.05 mM), an NADPH-generating system and either (A) α-mercaptopropionylglycine (0.1 mM) or (B) no α-mercaptopropionylglycine was injected into a C_{18} μBondapak column using 12.5% methanol/1% glacial acetic acid/86.5% water at a flow rate of 1.0 ml/min. Ultraviolet absorbance (top half) was measured at 254 nm; the arrow indicates a 5-fold increase in detector sensitivity. Radioactivity (bottom half) was measured by collecting fractions of the column eluate every 30 sec for liquid scintillation counting.

repeat experiment which again showed that cysteamine and cysteine reduced the total metabolism of acetaminophen to about 60 per cent of the control level (data not shown).

DISCUSSION

The concepts that the liver microsomal mixed function oxygenases metabolize acetaminophen to a highly reactive electrophile that subsequently binds to tissue macromolecules and that the amount of this metabolite covalently bound in the liver can be correlated with the severity of hepatic necrosis are well established. Glutathione levels in the liver must be low before a significant amount of covalent binding and necrosis occurs; thus, acetaminophen-induced hepatic necrosis appears to be dependent upon sulfhydryl levels in the liver [2,11]. These observations led to the first rational treatment of acetaminophen poisoning [7].

Sulfhydryl nucleophiles may inhibit acetaminophen-induced hepatic injury by several mechanisms. Glutathione, cysteine and *N*-acetylcysteine decrease the covalent binding of acetaminophen (Fig. 4) and form easily detectable conjugates. Glutathione is the naturally occuring sulfhydryl thought to be responsible for the detoxification of many highly electrophilic compounds [12–14]. It appears to act solely through nucleophilic conjugation with the electrophilic acetaminophen metabolite, and this reaction appears to be catalyzed by a group of soluble enzymes in the liver [9]. Although glutathione would appear to be an excellent choice in the treatment of acetaminophen poisoning, it penetrates the hepatocyte poorly and must be given in massive doses to be effective [15]. *N*-acetylcysteine has been shown to decrease the toxicity of acetaminophen in animals [16], apparently by acting in a manner similar to glutathione (Fig. 4).

Unlike glutathione and *N*-acetylcysteine, cysteine may be acting by a variety of mechanisms to decrease acetaminophen toxicity. Cysteine not only forms a conjugate with acetaminophen *in vitro*, but it also inhibits the NADPH-dependent metabolism of acetaminophen, thereby decreasing the production of the highly reactive acetaminophen metabolite. *In vivo*, cysteine is a direct precursor of glutathione and had been shown to completely restore liver glutathione after acetaminophen overdose [17]. This combination of actions may explain why cysteine is highly effective in preventing acetaminophen-induced liver necrosis in animals [2,3,11,17].

Even though cysteamine had been used effectively to prevent the liver injury associated with high doses of acetaminophen, its mechanism of action has not been clearly defined. Two proposals have been made: (1) that cysteamine forms a non-toxic sulfhydryl conjugate with the reactive acetaminophen metabolite, and (2) that cysteamine acts by inhibiting the cytochrome P-450-mediated metabolism of acetaminophen. Although cysteamine increases the plasma half-life of acetanilide (a compound that is metabolized primarily by liver hydroxylation) and decreases the proportion of glutathione-derived conjugates of acetaminophen [18], it does

