Metabolism of the Radioprotective Agent Triammonium 2-Dithiocarbamyl-3-dithiocarbonylthiopropanoate in the Mouse

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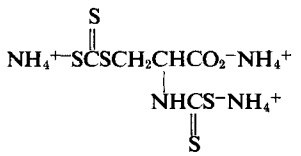
Abstract □ To determine whether one or both of the sulfur-containing functions of the dithiocarbamate trithiocarbonate (I) of cysteine is responsible for its radioprotective ability, ^{35}S-I was synthesized and its metabolism was studied in mice. The major metabolite was the dithiocarbamate of cystine, which was found in the liver, kidney, and spleen. The protein-bound component was also found in these tissues. Other metabolites found were intact I and possibly cystine in the liver and sulfate in the kidney and spleen. ^{35}S-I was rapidly eliminated, and radioactive compounds which appeared in the urine included the dithiocarbamate, unchanged I, sulfate, and an unidentified substance in small amount.

Keyphrases □ Triammonium 2-dithiocarbamyl-3-dithiocarbonylthiopropanoate, radiolabeled—metabolism studied, metabolites determined, mice □ Radioprotective agents—metabolism, metabolic products of triammonium 2-dithiocarbamyl-3-dithiocarbonylthiopropanoate determined, mice □ Radiolabeling—synthesis of triammonium ^{35}S-2-dithiocarbamyl-3-dithiocarbonylthiopropanoate for metabolism study

Appreciable radiation protection in mice, of a higher degree than that conferred by cysteine, has been found for the product (I) of carbon disulfide condensation of cysteine, the dithiocarbamate trithiocarbonate (1). To determine whether one or both of these sulfur-containing groups survives *in vivo*, and may thus be responsible for the radiation protection, a study of the metabolic products of I in mice was carried out. Metabolism studies of other dithiocarbamates showed that this function remains intact *in vivo* (2); little is known of the stability of the trithiocarbonate function to animal metabolism.



I

Distribution and excretion studies with other radiation-protective agents, notably the amino thiols, revealed sizable amounts of these substances bound to protein (3) within a very short time. Excretion is also rapid, 30% of the injected radioactivity of the S-labeled 2-mercaptoethylamine appearing in the urine of dogs in 6 hr. (4). Taurine and sulfate appear to be the major metabolites of 2-mercaptoethylamine (5), with both the thiol and disulfide appearing in the urine.

To determine the distribution of I in such radiosensitive tissues as the liver, kidney, and spleen, it was first necessary to synthesize the compound using ^{35}S-carbon disulfide.

Table I—R_f Values of Sulfur-Containing Compounds in *n*-Butanol–Pyridine–Water (5:3:5)

Compound	R_f	95% Confidence Level
Cystine	0.16	0.02
Cysteic acid	0.27	0.04
Taurine	0.42	0.02
Taurocholic acid	0.74	0.04
Thiourea	0.65	0.01
Sodium sulfate	0.19	0.04
Sodium thiosulfate	0.21	0.04
Potassium thiocyanate	0.71	0.03
Cysteine	0.67	0.03
	0.17	0.04
I	0.79	0.01

EXPERIMENTAL

Triammonium ^{35}S-2-Dithiocarbamyl-3-dithiocarbonylthiopropanoate—^{35}S-Carbon disulfide was obtained[1] and had a radioactivity of 10 mc. ml.$^{-1}$ at the time of use. The ^{35}S-carbon disulfide (1 ml.) was cooled prior to use, in an ice bath, and a small piece of iron rod was placed in the top of the container and held away from the glass seal by a magnet. The container with the ^{35}S-carbon disulfide was attached to a three-necked flask which was evacuated to 20 mm. pressure; the magnet was withdrawn, allowing the rod to break the glass seal, and the ^{35}S-carbon disulfide slowly distilled into the ice-cooled flask and condensed. A solution of L-cysteine (0.968 g.) in 3 ml. of concentrated ammonium hydroxide and 5 ml. of water was added dropwise, and the reaction mixture was stirred for 1 hr. at ice bath temperature. Absolute ethanol (50 ml.) was added, giving a bright-yellow precipitate, which was isolated, washed with cold ethanol, and dried *in vacuo*. A 49% yield was obtained of material having a specific activity of 1.25 mc./mmole.

