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Studies of the Mechanism of Metabolism of Thioacetamide S-Oxide by Rat Liver Microsomes

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SUMMARY

The major amino acid adduct formed during microsomal metabolism of thioacetamide *S*-oxide has been identified as *N*-ε-acetyllysine. This same product is formed *in vivo* in the livers of rats administered thioacetamide [Dyroff, M. C., and R. A. Neal, *Cancer Res.* **41**:3430–3435 (1981)]. Acetamide, another product of tioacetamide *S*-oxide metabolism by rat liver microsomes, and the amino acid adduct, *N*-ε-acetyllysine, appear to be derived from a common intermediate formed during oxidative metabolism of thioacetamide *S*-oxide. The microsomal, flavin-containing monooxygenase (EC 1.14.13.8; *N*,*N*-dimethylaniline *N*-oxidizing) is the enzyme predominantly responsible for the metabolism of thioacetamide *S*-oxide by rat liver microsomes. The addition of 1 mM concentrations of glutathione or *N*-acetylcysteine to the *in vitro* incubations had only a small effect on the covalent binding of radioactivity from thioacetamide *S*-oxide to protein. On the other hand, 1 mM concentrations of spermidine or spermine significantly decreased the levels of protein binding. Experiments using $H_2{}^{18}O$ or $^{18}O_2$ indicated that the oxygen atoms present in the metabolite acetamide and the acetyl group of *N*-ε-acetyllysine were derived predominantly, if not solely, from water. Additional studies have shown that the formation of *N*-ε-acetyllysine resulting from the incubation of thioacetamide *S*-oxide with rat liver microsomes and bovine serum albumin does not proceed by way of an *N*-ε-acetamidinolysine intermediate. Oxidation of thioacetamide *S*-oxide by hydrogen peroxide in the presence of bovine serum albumin resulted in covalent binding of radioactivity to the bovine serum albumin. The amino acid adducts generated during the oxidation of thioacetamide *S*-oxide by hydrogen peroxide were pH-dependent, with *N*-ε-acetyllysine being the predominant product formed at pH 7.4 and *N*-ε-acetamidinolysine being the major product formed at pH 10.5.

INTRODUCTION

The thiono sulfur-containing compound, thioacetamide, causes centrilobular hepatic necrosis in rats after acute administration (1, 2). Chronic oral administration of thioacetamide causes bile duct proliferation (3, 4), liver cirrhosis (3, 5), and nodular liver tumors (3, 5). Thioacetamide is rapidly metabolized *in vivo* in the rat to sulfate (6), acetamide (7), and thioacetamide *S*-oxide (8). Although the metabolite acetamide also causes liver tumors in rats, much larger doses are required as compared to thioacetamide (9). Additionally, liver cirrhosis is not observed in acetamide-treated animals (9). The metabolite thioacetamide *S*-oxide produces a more severe and earlier onset of necrosis than does an equimolar dose of thioacetamide (2). Chronic administration of thioacetamide *S*-oxide also causes toxic responses similar to those observed with thioacetamide (10).

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The available evidence indicates that thioacetamide *S*-oxide is an obligatory intermediate in the formation of acetamide from thioacetamide both *in vivo* (8) and *in vitro* (11). These data also indicate that thioacetamide *S*-oxide must be further metabolized in order to form acetamide (11), covalently bind to macromolecules (11, 12), and exert its hepatotoxic effects (2). Covalent binding of radioactivity to rat liver macromolecules has been detected following administration of tritium- and carbonyl carbon-labeled thioacetamide (7, 8, 11). However, negligible covalent binding of radioactivity is detected after the administration of [^{35}S]thioacetamide to rats (8). The radioactivity which becomes covalently bound to liver macromolecules following administration of [1-^{14}C]thioacetamide or [^{3}H]thioacetamide has been shown not to be the result of hydrolysis of the metabolite acetamide to acetate with subsequent incorporation into endogenous macromolecules by way of intermediary metabolism (8).

The structure of the major amino acid adduct formed *in vivo* in rat liver after thioacetamide administration

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has recently been identified as *N*-ε-acetyllysine (13). In the present study, we have examined the structure of the major amino acid adduct formed with bovine serum albumin during the rat liver microsomal metabolism of thioacetamide *S*-oxide. Both the cytochrome P-450-containing monooxygenases (14) and the flavin-containing monooxygenase (15, 16) have been reported to oxidize sulfur-containing compounds, including thioacetamide. We have examined the relative contribution of these two enzymes to the microsomal metabolism of thioacetamide *S*-oxide to acetamide and to a product which is covalently bound to bovine serum albumin. The chemical mechanisms of metabolism leading to the formation of acetamide and the major amino acid adduct, *N*-ε-acetyllysine, have also been investigated.

MATERIALS AND METHODS

Chemicals. [^{3}H]Thioacetamide *S*-oxide (specific activity 2.18 mCi/mmole) was prepared by the oxidation of [^{3}H]thioacetamide with hydrogen peroxide as described previously (8). The [^{3}H]thioacetamide (specific activity 25 mCi/mmole) was synthesized from [^{3}H]acetonitrile (3.75 Ci/mmole; New England Nuclear Corporation, Boston, Mass.) as described by Porter and Neal (11). The radiopurity of the [^{3}H]thioacetamide *S*-oxide was greater than 98% as determined by thin-layer chromatography (11). Ethyl [^{3}H]acetimidate hydrochloride (specific activity 2.27 mCi/mmole) was synthesized from [^{3}H]acetonitrile (3.42 Ci/mmole; New England Nuclear Corporation) by the method of Pinner (17). ^{18}O-Enriched water (30.3 atom %, normalized) was obtained from ICN Pharmaceuticals (Irvine, Calif.), and ^{18}O-enriched gas (99 atom %) was the product of Miles Biochemicals (Elkhart, Ind.). Dimethylformamide dimethylacetal and 3% OV17 on Chromosorb W(HP) were obtained from Pierce Chemical Company (Rockford, Ill.). TENAX GC (60/80 mesh) was obtained from Supelco Inc. (Bellefonte, Pa.), and Type 13X Molecular Sieve was the product of Packard Instrument Company, Inc. (Downers Grove, Ill.). LK5D silica gel thin-layer chromatography plates were obtained from Whatman Inc. (Clifton, N. J.). *N*-ε-Acetyllysine was obtained from Vega Biochemicals (Tucson, Ariz.), 7-ethoxycoumarin was from Aldrich Chemical Company (Milwaukee, Wisc.), and glucose 6-phosphate was the product of Boehringer Mannheim (Indianapolis, Ind.). Metyrapone, cadaverine, putrescine, spermidine, spermine, glutathione, *N*,*N*-dimethylaniline, NADP$^+$, pronase, microsomal leucine aminopeptidase, bovine serum albumin, and glucose 6-phosphate dehydrogenase were from Sigma Chemical Company (St. Louis, Mo.).