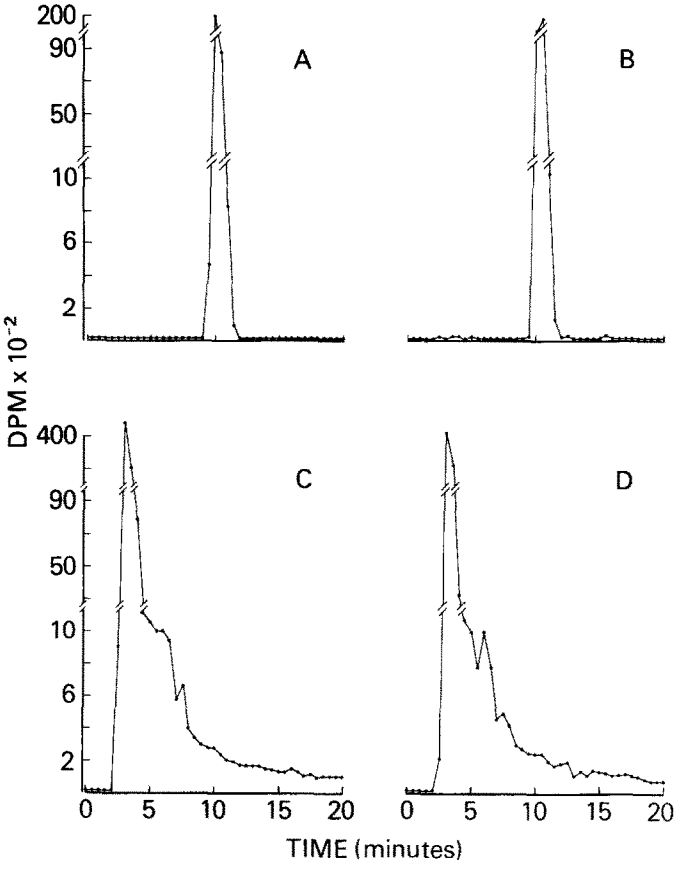

Fig. 3. Radioactive elution profiles of samples from incubations of [^{14}C]acetaminophen and [^{14}C]methionine. One hundred μl of a sample prepared from a microsomal incubation containing an NADPH-generating system and (A) [^{14}C]acetaminophen (0.5 mM) and methionine (0.5 mM), (B) [^{14}C]acetaminophen (0.5 mM) and no methionine, (C) acetaminophen (0.5 mM) and [^{14}C]methionine (0.5 mM), and (D) no acetaminophen and [^{14}C]methionine (0.5 mM) was injected onto a C_{18} μBondapak column under the same conditions as described in Fig. 2. Fractions of the column eluate were collected every 30 sec. for liquid scintillation counting.

not inhibit the cytochrome P-450-dependent demethylation of ethylmorphine [4]. Our results (Figs. 1 and 4) show that cysteamine forms a previously undetected conjugate with acetaminophen, and in high concentrations also decreases the rate of acetaminophen metabolism. Thus, both mechanisms should be operative *in vivo*, with thier respective importance dependent on the hepatic concentration of cysteamine relative to the concentration of acetaminophen.

Recently, Labadarios *et al* [19,20] reported that α-mercaptopropionylglycine also affords significant protection against the hepatotoxic effects of acetaminophen in mice without forming a detectable conjugate with the acetaminophen metabolite. Thus, this thiol appeared to have a potentially very interesting mechanism of protection. From our studies, however, α-mercaptopropionylglycine appears to act similarly to glutathione or *N*-acetylcysteine. α-Mercaptopropionylglycine inhibits the covalent binding of acetaminophen by forming a sulfhydryl conjugate not detected *in vivo*, and it has no effect on the rate of acetaminophen metabolism.

Although methionine appears to be mildly effective in reducing the hepatotoxicity of overdoses of acetaminophen [21,22], it did not reduce the rate of covalent binding of the toxic metabolite *in vitro* (Fig. 4), decrease the rate of metabolism of acetaminophen, or form detectable amounts of conjugate (Fig. 3). These results provide an explanation for the inferior protection against acetaminophen liver injury in overdosed patients provided by methionine relative to cysteamine and *N*-acetylcysteine [21–23] because methionine apparently does not act directly but serves only as a presursor for the synthesis of glutathione and sulfate.

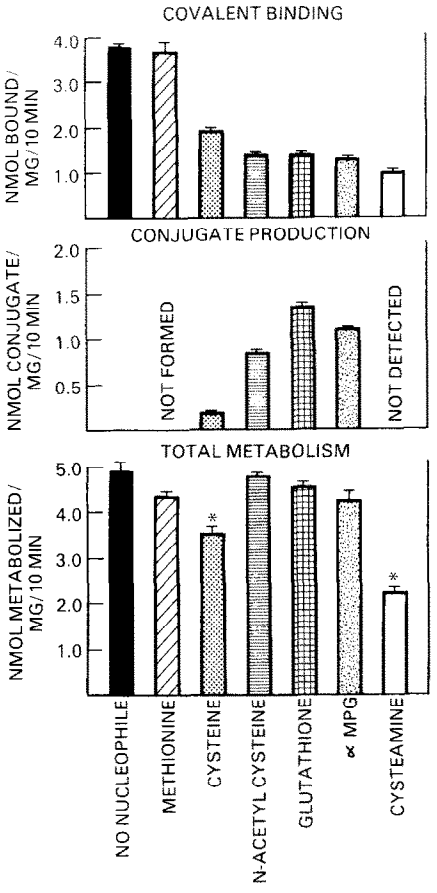

Fig. 4. Effects of several nucleophiles on the rates of covalent binding, conjugate production and metabolism of [^{14}C]acetaminophen. Liver microsomes and [^{14}C]acetaminophen (0.05 mM) were incubated with or without the indicated nucleophile (0.1 mM) as described in Materials and Methods. All incubations were performed simultaneously. The data are the means ± S.E. of three incubations.

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