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METABOLISM OF BIS-β-CHLOROETHYL SULFIDE (SULFUR MUSTARD GAS)*

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Abstract—The metabolism of bis-β-chloroethyl sulfide-^{35}S has been investigated in mice, rats and human subjects. In the rodents, the major portion of radioactivity was excreted in the urine within the first 24 hr, whereas in the human considerable radiosulfur was retained for long periods. In the rat only traces of radioactivity were expired, while small amounts were found in the feces. Large amounts of thiodiglycol and of bis-β-chloroethyl sulfone have been detected in the urine, largely as conjugates. Evidence is presented that much of the drug reacts immediately with glutathione, and this complex is excreted. It is concluded that the majority of the radioactivity excreted in the urine represents compounds formed from alkylation by the drug, rather than metabolites formed by enzymic action.

INTRODUCTION

BIS-β-chloroethyl sulfide (mustard gas, Yperite) represents the oldest of the so-called alkylating agents, having been synthesized first in 1822. It received usage as a vesicant during the first World War. Its clinical employment as a tumor inhibitor has been minimal, probably resulting from the inconvenience of handling the volatile, water-insoluble drug, rather than from any lack of effectiveness, since reports[1, 2] suggest that it possesses tumor-inhibiting activity similar to that of the various other "alkylating" compounds.

While a variety of investigators have studied *in vitro* reactions of bis-β-chloroethyl sulfide with various amino acids,[3, 4] proteins[5] and nucleic acid constituents,[6, 7] the degradation of this compound within the mammalian body has been largely neglected.

The present studies of its metabolism were undertaken not only because of an interest in the metabolism of alkylating agents, but also in the belief that such information might give some insight into the mechanism of action of this general class of compounds. It is believed because of its simplicity of structure that bis-β-chloroethyl sulfide represents the ideal compound for such studies.

MATERIALS AND METHODS

Synthesis of sulfur-labeled bis-β-chloroethyl sulfide and possible metabolites

Radioactive bis-β-chloroethyl sulfide was synthesized according to modifications of the method of Boursnell *et al.*[8] by reaction of hydrogen sulfide-^{35}S with stoichiometric amounts of ethylene oxide to yield thiodiglycol, followed by chlorination with

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concentrated hydrochloric acid. The technique differed from that of Boursnell *et al.* in that the hydrogen sulfide generated from barium sulfide-^{35}S was permitted to diffuse into the reaction vessel in a closed system rather than being flushed over with hydrogen, losses being minimized in this way. Usually about 1 m-mole of barium sulfide, containing 10 mC of radioactive sulfide (from the Oak Ridge National Laboratory), was used. The bis-β-chloroethyl sulfide formed was extracted from the acidic solution with low-boiling petroleum ether and, after removal of the organic solvent, was sublimed *in vacuo*. Yields ranged up to 91 per cent. The radioactive product was frozen in sealed ampules until used. The purity of the product was established by melting point, and by ascending chromatography on Whatman no. 1 paper in two non-aqueous systems, chloroform on formamide-saturated paper (R_f, 0·78), and petroleum ether on silicone-impregnated paper (R_f, 0·75). To prevent volatilization of the compounds during counting, the chromatograms were sprayed with collodion-ether.

Radioactive thiodiglycol was prepared by shaking the bis-β-chloroethyl sulfide-^{35}S in 0·1 N sodium hydroxide for 1 hr. Its purity was established by ion exchange chromatography on Dowex-1, as described below.

A number of possible metabolites of bis-β-chloroethyl sulfide were synthesized: sulfoacetic acid;[9] potassium isethionate;[10] bis-β-chloroethyl sulfoxide and sulfone;[11] and thiodiglycolic acid-sulfone.[12]