Preparation of microsomes. Liver microsomes were prepared from unfasted, male, Sprague-Dawley rats weighing 150–200 g. The animals were killed by decapitation, and the livers were perfused *in situ* with cold 1.15% KCl, excised, and placed in ice-cold 1.15% KCl. Because of the sensitivity of the microsomal flavin-containing monooxygenase to thermal inactivation (16), all subsequent steps in the isolation of microsomes were carried out at 0°–4°. The microsomal pellet obtained by the previously described method (18) was resuspended in 0.1 M potassium pyrophosphate buffer (pH 7.4) containing 1 mM EDTA and 20 μM butylated hydroxytoluene, and recentrifuged at 105,000 × *g* for 60 min. The resulting pellet was overlaid with 10 mM Tris-HCl (pH 7.4) containing 20% glycerol and 1 mM EDTA, and was stored at −70°.

In vitro incubations. All incubations were carried out at 37° for 60 min in a total volume of 1 ml in 50 mM sodium phosphate buffer (pH 7.4) containing 5 mM $MgCl_2$ and an NADPH-generating system consisting of 10 mM glucose 6-phosphate, 0.5 mM NADP$^+$, and glucose 6-phosphate dehydrogenase (1 unit/ml). The [^{3}H]thioacetamide *S*-oxide concentration in the incubation media was 2 mM unless otherwise noted. All reactions were started by the addition of microsomes (2 mg of microsomal protein per milliliter of incubation medium). For the incubations carried out in the presence of $H_2{}^{18}O$, all reagents except the microsomes were dissolved in the ^{18}O-enriched water. The incubations in the presence of $^{18}O_2$ utilized Thunberg tubes.

Analytical methods. Protein concentrations were determined by the biuret method (19), using bovine serum albumin as standard. Cytochrome P-450 was quantitated by the method of Omura and Sato (20), and cytochrome P-450 activity was assayed by measuring 7-ethoxycoumarin deethylation as described by Greenlee and Poland (21). Activity of the microsomal flavin-containing monooxygenase was assayed by measuring *N*,*N*-dimethylaniline *N*-oxidation at pH 8.4 as described by Gold and Ziegler (22). Metabolites of thioacetamide *S*-oxide were separated and quantitated by the thin-layer chromatographic procedure of Porter and Neal (11). Covalent binding of radioactivity to microsomal protein and bovine serum albumin was measured by liquid scintillation counting of samples which had been dialyzed against 50 mM sodium phosphate buffer (pH 7.4) containing 1% sodium dodecyl sulfate until the radioactivity in the dialysate was at background levels.

Identification of the amino acid adduct of thioacetamide S-oxide formed with bovine serum albumin. [^{3}H] Thioacetamide *S*-oxide and rat liver microsomes were incubated for 60 min in the presence of bovine serum albumin (10 mg/ml). At the termination of the incubation, the microsomes were removed by centrifugation (105,000 × *g* for 60 min), and the supernatant was dialyzed aganst 50 mM *N*-ethyl morpholine buffer (pH 7.4). The major amino acid adduct of thioacetamide *S*-oxide bound to bovine serum albumin was isolated by enzymatic digestion to constituent amino acids and subsequent ion-exchange chromatography as described previously (13). The adduct was identified by gas chromatography/mass spectrometry after derivatization with dimethylformamide dimethylacetal (23). The mass spectrum of the adduct was obtained using a Ribermag 10-10b GC/MS[2] and a column (2 mm × 6 foot) of 3% OV-17 on Chromosorb W(HP). The chromatographic conditions were as follows: injector, 150°; column, 200° for the first 5 min, then temperature programmed at 15°/min to a final temperature of 250°. The carrier gas of He was maintained at a flow rate of 20 ml/min. The mass spectral analysis was performed with an electron energy of 70 eV,

[2] The abbreviation used is: GC/MS, gas chromatograph/mass spectrometer.

an ionizing current of 200 μamp, and an ion source temperature of 180°.