Tetrasodium Bis(2-dithiocarbamyl-3-thiopropanoate)—Cystine (5 g., 0.02 mole) was dissolved in 25 ml. (0.03 mole) of 5% sodium hydroxide solution, and 20 ml. (0.33 mole) of carbon disulfide was added slowly with stirring at room temperature. After it was stirred for 4 hr., the mixture was allowed to evaporate at room temperature. A 50% yield of yellow solid was obtained, which was washed well with ethanol and dried ($CaCl_2$) and did not give a melting point; R_f (butanol–pyridine–water, 5:3:5) 0.57; $\lambda_{max.}^{H_2O}$ 270 nm. (log $E_{max.}$ = 3.20); $\nu_{max.}^{KBr}$ 1275, 1050, and 945 cm.$^{-1}$.

Anal.—Calc. for $C_8H_8N_2Na_4O_4S_6$: C, 20.00; H, 2.56; N, 5.83. Found: C, 20.02; H, 2.91; N, 5.87.

Paper Chromatography—Paper chromatography was carried out in an ascending system using Whatman No. 1 paper. Samples of known quantity (50–100 mcg./10 λ) were applied to a 25 × 21-cm. sheet and dried by hot air. After being dried, the sheet was rolled into a cylinder and placed in a jar for developing; it was developed at 4° for 24 hr. The solvent system was composed of *n*-butanol, pyridine, and water (5:3:5) (Table I).

Determination of I Stability *In Vitro*—Compound I (100 mcg./10 λ) was dissolved in phosphate buffer at pH values of 6.1, 7.4, and 8.4. The solutions were chromatographed immediately on formation and after standing at room temperature for 1 hr. (Table II). Compounds known to be end-products of cysteine and sulfur metabolism were also chromatographed, and R_f values were determined (Table I). To locate the spots, the chromatogram sheet was cut into strips, each of which was treated with one of the following reagents by dipping: ninhydrin–collidine reagent (6), Grote's reagent (7), *N*-ethylmaleimide–KOH (8), ammoniacal $CuCl_2$–hydroxylamine (9), phosphomolybdic acid (10), $BaCl_2$–$KMnO_4$ (11), and $Fe(NO_3)_3$ (12).

[1] New England Nuclear Corp., Boston, Mass.

Table II—Stability of I *In Vitro*: R_f Values of Breakdown Products

pH	R_f	95% Confidence Level
6.1 and 7.4	0.59	0.04
	0.48	0.02
	0.36	0.04
	0.16	0.02
8.4	0.81	0.03
	0.64	0.08
	0.47	0.03
	0.39	0.01
	0.23	0.01

Table III—Distribution of Radioactivity in Mice

Hours	Percent of Administered Radioactivity—Liver	Kidney	Spleen	Urine	Feces
0.5	17.6 (5.8)[a]	4.7 (1.9)	1.1 (0.6)	53.9 (8.8)	— —
1	9.4 (1.4)	3.3 (1.1)	0.7 (0.3)	51.2 (5.1)	— —
2	9.4 (3.3)	1.4 (0.7)	0.8 (0.7)	52.3 (7.1)	6.3 (3.5)

[a] Numbers in parentheses are standard deviations.

Areas on the I strips giving positive ninhydrin–collidine reagent and Grote's reagent reactions were eluted with 90% ethanol and with 0.1 *N* alcoholic KOH. UV spectra of the eluates were obtained with a spectrophotometer[2].

Determination of I Stability *In Vivo*—The distribution and excretion of ^{35}S-labeled I were investigated at three time intervals after a given dose of compound: at 0.5, 1, and 2 hr. Male Swiss mice (Webster strain) were used; a group of five was used for each determination, and 20–30 were used for each experiment.