PROCEDURE FOR METABOLISM STUDIES

Rats

Twenty-two male Wistar rats weighing from 150 to 300 g were injected intravenously with radioactive bis-β-chloroethyl sulfide in one of four solvents. The solvents, propylene glycol, 95% ethanol, dioxane or 20% ethanol–80% Lipomul*, did not cause appreciable hydrolysis of the drug during the period of administration. The dosages employed were from 1 to 5 mg of drug per kg in 1 ml of solvent per kg, solutions being used within 1 min of preparation. The solvents themselves did not produce grossly evident toxicity, except for a brief hematuria that occurred in certain of the animals given propylene glycol. Toxicity resulting from the drug during the period of observation of up to 72 hr consisted of diarrhea, emaciation, convulsions and sloughing at the site of injection. Since toxicity seemed least in the ethanol–Lipomul solvent this solvent was employed in all the later experiments. Urine collections were made by restraining the animals in a rigid frame suspended over the collecting tube. Collections were made during the periods of 0–6, 6–12, 12–24, 24–48 and 48–72 hr and rat urine and feces were analyzed by chromatographic and chemical techniques. In one animal the expired air was passed through successive traps of lead acetate, potassium permanganate, and Pirie's reagent[13] in an effort to collect volatile sulfur compounds, such as sulfides, mercaptans and sulfones.

Mice

Twenty male CAF_1 mice were injected intravenously with radioactive bis-β-chloroethyl sulfide in propylene glycol at a dose of 2 mg/kg. Toxic reactions were observed as with rats. Urine was collected in a metabolism cage at 0–6, 6–12, 12–24 and 24–48 hr and the radioactivity of the urine fractions was measured.

* Lipomul is an emulsion containing 15% (w/v) vegetable oil and 4% anhydrous dextrose, manufactured by the Upjohn Company, Kalamazoo, Mich.

Human subjects

Two human terminal cancer patients were injected intravenously with 5 mg of radioactive bis-β-chloroethyl sulfide dissolved in 5 ml of 95% ethanol. The solution was injected over a 10-min period into the tubing of an infusion apparatus during the administration of isotonic saline.* Total urine output was collected at 6, 12, 24 and 48 hr; aliquots were subjected to chromatography in the usual manner and their radioactivity was measured. Blood samples were taken 1, 3, 5, 15 and 60 min and 6, 12, 24 and 48 hr after termination of the administration. The plasma and cells were checked for total radioactivity.

Separation of urinary metabolites

Two ion exchange resins, Dowex 50-X8, 200–400-mesh, and Dowex 1-X8, 200–400-mesh, were employed for chromatography of the radioactive urine. Dowex-50 cation exchange columns (1 cm × 50 cm) were washed with 4 N NH_4OH, then equilibrated with 0·1 HCl and suitable aliquots of urine were eluted successively with 0·1 N HCl, pH 1; 0·1 M citric acid, pH 5·5; and 4 N NaOH. Volumes of 10–20 ml were collected and suitable aliquots were counted.

Dowex-1 anion exchange columns (1 cm × 50 cm) were washed with 4 N HCl and were equilibrated with 0·1 N NH_4OH, and urine samples were eluted successively with 0·1 N NH_4OH, pH 11; 0·1 N ammonium acetate, buffered to pH 5 with acetic acid; 0·1 N acetic acid, pH 2·8; 0·1 N formic acid, pH 2·3; 0·1 N HCl, pH 1 and finally 4 N HCl (pH about 0).

RESULTS

Blood levels in the human

The plasma and red cells showed only slight radioactivity. Calculation revealed that after several minutes from 80 to 90 per cent of the radioactivity disappeared from the blood. The residual level of radioactivity remained constant in both plasma and cells for at least 2 days, indicating a relatively permanent binding to some blood constituent, possibly protein. Other workers have also found that the alkylating agents disappear very rapidly from blood.[14]

Excretion of radioactivity

Table 1 presents the percentage of radioactivity from bis-β-chloroethyl sulfide excreted over various intervals in the three species studied. The trace of activity found in expired air was too little to permit any chemical identification. It is apparent from Table 1 that the major portion of the drug emerges rather rapidly from the rodent body, either due to an excessive amount over the available reaction sites, or due to the formation of conjugates with small, rapidly-excreted molecules, or possibly by the excretion of large molecules from damaged renal tubules. In the human subject, in which the dosage was of the order of 0·1 mg/kg, rather than the higher amounts (1–5 mg/kg) given the small animals, the urinary excretion of radioactivity was relatively poor over the 48-hr period. This may reflect the lower overall rate of metabolism of the human, or the fact that at lower dosages the molecules reacting first are those more slowly turned over, i.e. proteins, nucleoproteins, etc. Moreover, the patients were in terminal stages of cancer and may well have had pre-existing metabolic disorders, especially anuria and ascites.