^{18}O Studies. Following a 60-min incubation of thioacetamide *S*-oxide with rat liver microsomes in a medium enriched with $H_2{}^{18}O$ or an atmosphere enriched with $^{18}O_2$, acetamide was separated from the substrate and other metabolites by the thin-layer chromatographic procedure of Porter and Neal (11). The acetamide was extracted from the silica gel of the thin-layer plate with 2 ml of methanol. This solution was concentrated to 20 μl under a gentle stream of nitrogen, and 1 μl was analyzed for ^{18}O enrichment of acetamide by gas chromatography/mass spectrometry. The analysis was performed on a column (2 mm × 6 foot) of TENAX (60/80 mesh) in a Ribermag 10-10b GC/MS operated in the electron impact mode with the following conditions: injector, 160°; column 150° (isothermal). The carrier gas of He was maintained at a flow rate of 20 ml/min. The mass spectral analysis was performed using an electron energy of 70 eV, an ionizing current of 100 μamp, and an ion source temperature of 140°. The incubation medium and headspace of the incubations conducted in the Thunberg tubes were analyzed separately by gas chromatography/mass spectrometry to determine the percentage enrichment with $H_2{}^{18}O$ and $^{18}O_2$, respectively. ^{18}O Enrichment of water was analyzed on a column (2 mm × 6 foot) of TENAX (60/80 mesh). The chromatographic conditions were as follows: injector, 110°; column 80° (isothermal). The carrier gas of He was maintained at a flow rate of 20 ml/min. The mass spectral analysis was performed with an electron energy of 70 eV, ionizing current of 200 μamp, and an ion source temperature of 140°. Analysis of $^{18}O_2$ enrichment in the headspace over the incubation utilized a column (2 mm × 6 foot) of Molecular Sieve 13X. The chromatographic conditions were as follows: injector, 90°; column, 70° (isothermal). The carrier gas of He was maintained at a flow rate of 20 ml/min. The mass spectral analysis was performed using the conditions described above for analysis of $H_2{}^{18}O$.

Incorporation of ^{18}O into the adduct *N*-ε-acetyllysine formed with bovine serum albumin during incubation of thioacetamide *S*-oxide with rat liver microsomes in an incubation medium enriched with $H_2{}^{18}O$ was also examined using gas chromatography/mass spectrometry. In these experiments *N*-ε-acetyllysine was isolated from the bovine serum albumin and analyzed for ^{18}O incorporation using an OV-17 column and the Ribermag 10-10b GC/MS as described above.

Hydrogen peroxide-generated covalent binding of [^{3}H]thioacetamide S-oxide to bovine serum albumin. Hydrogen peroxide (2 mM) was incubated for 24 hr with 2 mM [^{3}H]thioacetamide *S*-oxide in the presence of 1% bovine serum albumin in buffers of pH 7.4 (50 mM Tris-HCl), 9.2 (50 mM sodium borate), and 10.5 (50 mM sodium borate). Covalent binding to protein was determined by dialysis as described above. The modified proteins isolated from the incubations at the three pH values were also subjected to acid hydrolysis and enzymatic digestion in order to determine the structure of the amino acid adducts which were formed. Aliquots of the modified proteins were hydrolyzed *in vacuo* with 6 N HCl for 24 hr at 110°. Nonvolatile radioactivity present after acid hydrolysis was determined by evaporating aliquots of the samples to dryness under a stream of nitrogen, resuspending the residue in 1 ml of water, and liquid scintillation counting of the samples after adding 10 ml of ACS scintillation cocktail. The enzymatic digests of the [^{3}H] thioacetamide *S*-oxide-modified bovine serum albumin were analyzed for amino acid adducts using an amino acid analyzer as described previously (13).

RESULTS

Bovine serum albumin incubated with [^{3}H]thioacetamide *S*-oxide and rat liver microsomes was enzymatically digested to constituent amino acids as described under Materials and Methods. The enzymatic digest was analyzed on an amino acid analyzer for ninhydrin-positive material and for radioactivity (Fig. 1). Of the radioactivity which was covalently bound to bovine serum albumin, 93% eluted as a single symmetrical peak in the region of the acidic amino acids. The fractions comprising this peak of radioactivity were pooled and lyophilized. The

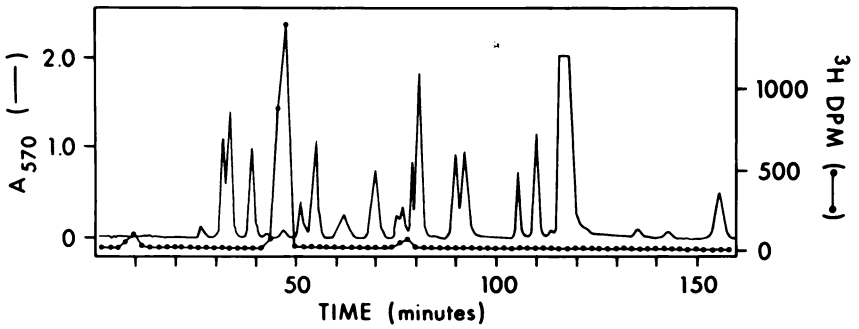

FIG. 1. *Ion-exchange chromatography on a Beckman 121 amino acid analyzer of the amino acid adducts of thioacetamide S-oxide formed with bovine serum albumin during incubation with rat liver microsomes*

The incubation conditions and the procedures used to isolate the amino acid adducts are described under Materials and Methods. One aliquot of the enzymatic digest of bovine serum albumin was applied to the amino acid analyzer and monitored for ninhydrin-positive material (——). A separate aliquot was subsequently applied to the analyzer column, effluent fractions were collected, and radioactivity was determined by liquid scintillation counting (●——●). The standardization of the timed fraction collection to the automated ninhydrin analysis was established by injection of a reference amino acid of known retention time (aspartic acid, 28 min), collection of 1-min timed fractions, and examination of these fractions for ninhydrin-positive material. The radioactivity profile was then adjusted for the difference in retention times observed for automated analysis and manual fraction collection.

residue was derivatized for analysis by gas chromatography and gas chromatography/mass spectrometry using the procedures described prviously (13). Gas chromatography/mass spectrometry (Fig. 2) indicated that this major amino acid adduct formed with bovine serum albumin during the *in vitro* microsomal metabolism of thioacetamide *S*-oxide is *N*-ε-acetyllysine.

Thioacetamide is a substrate for the microsomal flavin-containing monooxygenase (15) and for the cytochrome P-450-containing monooxygenase systems (11, 14). In these studies, the enzyme which is responsible for the metabolism of thioacetamide *S*-oxide by hepatic microsomes isolated from untreated rats has been examined. The cytochrome P-450 monooxygenase can be selectively inhibited by incubation in an atmosphere enriched with CO or by the use of competitive inhibitors such as metyrapone or SKF 525-A. The flavin-containing monooxygenase, which is exceptionally heat-sensitive (16), can preferentially be inactivated by preincubating hepatic microsomes at 37° for 45 min in the absence of NADPH (24).

The metabolism of thioacetamide *S*-oxide to acetamide and to a product which covalently binds to bovine serum albumin was determined under the various conditions intended to decrease preferentially the activity of either the cytochrome P-450-containing or the flavin-containing monooxygenase systems. The results of these experiments (Table 1) indicated that preincubation of the microsomes at 37° for 45 min had no effect on cytochrome P-450 monooxygenase activity (ethoxycoumarin deethylation) as compared to control values, but that the formation of acetamide and covalent binding of radioactivity to bovine serum albumin were inhibited, as was the flavin-containing monooxygenase activity (*N*-N-dimethylaniline oxidation). Ethoxycoumarin deethylation was inhibited by approximately 80% in an atmosphere of 80:20 CO/O_2 and by 45% in the presence of 0.5 mM metyrapone. However, neither the carbon monoxide-containing atmosphere nor the presence of metyrapone decreased *N,N*-dimethylaniline oxidation, acetamide formation, or covalent binding of radioactity from thioacetamide *S*-oxide to bovine serum albumin when compared to control incubations.

Increasing the concentration of bovine serum albumin in the microsomal incubations (Fig. 3) resulted in an increase in the amount of covalent binding of radioactivity to bovine serum albumin and a corresponding decrease in the amount of acetamide formation. No alteration in the total amount of thioacetamide *S*-oxide metabolized was detected with increasing concentrations of bovine serum albumin.

In order to determine whether the oxygen atom of the metabolite acetamide was derived from molecular oxygen or from water, thioacetamide *S*-oxide and rat hepatic

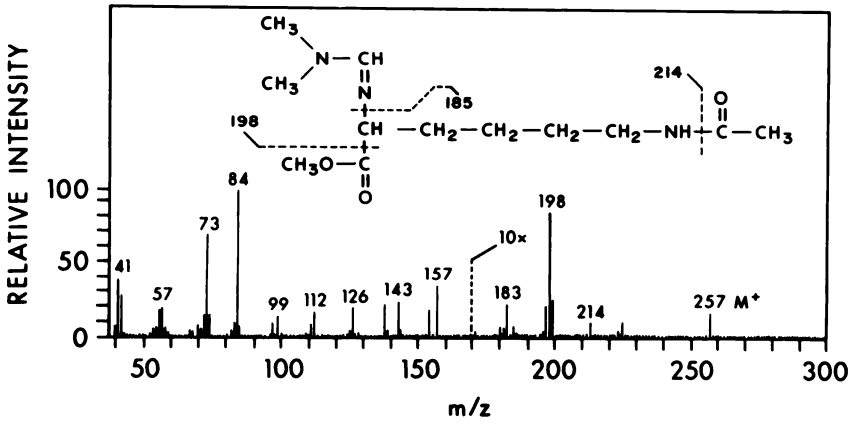

FIG. 2. *Mass spectrum of the dimethylformamide dimethylacetal-derivatized ^3H-labeled amino acid adduct of [^3H]thioacetamide S-oxide isolated from bovine serum albumin*

The major amino acid adduct of bovine serum albumin formed during microsomal metabolism of [^{3}H]thioacetamide *S*-oxide was isolated as described in Fig. 1, derivatized, and subjected to gas chromatography/mass spectrometry as described under Materials and Methods. The mass spectrum is identical with authentic *N*-α-(*N'*,*N'*-dimethylaminomethylene)-*N*-ε-acetyllysine methyl ester (structure shown). The relative intensity scale has been expanded 10 times above m/z 170.

TABLE 1

Examination of the metabolism of thioacetamide S-oxide by the cytochrome P-450 and the microsomal flavin-containing monooxygenase systems

Ethoxycoumarin deethylation and *N,N*-dimethylaniline oxidation were determined as described under Materials and Methods in separate incubations in the absence of thioacetamide *S*-oxide at pH 7.4 and 8.4, respectively. Acetamide formation and covalent binding of radioactivity from [^{3}H]thioacetamide *S*-oxide to macromolecules were determined in the same incubation, as described under Materials and Methods. All incubations contained bovine serum albumin (10 mg/ml). The values shown are the means ± standard deviations for three separate incubations.

Treatment	Ethoxycoumarin deethylation	Dimethylaniline *N*-oxidation	Acetamide formation	Covalent binding to macromolecules
		% control		
Control	100 ± 18.1	100 ± 12.5	100 ± 2.0	100 ± 2.2
Preincubation (37°/45 min)	98.9 ± 13.8	30.8 ± 6.1	48.3 ± 4.6	15.4 ± 4.3
Metyrapone, 0.5 mM	55.3 ± 17.8	140.9 ± 36.9	113.8 ± 10.8	117.6 ± 8.7
CO/O_2 (80:20)	19.8 ± 2.8	90.4 ± 6.0	117.2 ± 18.9	102.1 ± 16.1