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Boursnell *et al.*[15] have reported excretion of radioactivity in bile after administration of bis-β-chloroethyl sulfide-^{35}S. Bile cannulation was performed in two rats, and the bile collected was analyzed by paper chromatography. Radioactivity was detected in neither taurine nor taurocholate.

TABLE 1. EXCRETION OF RADIOACTIVITY AFTER INTRAVENOUS ADMINISTRATION OF BIS-β-CHLOROETHYL SULFIDE-^{35}S

Route of excretion	Period after drug (hr)	Per cent of injected radioactivity		
		Rat	Mouse	Man
Expired air	0–6	0·05	—	—
Feces	0–12	6	—	—
Urine	0–6	48	48	6·4
	6–12	25	22	10·6
	12–24	5	11	5·5
	24–48	9	5	4·0
	48–72	2	4	—

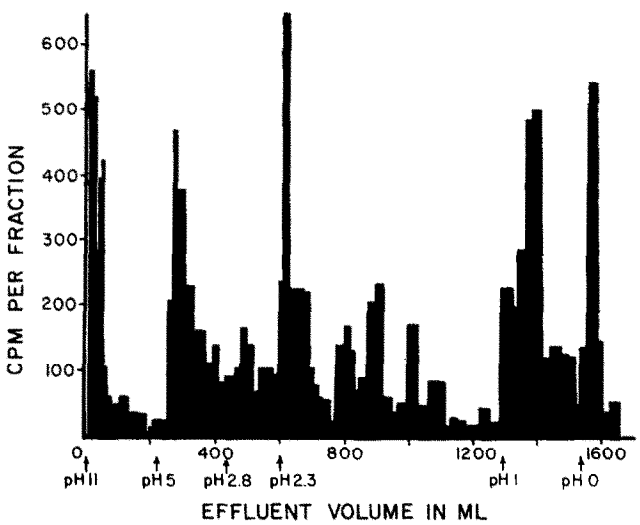

FIG. 1. Eluant pattern from Dowex-1 of sulfur-35 excretion after administration of bis-β-chloroethyl sulfide-^{35}S. Forty-eight-hour sample of human urine.

Chromatographic studies with urine

Preliminary chromatography of rat and human urine on Dowex-50 revealed that 98 per cent of the radioactivity emerged immediately from the column at pH 1; most of the remainder emerged at pH 5·5, a finding which implied that most of the metabolites were present in a neutral or anionic form.

Chromatography on Dowex-1 revealed an excretory pattern characteristic of a host of metabolites. Fig. 1 shows the elution pattern derived from a sample of human urine. The patterns derived from rat and mouse urine were essentially similar to each other but differed from those of the human subjects in showing somewhat lower peaks at pH 2·8 and 2·3. Fig. 2 portrays a representative diagram of radioactivity in rat urine after the administration of either bis-β-chloroethyl sulfide-^{35}S or of an identical

dose of its hydrolysis product thiodiglycol-^{35}S. It appears that some but not all of the metabolites may be derived from the latter rather than from the sulfur mustard.

Indentification of urinary metabolites in the rat

Chromatography on Dowex-1 had revealed the presence, in appreciable amounts, of at least six substances in rat urine. The major radioactive compounds were a neutral or very weakly acidic substance emerging from the column at pH 11 and a more anionic substance emerging at pH 5. A number of likely sulfur compounds were subjected to the same chromatographic scheme in order to determine their elution characteristics.