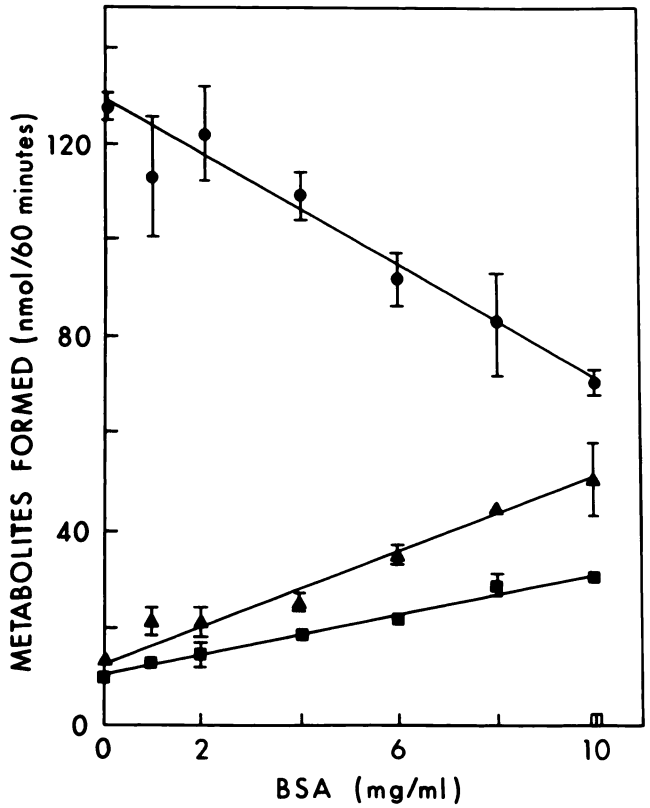

FIG. 3. *Effect of increasing the concentration of bovine serum albumin (BSA) on the microsomal metabolism of [^{3}H]thioacetamide S-oxide to acetamide and to radioactivity covalently bound to bovine serum albumin*

The formation of acetamide (●——●), polar metabolites (▲——▲), and radioactivity covalently bound to bovine serum albumin (■——■) were measured as described under Materials and Methods. Covalent binding of [^{3}H]thioacetamide S-oxide to bovine serum albumin was also determined in incubations lacking NADP$^+$ (□——□). The values shown are the means ± standard deviations of triplicate incubations.

microsomes were incubated in a buffer enriched with $H_2{}^{18}O$ or in an atmosphere enriched with $^{18}O_2$. Gas chromatography/mass spectrometry of the acetamide isolated from these incubations (Table 2) indicated that, in the presence of $H_2{}^{18}O$, 81% of the maximal theoretical incorporation of ^{18}O into the product was obtained. Acetamide formed in the presence of an atmosphere enriched with $^{18}O_2$ did not show any evidence of ^{18}O incorporation.

In order to determine the source of the oxygen atom present in the acetyl group of the amino acid adduct, *N*-ε-acetyllysine, bovine serum albumin was incubated with hepatic microsomes and thioacetamide *S*-oxide in a medium enriched with $H_2{}^{18}O$. *N*-ε-Acetyllysine was isolated from the bovine serum albumin as described under Materials and Methods and analyzed for ^{18}O incorporation by gas chromatography/mass spectrometry. The mass spectrum obtained was compared with the spectrum of the same adduct formed in an incubation carried out in the absence of any ^{18}O enrichment. The result of this experiment (Table 2) indicated that 105% of the maximal theoretical incorporation of ^{18}O into *N*-ε-acetyllysine occurred in the presence of $H_2{}^{18}O$. Because of this theoretical incorporation of ^{18}O from $H_2{}^{18}O$ into *N*-ε-acetyllysine, the corresponding experiment utilizing enrichment of the incubation with $^{18}O_2$ was not performed.

The effect of various thiol- and amine-containing compounds on the binding of radioactivity from [^{3}H]thioacetamide *S*-oxide to bovine serum albumin was also investigated. Increasing the concentration of glutathione present in the incubation medium from 1 μM to 1 mM resulted in only a slight decrease in the amount of covalent binding of radioactivity to bovine serum albumin as compared to control values (Table 3). A higher concentration of glutathione (10 mM) resulted in a reduction of covalent binding to less than 25% of control values. This inhibition of binding was subsequently shown to be the result of a non-enzymatic reduction by glutathione of thioacetamide *S*-oxide to thioacetamide (data not shown).

Incubation of thioacetamide *S*-oxide with rat liver microsomes and bovine serum albumin in the presence of 1 mM concentrations of the polyamines putrescine, spermidine, and spermine resulted in decreased levels of covalent binding (Table 4). There was no detectable decrease in the amount of acetamide formed in these

TABLE 2

^{18}O Enrichment of acetamide and N-ε-acetyllysine during rat hepatic microsomal metabolism of thioacetamide S-oxide

The incubation procedures and analysis of ^{18}O incorporation are described under Materials and Methods.

Expt.	Source (atom % excess ^{18}O)	Product analyzed	Observed atom % enrichment (% of theoretical atom % excess)
1	$H_2{}^{18}O$ (20.1)	Acetamide	16.3 (81.4%)
2	$^{18}O_2$ (42.9)	Acetamide	0 (0%)
3[a]	$H_2{}^{18}O$ (20.6)	*N*-ε-acetyllysine	21.7 (105.1%)

[a] Incubation mixture included bovine serum albumin (10 mg/ml).

TABLE 3

Effect of glutathione and N-acetyl cysteine on the covalent binding of radioactivity from [^{3}H]thioacetamide S-oxide to bovine serum albumin and microsomal macromolecules

The incubation procedures are described under Materials and Methods. Each incubation contained bovine serum albumin (10 mg/ml). The values shown are the means ± standard deviation of three separate incubations. The statistical significance was estimated by Student's *t*-test.

Addition	Covalent binding (nmoles bound/60 min)[a]
None	43.7 ± 1.5 (100 ± 3.4)[b]
N-Acetylcysteine, 1 mM	45.4 ± 5.0 (104 ± 11.4)
GSH	
1 μM	45.8 ± 0.5 (104 ± 1.1)
10 μM	40.9 ± 0.9[c] (94 ± 2.0)
100 μM	41.3 ± 1.0[c] (94 ± 2.3)
1 mM	39.6 ± 0.8[d] (91 ± 1.0)
10 mM	10.1 ± 0.1[e] (23 ± 0.2)
Boiled microsomes	1.8 ± 0.1[e] (4 ± 0.2)

[a] Calculated on the basis of the specific activity of the [^{3}H]thioacetamide *S*-oxide.

[b] Values expressed as percentage of control.

[c] Statistically significant ($p < 0.05$) as compared with controls.

[d] Statistically significant ($p < 0.01$) as compared with controls.

[e] Statistically significant ($p < 0.005$) as compared with controls.

TABLE 4

Effect of various amine-containing compounds on the covalent binding of radioactivity from [^{3}H]thioacetamide S-oxide to bovine serum albumin and microsomal macromolecules

The incubation procedures are described under Materials and Methods. Each incubation medium contained bovine serum albumin (10 mg/ml). The values shown are the means ± standard deviation of three separate incubations. The statistical significance was estimated by Student's *t*-test.

Addition	Covalent binding (nmoles bound/60 min)[a]	
None	49.2 ± 2.8	(100 ± 5.7)[b]
N-α-Acetyllysine, 1 mM	58.9 ± 3.5	(109 ± 7.1)
Cadaverine, 1 mM	49.0 ± 1.4	(100 ± 2.8)
Putrescine, 1 mM	43.0 ± 3.0[c]	(87 ± 6.1)
Spermidine, 1 mM	28.9 ± 0.9[d]	(59 ± 1.8)
Spermine, 1 mM	30.8 ± 8.0[d]	(63 ± 16.3)
Boiled microsomes	1.6 ± 0.2[d]	(3 ± 0.4)

[a] Calculated on the basis of the specific activity of the [^{3}H]thioacetamide *S*-oxide.

[b] Values expressed as percentage of control.

[c] Statistically significant ($p < 0.01$) as compared with controls.

[d] Statistically significant ($p < 0.005$) as compared with controls.

incubations as compared to the control incubation (data not shown).

A possible intermediate in the formation of the amino acid adduct, *N*-ε-acetyllysine, is *N*-ε-acetamidinolysine. This acetamidine-containing intermediate could conceivably be formed by reaction of the ε-amino group of lysine with the postulated reactive metabolite of thioacetamide *S*-oxide, thioacetamide *S*-dioxide (2, 11). In order to examine the possible involvement of an acetamidine intermediate in the formation of *N*-ε-acetyllysine, bovine serum albumin was amidinated with ethyl [^{3}H]acetimidate hydrochloride using the procedure of Wofsy and Singer (25). An aliquot of the [^{3}H]amidinated bovine serum albumin was subjected to the same enzymatic digestion procedure which was used in the isolation of the thioacetamide *S*-oxide-derived adduct, *N*-ε-acetyllysine. A second portion of the amidinated bovine serum albumin was subjected to 24-hr acid hydrolysis *in vacuo* using 6 N HCl at 110°. Under these conditions, radioactivity bound to liver cytosolic protein following *in vivo* administration of [^{3}H]thioacetamide is released as acetate, and *N*-ε-acetyllysine is converted to lysine and acetate (13). Both the enzymatic digestion and the acid hydrolysate of the amidinated protein were analyzed for ninhydrin-positive material and for radioactivity using an amino acid analyzer. All of the radioactivity present in the acid hydrolysate of bovine serum albumin eluted from the amino acid analyzer column in a symmetrical peak after lysine, in the reported position of *N*-ε-acetamidinolysine (25). Analysis of the enzymatic digest of the amidinated protein indicated that 80% of the radioactivity bound to bovine serum albumin eluted from the column in the position of *N*-ε-acetamidinolysine. Less than 3% of the radioactivity eluted from the amino acid analyzer column in the position of authentic *N*-ε-acetyllysine.

Work by Browne and Kent (26) has indicated that the product formed during ethyl acetimidate modification of protein lysyl groups is highly pH-dependent. At a pH of 10, *N*-ε-acetamidinolysine is the predominant product. At a lower pH of 7 or 8, *N*-ε-ethylacetimidate-lysine is formed which subsequently is either hydrolyzed to regenerate free lysine or can undergo nucleophilic attack by ammonia to form the acetamidine derivative of lysine. Thioacetamide *S*-dioxide, the postulated reactive metabolite of thioacetamide and thioacetamide *S*-oxide (2, 11), is similar in structure to ethyl acetimidate with the SO_2^- moiety of thioacetamide *S*-dioxide substituting for the ethoxy group of ethyl acetimidate. If the reaction of thioacetamide *S*-dioxide with protein lysyl groups is analogous to the reaction of ethyl acetimidate with protein, then the products formed between bovine serum albumin and the reactive oxidative metabolite of [^{3}H]thioacetamide *S*-oxide should be pH-dependent. Incubation of 2 mM [^{3}H]thioacetamide *S*-oxide and 2 mM hydrogen peroxide in the presence of bovine serum albumin (10 mg/ml) for 24 hr at 37° resulted in the covalent binding of 154, 732, and 1050 nmoles of radioactivity per 10 mg of bovine serum albumin in incubations conducted at a pH of 7.4, 9.2, and 10.5, respectively. Incubation of bovine serum albumin with [^{3}H]thioacetamide *S*-oxide in the absence of hydrogen peroxide resulted in the binding of 37 and 738 nmoles at pH values of 7.4 and 10.5, respectively. The stability of the bound radioactivity to acid hydrolysis was examined. *N*-ε-Acetyllysine is hydrolyzed under acid conditions to acetate and lysine (13), but *N*-ε-acetaminidinolysine is stable (25). The amounts of radioactivity which remained bound to bovine serum albumin after acid hydrolysis were 22%, 52%, and 72% of the total amounts bound for the incubations conducted at pH values of 7.4, 9.2, and 10.5, respectively. The [^{3}H]thioacetamide *S*-oxide-modified bovine serum albumin was also enzymatically digested and examined for modified amino acids using an amino acid analyzer (Fig. 4). Of the radioactivity bound to bovine serum albumin, 66%, 55%, and 35% was recovered as a product having the same retention time as authentic *N*-ε-acetyllysine from the incubations carried out at pH values of 7.4, 9.2, and 10.5, respectively. Radioactivity eluting in the position of *N*-ε-acetamidinolysine accounted for 14%, 24%, and 40% of the total amount bound to bovine serum albumin in the incubations carried out at pH 7.4, 9.2, and 10.5, respectively.