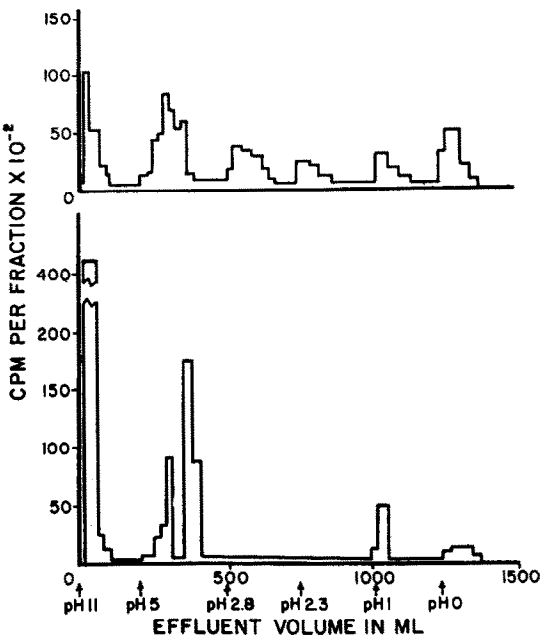

FIG. 2. Eluant pattern from Dowex-1 of sulfur-35 excretion in 6-hr samples of rat urine. Upper: after administration of bis-β-chloroethyl sulfide-^{35}S. Lower: after administration of bis-β-hydroxyethyl sulfide-^{35}S.

Identification of thiodiglycol

Since thiodiglycol emerged immediately from the Dowex-1 column at pH 11, the corresponding major component of urine was analyzed for thiodiglycol by ascending paper chromatography on Whatman no. 1. In four solvent systems: (1) phenol saturated with water (R_f, 0·88); (2) isoamyl alcohol saturated with water (R_f, 0·69); (3) 95% ethanol–NH_4OH 95 : 5 (v/v) (R_f, 0·82); (4) 95% ethanol-concentrated HCl 95 : 5 (v/v) (R_f, 0·88); a portion of the radioactivity present in this fraction had the same R_f as thiodiglycol.

Quantitation of the amount of thiodiglycol present was effected by inverse isotope dilution. To suitable samples of the whole urine was added 0·2 m-moles of carrier thiodiglycol, and the di-*p*-nitrobenzoyl ester was prepared according to McElvain[16]. The ester was identified by melting point and mixed melting point, and was repeatedly recrystallized from absolute ethanol until constant specific activity was reached. In repeated determinations free thiodiglycol accounted for about 6 per cent of the first

fraction or 1·4 per cent of the total radioactivity. A similar value was obtained upon preparation of the di-α-naphthyl carbamate of thiodiglycol.

To detect conjugates of thiodiglycol in urine, whole urine was hydrolyzed by refluxing with dilute hydrochloric acid for 2 hr, after carrier thiodiglycol had been added. The di-α-naphthyl carbamate ester was synthesized and purified from ethanol as above. Thiodiglycol accounted for up to 14·4 per cent of the total radioactive metabolites, a tenfold increase over the free form. Hydrolyses in base and by β-glucuronidase gave 3·0 and 2·6 per cent, respectively, findings which suggest conjugation with glucuronic acid, as well as with other compounds.

Identification of bis-β-chloroethyl sulfoxide

Partial oxidation of the sulfur of bis-β-chloroethyl sulfide produces the sulfoxide. Carrier sulfoxide added to the urine and recrystallized to constant specific activity, as well as carrier sulfoxide added with the isolation of the phenyl mercaptan derivative,[11] showed that up to 0·5 per cent of the activity of whole urine can be accounted for as the sulfoxide.

Identification of bis-β-chloroethyl sulfone or derivatives

Further oxidation of the sulfoxide would yield the sulfone. Carrier sulfone isolated directly from radioactive urine contained 1·0 per cent of the total radioactivity. The urine was then examined for the compound by dehydration to the thioxane, according to a modification of the procedure of Cashmore[17]. Whole urine was refluxed for 6 hr with carrier sulfone in 1·7 N NaOH. The solution was then concentrated and extracted with chloroform. The chloroform was evaporated and the residue recrystallized to constant activity from ethanol. The thioxane formed accounted for 7·4 per cent of the total radioactivity of the rat urine, indicating that the major portion of the sulfone is present as conjugates. A similar experiment in which the thioxane itself (rather than sulfone) was added directly and isolated, revealed that no radioactivity was present as this compound, suggesting that this compound is not normally formed.

Identification of isethionic acid

This substance results from oxidation and splitting of the sulfide with simple hydrolysis of the halogen atoms. It emerged from the columns along with sulfoacetic acid. Whole urine, as well as this fraction, was analyzed for isethionate by inverse isotope dilution, isolating the potassium acetyl derivative.[10] This metabolite accounted for 0·2 per cent of the total.

Identification of inorganic sulfate

Complete oxidation of bis-β-chloroethyl sulfide leads to sulfate formation. This ion emerged, together with the sulfonic acids, from the columns in the pH 0 fraction. Whole urine was examined for sulfate by addition of carrier and recovery as benzidine sulfate after phosphate precipitation with magnesium carbonate–ammonium chloride.[18] Since benzidine sulfate does not recrystallize readily, purification was effected by repeatedly dissolving the precipitate in base, removing the insoluble benzidine, acidification of the supernatant fraction and reprecipitation with fresh benzidine. Again, only traces of activity were detected (0·5 per cent). No appreciable increase in radioactivity as sulfate was observed upon acid hydrolysis, a finding which indicates that no sulfur-35 was present as ethereal sulfate.

Identification of sulfoacetic acid

It was proposed that sulfoacetic acid might result from oxidation of the sulfur and terminal carbon of the molecule after splitting of one carbon–sulfur bond. This highly acidic compound emerged from Dowex-1 with 4 N HCl. Accordingly, that fraction and whole urine were tested for sulfoacetic acid by inverse isotope dilution, and isolation of the aniline salt of sulfoacetic acid–anilide according to McElvain[19]. The compound was identified by melting point and recrystallized repeatedly from 95 per cent ethanol. Only a trace (0·2 per cent) of sulfoacetate was detected. This represented about 10 per cent of the eluted fraction. No appreciable increase in recovery of radioactive sulfoacetate was observed upon acid hydrolysis.

Identification of thiodiglycolic acid

Thiodiglycolic acid, formed by oxidation of both β-carbons of the otherwise intact bis-β-chloroethyl sulfide molecule, was eluted from the Dowex-1 columns at pH 1. The acid was isolated from this fraction and from whole urine as the dianilide derivative after the addition of carrier. It accounted for only 0·2 per cent of the total urinary activity. Isolation of thiodiglycolate after acid hydrolysis accounted for 1·0 per cent, so that apparently 0·8 per cent is present as conjugates.

Identification of sulfone of thiodiglycolic acid

In view of the recovery of bis-β-chroloethyl sulfone and of small amounts of thiodiglycolic acid, it was suspected that the sulfone of thiodiglycolic acid might be detected. Carrier amounts of thiodiglycolic acid sulfone were added to urine which was then extracted repeatedly with ether. After evaporation of the ether the acid was repeatedly recrystallized from hot toluene by adding glacial acetic acid until the acid just dissolved, and then cooling the solution. The sulfone accounted for 4·8 per cent of the urinary activity.

Identification of the reaction products of bis-β-chloroethyl sulfide and its sulfone with cysteine

Since the comparatively simple organic compounds accounted for only one-quarter of the excretion of radioactivity after bis-β-chloroethyl sulfide administration, conjugates with cysteine were investigated. Cysteine was selected for study since it possesses the reactive sulfhydryl group and since its reaction products had already been reported by Hartwell[3]. These two derivatives were isolated as white solids, the formol titration values of which corresponded to a product of two molecules of cysteine reacting with one of bis-β-chloroethyl sulfide or its sulfone. Carrier amounts of the sulfide or sulfone conjugate were mixed with urine, dissolved by the dropwise addition of concentrated HCl and precipitated by the addition of ammonium hydroxide. Reprecipitations were from sodium hydroxide solutions by the addition of acid, and by acetone precipitation. Thirteen precipitations were performed until constant specific activity was reached. No radioactivity was detectable in the cysteine–sulfide conjugate, while a trace (0·2 per cent) was found in the conjugate with the sulfone.