DISCUSSION

The major amino acid adduct formed during the rat hepatic flavin-containing monooxygenase-catalyzed metabolism of thioacetamide *S*-oxide *in vitro* is *N*-ε-acetyllysine. *N*-ε-Acetyllysine accounts for at least 93% of the radioactivity bound to bovine serum albumin under the conditions of these experiments. This same adduct accounts for approximately 70% of the radioactivity covalently bound to liver cytosolic protein 12 hr following administration of thioacetamide to rats (13).

The addition of increasing amounts of bovine serum albumin to incubations containing rat liver microsomes, [^{3}H]thioacetamide *S*-oxide and an NADPH-generating system, resulted in higher levels of covalent binding of radioactivity to bovine serum albumin and a corresponding decrease in the amount of acetamide formed. However, there was no detectable decrease in the total

amount of thioacetamide *S*-oxide metabolized. These results indicate the existence of a metabolite of thioacetamide *S*-oxide which can either be hydrolyzed to form acetamide or react with protein forming predominantly *N*-ε-acetyllysine. The previously postulated thioacetamide *S*-dioxide (2, 11) could be this reactive intermediate.

The chemical mechanism of the metabolism of thioacetamide *S*-oxide to acetamide and to *N*-ε-acetyllysine is of interest. There are two most probable routes to the formation of acetamide. One of these involves the hydrolysis of a reactive intermediate of thioacetamide *S*-oxide such as thioacetamide *S*-dioxide (2, 11). Alternatively, one could postulate an intramolecular rearrangement of thioacetamide *S*-dioxide forming a transient oxathiirane cyclic intermediate which could decompose to form acetamide and release an oxidized species of sulfur. This latter mechanism has been proposed by Walter and Bauer (27) for the decomposition of *N*-substituted thioamide *S*-dioxides to *N*-substituted amides. In the case of hydrolysis of thioacetamide *S*-dioxide to acetamide, one would expect the oxygen of acetamide to be derived from water. In the case of rearrangement of an oxathiirane intermediate of thioacetamide *S*-dioxide to acetamide, one would *a priori* anticipate that half of the acetamide molecules would contain an oxygen atom derived from the substrate thioacetamide *S*-oxide and the other half would contain an oxygen atom derived from atmospheric oxygen which was metabolically incorporated into thioacetamide *S*-oxide to form thioacetamide *S*-dioxide. The ^{18}O incorporation experiments described in Table 2 indicate that water is the predominant (if not the sole) source of the oxygen atom which becomes incorporated into acetamide. Figure 5 shows the proposed chemical mechanism for the flavin-containing monooxygenase-catalyzed metabolism of thioacetamide *S*-oxide to acetamide. The first step is the oxidation of thioacetamide *S*-oxide to the hypothetical intermediate thioacetamide *S*-dioxide. Water attack on the thioacetamide *S*-dioxide with loss of HSO_2^- (sulfoxylate anion) would lead to the formation of acetamide. The postulated sulfoxylate anion would probably be rapidly oxidized to sulfate by an, as yet, undetermined mechanism. Sulfate is the major sulfur-containing metabolite of thioacetamide excreted in the urine (6).

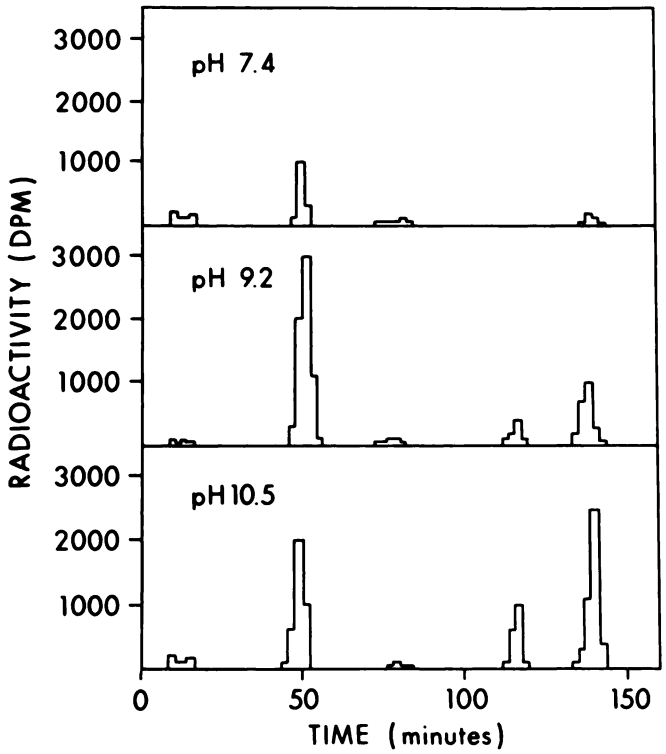

FIG. 4. *Ion-exchange chromatography on a Beckman 121 amino acid analyzer of the enzyme digest of bovine serum albumin incubated with hydrogen peroxide and thioacetamide S-oxide at pH values of 7.4, 9.2, and 10.5*

The incubation conditions and experimental procedures are described under Materials and Methods. Radioactivity was determined in timed fractions of the column effluent. Equal amounts of the enzyme digests of bovine serum albumin incubated with thioacetamide *S*-oxide and H_2O_2 at the three different pH values were applied to the amino acid analyzer column.