Identification of the reaction products of bis-β-chloroethyl sulfide with glutathione

Since glutathione also has the reactive sulfhydryl group and is found in relatively high concentration in the plasma, the possibility of its interaction with bis-β-chloroethyl sulfide was studied. Several preparations were made with various ratios of glutathione to the drug, but in agreement with Hartwell[3] a gummy oil was repeatedly

obtained. The reaction products, upon paper chromatography in 60% ethanol saturated with potassium chloride or in pyridine–isoamyl alcohol–water (80 : 40 : 70 v/v), seemed to have appreciable amounts of glutathione remaining. The products were largely freed of glutathione by column chromatography on Dowex-1 anion exchange resin. Glutathione conjugates emerged at pH 5, whereas the glutathione itself was eluted with 0·1 N acetic acid. Preparations of the product were mixed with radioactive urine and subjected to such chromatography. The fractions obtained at pH 5 were lyophilized, taken up in minimal volumes of water and repeatedly reprecipitated with acetone or acetone–chloroform to constant specific activity. About 45 per cent of the total radioactivity was recovered in the oil after seven precipitations. Examination of the total urinary excretion curve reveals (Fig. 2) that the pH 5 peak could well contain such a large percentage. Because of the unknown composition of these reaction products, and their hygroscopic nature, the accuracy of this quantitative value must remain in question; both column and paper chromatography failed to separate the radioactivity from the reaction products.

TABLE 2. URINARY METABOLITES 6–12 HR AFTER ADMINISTRATION OF BIS-β-CHLOROETHYL SULFIDE-^{35}S TO RAT

Urinary fraction	Proposed metabolite	Per cent of total urinary radioactivity
Whole urine	Glutathione-bis-β-chloroethyl sulfide conjugates	45·0
Whole urine	Glutathione-bis-β-chloroethyl sulfone conjugates	7·0
Whole urine	Thiodiglycol and conjugates (acid hydrolysis)	14·4
pH 11-eluate	Thiodiglycol	1·4
Whole urine	Bis-β-chloroethyl sulfone and conjugates	7·4
Whole urine	Bis-β-chloroethyl sulfone	1·0
Whole urine	Sulfone of thiodiglycolic acid	4·8
Whole urine	Thioglycolic acid and conjugates	1·0
pH 1-eluate	Thiodiglycolic acid	0·2
Whole urine	Bis-β-chloroethyl sulfoxide	0·5
pH 0-eluate	Inorganic sulfate	0·5
pH 0-eluate	Isethionic acid	0·2
pH 0-eluate	Sulfoacetic acid	0·2
Whole urine	Cysteine-bis-β-chloroethyl sulfone conjugate	0·2

Identification of the reaction product of bis-β-chloroethyl sulfone with glutathione

Since it appeared from the earlier studies that a portion of bis-β-chloroethyl sulfide could be oxidized to a sulfone, the reaction product of this sulfone with glutathione was also investigated. This product was a white crystalline substance similar to that described by Hartwell[3]. Carrier amounts were mixed with radioactive urine and repeatedly precipitated with ethanol and acetone to constant specific activity. About 7 per cent of the activity was accounted for as this substance.

The values for all the identified substances are summarized in Table 2.

Compounds not detected in rat urine

An attempt to detect unchanged bis-β-chloroethyl sulfide in urine by extraction into petroleum ether and application of the reaction of Harley-Mason[20] met with no success, a finding which indicated that amounts of the drug in excess of 5 μg are not present.

It seemed possible that one end of the thiodiglycol molecule might have been oxidized to the carboxylic acid, whereas the other end was not. Accordingly, the

lactone of this acid, hydroxyethylmercaptoacetic acid was prepared according to Hellström[21]. Attempts to isolate this product from rat urine were unsuccessful. Likewise, an attempt to synthesize hydroxyethylsulfonyl acetic acid[22] was unsuccessful. Since the substances seemed of minor interest, these experiments were abandoned.

DISCUSSION

Fig. 3 presents the structure of the various substances which have been found. The metabolites present in largest amount are thiodiglycol, chiefly as acid-labile

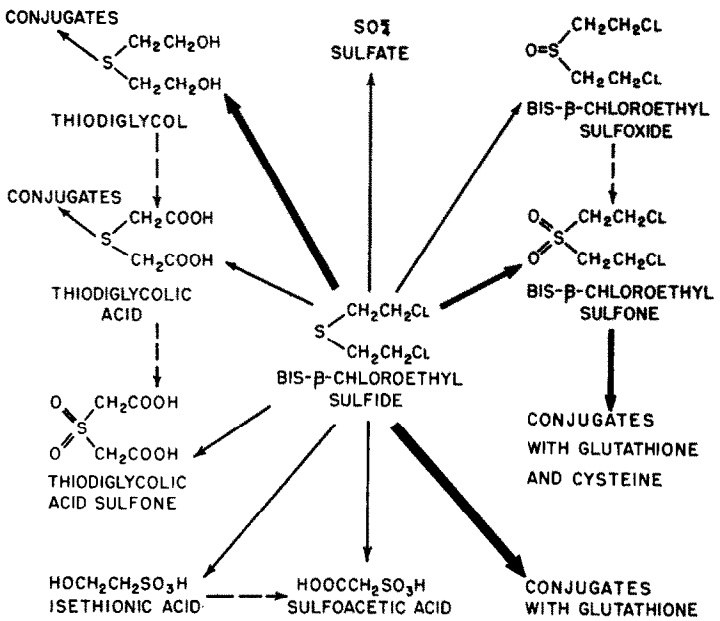

FIG. 3. Structures of the identified urinary metabolites. Heavy arrows indicate major pathways. Dashed arrows indicate possible pathways.

conjugates, and one or more reaction products with glutathione. Since the latter substances might very well be hydrolyzed to thiodiglycol, the two types are not mutually exclusive. In a similar way, conjugates of bis-β-chloroethyl sulfide with proteins, nucleic acids, etc., might also be hydrolyzed to thiodiglycol. The formation of thiodiglycol may very well be non-biochemical in origin, since it is well known that bis-β-chloroethyl sulfide hydrolyzes extensively within minutes in water. As shown in Fig. 2, thiodiglycol itself is metabolized within the mammalian body and yields a peak at pH 11, two peaks emerging at pH 5 and small amounts of highly acidic substances. The finding of trace amounts of sulfate and the less highly oxidized substances, sulfoacetic acid and isethionate, might be merely an indication that some of the thiodiglycol formed is partially or completely oxidized, both at the central sulfur atom and at the terminal carbon atoms. Because of the highly water-soluble nature of thiodiglycol and its conjugates, however, there is apparently little physiological requirement for such oxidation. The failure to detect significant amounts of sulfate rules out any extensive conversion of the drug to hydrogen sulfide, since the mammalian body oxidizes hydrogen sulfide to sulfate.[23]

The evidence suggests that a portion of the drug is oxidized to sulfones, possibly by way of sulfoxides, since two different sulfones and several conjugates have been

detected. These results are in agreement with the finding of the sulfoxide as a metabolite of promazine[24] and of methyl ethyl sulfone as a metabolite of ethanethiol.[25]

Since Kinsey and Grant[26] have shown that up to 50 per cent of bis-β-chloroethyl sulfide reacting with yeast does so with glutathione, the apparently large amount conjugated here is not unexpected. The finding of larger amounts of conjugates of sulfur mustard with glutathione than with cysteine is probably merely indicative of the relatively higher concentration of glutathione in plasma. It is reported that whole rat blood contains 40 mg of reduced glutathione per 100 ml, and red cells even more. This is far in excess of the amount required to react with the dose of drug given. It must be recalled that the glutathione conjugate is not a well-defined substance. In contrast, cysteine is present to the extent of only 0·3 mg per cent in blood. The failure to find appreciable amounts of cysteine conjugates also suggests that if these drugs do react with proteins through the sulfhydryl group, these proteins are not hydrolyzed completely prior to excretion or that they are retained for long periods. The relatively constant level of radioactivity in blood favors the latter viewpoint.

The major metabolites so far identified seem to reveal little with regard to the mechanism of action of the drug since they reflect simply hydrolytic processes, or perhaps an immediate non-specific reaction with glutathione in the circulatory system. There remain large amounts of radioactivity unaccounted for, much of it in highly acidic forms. The nature of conjugates with other amino acids, purines, pyrimidines, etc., is being undertaken by investigations *in vivo*.

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