The results obtained using ethyl acetimidate-modified bovine serum albumin indicate that the formation of the protein adduct, *N*-ε-acetyllysine, does not proceed via an *N*-ε-acetamidinolysine intermediate. The data in Table 2 indicate that the oxygen atom of the acetyl group of *N*-ε-acetyllysine formed in an incubation of thioacetamide *S*-oxide with rat liver microsomes in the presence of bovine serum albumin is derived from water. The experiments utilizing hydrogen peroxide oxidation of thioacetamide *S*-oxide in the presence of bovine serum albumin indicated that the structures of the amino acid adducts formed are pH-dependent. The results of these experiments further suggest that the chemical mechanism of the flavin monooxygenase-catalyzed metabolism of thioacetamide *S*-oxide to form *N*-ε-acetyllysine derivatives of bovine serum albumin appears to be somewhat analogous to the reaction of ethyl acetimidate with protein at neutral pH (26). Figure 6 shows the proposed mechanism for the formation of *N*-ε-acetyllysine during incubation of thioacetamide *S*-oxide with rat liver microsomes in the presence of NADPH and bovine serum albumin. The initial reaction involves the formation of the hypothetical thioacetamide *S*-dioxide intermediate. Thioacetamide *S*-dioxide is then subjected to nucleophilic attack by the ε-amino group of lysine to form a tetrahedral intermediate which subsequently breaks down with the elimination

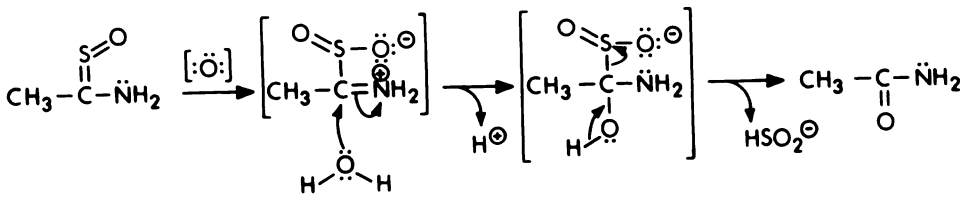

FIG. 5. *Proposed mechanism for the formation of acetamide during rat hepatic microsomal metabolism of thioacetamide S-oxide*

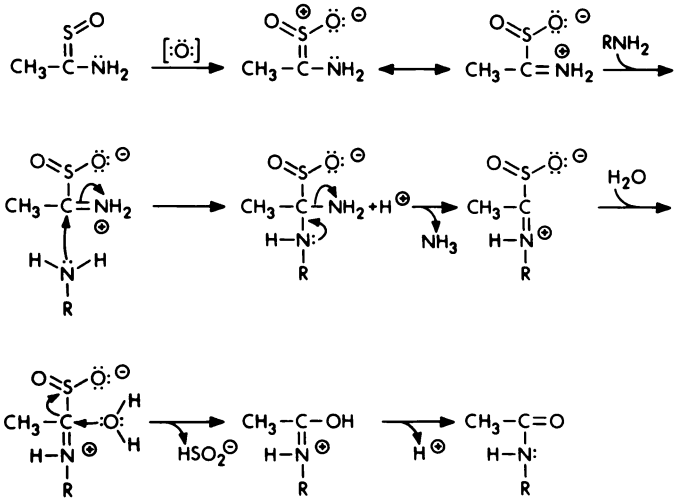

FIG. 6. *Proposed mechanism for the formation of N-ϵ-acetyllysine derivatives of bovine serum albumin during rat hepatic microsomal metabolism of thioacetamide S-oxide*

of NH_2. Attack by water on this imine *S*-dioxide intermediate leads to the formation of *N*-ϵ-acetyllysine with the loss of sulfoxylate anion.

Identification of lysine as the predominant amino acid involved in the covalent binding of the reactive metabolite of thioacetamide *S*-oxide to liver protein *in vivo* (13) and to bovine serum albumin *in vitro* was somewhat unexpected in light of the presence in liver proteins and bovine serum albumin of the more nucleophilic amino acid, cysteine. Because of its increased nucleophilicity in relation to the ϵ-amino groups of lysine, it was hypothesized that glutathione might react with the reactive metabolite of thioacetamide *S*-oxide and decrease the covalent binding to protein. Such a phenomenon has been observed with other xenobiotics which are capable of binding to macromolecules during metabolism (28). In the present experiments, glutathione, over a broad range of concentrations, caused only a slight decrease in the levels of covalent binding of radioactivity from [^{3}H]thioacetamide *S*-oxide to bovine serum albumin. High concentrations of glutathione (10 mM) did result in a marked decrease in the covalent binding of radioactivity to protein, but this was shown to be the result of non-enzymatic reduction of thioacetamide *S*-oxide to thioacetamide, rather than the result of the reaction of glutathione with the reactive intermediate of thioacetamide *S*-oxide. The reduction of thioacetamide *S*-oxide to thioacetamide decreased the amount of the *S*-oxide available for metabolism to the species responsible for the observed covalent binding. In addition, the thioacetamide formed competitively inhibited the further metabolism of thioacetamide *S*-oxide (11).

The addition of 1 mM concentrations of various primary aliphatic amine-containing compounds to the microsomal incubation did result in decreased levels of covalent binding of the metabolic product of thioacetamide *S*-oxide to protein without any change in the amounts of acetamide formed. Increased acetylation of the polyamines putrescine and spermidine is detectable *in vivo* after thioacetamide administration to rats (29). In addition to the enhanced enzymatic acetylation of these aliphatic amines which is detectable after thioacetamide administration to rats (29, 30), it seems possible that some of the increased acetylation observed in these studies is the result of reaction of the polyamines with the reactive metabolite of thioacetamide.

It is interesting to speculate that the covalent binding of the reactive metabolite of thioacetamide and thioacetamide *S*-oxide (perhaps thioacetamide *S*-dioxide) to the ϵ-amino group of lysine may be responsible for some of the toxic effects seen on administration of these compounds *in vivo*. However, with the data currently available it is not possible to detemine what role (if any) the formation of *N*-ϵ-acetyllysine or acetylation of endogenous polyamines play in the acute and chronic toxicity seen on administration of thioacetamide and thioacetamide *S*-oxide to rats.

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