^{35}S-I was dissolved in water immediately before use. The mice were given a dose of 10 mg. (0.2 ml.) i.p. of labeled compound by injection and were put into a metabolism cage equipped to separate urine and feces. Food and water were not restricted during the experiment. Urine samples for the first two intervals were collected directly after the mice were sacrificed; all other excreta were collected from the cage.

After the animals were killed at the designated times, the livers, kidneys, and spleens were removed and kept frozen until used. Individual organs from five mice were pooled, and the pooled tissues were homogenized with a small amount of ice-cold distilled water (liver with 4 ml., kidneys with 1 ml., and spleen with 0.5 ml.). The homogenates were centrifuged at 10,000 to 15,000 r.p.m. in a refrigerated centrifuge. Urine was diluted to 1.5 ml. with cold distilled water, and feces were extracted with 0.5 ml. Ten lambdas of each preparation was chromatographed. After the chromatogram was developed, it was treated with ninhydrin–collidine reagent. It was then cut into strips 25 × 2.5 cm., and the strips were scanned for radioactivity using a Scanogram II[3].

RESULTS AND DISCUSSION

The I molecule bears two sulfur-containing functions, R—S—C(=S)—S$^-$ and R—NH—C(=S)—S$^-$, both containing C(=S) and S$^-$. They are, therefore, not distinguishable by chemical color reagents, but the UV absorption spectra of a sufficient number of examples of each have been recorded to allow these functions to be distinguished. Janssen (13) assigned peaks (in water) at 253 and 279 nm. to N—C(=S)—S$^-$ and peaks at 303 and 333 nm. to S—C(=S)—S$^-$. Also, using 95% ethanol, Foye *et al.* (14) found trithiocarbonate absorption peaks at 303 and 333 nm. and the dithiocarbamate absorption peaks at 255–257 and 290–295 nm. with a much weaker peak at 342–345 nm.

[2] Beckman model DU.
[3] Baird-Atomic Instrument Co.

***In Vitro* Determinations**—Dissolution of I in acidic medium resulted in immediate decomposition with evolution of gas (detected by lead acetate paper). At pH 7.4, gas was not detected for 0.5 hr.; at pH 8.4, no sign of decomposition was evident for 1 hr. UV absorption of the freshly prepared solutions showed peaks at 255, 292, and 333 nm., characteristic of both dithiocarbamate and trithiocarbonate groups. After 1 hr., the spectra of the solutions at pH 6.1 and 7.4 showed a broad band at 253–275 nm. For the solution at pH 8.4, a sharper peak at 268 nm. appeared. These peaks are indicative of a dithiocarbamate but not a trithiocarbonate group. It was apparent that the trithiocarbonate group had suffered decomposition.

Paper chromatograms of I at these three pH values were quite similar (Table II). A spot at R_f 0.59, positive to ninhydrin–collidine reagent, *N*-ethylmaleimide–KOH reagent, $CuCl_2$–hydroxylamine reagent, and Grote's reagent, indicative of both C=S and S=H, was eluted with 90% ethanol and showed a broad peak in the UV at 256–274 nm. This indicated a dithiocarbamate with loss of the trithiocarbonate group. Two spots with R_f values of 0.48 and 0.36 were positive to ninhydrin–collidine reagent but to none of the other reagents; these spots did not appear in the *in vivo* measurements and were not identified. Another spot at R_f 0.16, positive to Grote's reagent, agrees with the R_f value of cystine.

The chromatogram of the I solution at pH 8.4 showed small differences in R_f values from those of solutions at pH 6.1 and 7.4 and revealed an additional spot at R_f 0.81, which was positive to ninhydrin–collidine reagent, $CuCl_2$–hydroxylamine reagent, and Grote's reagent. UV absorption of the eluate of this spot was found at 254 and 332 nm., characteristic of both the dithiocarbamate and trithiocarbonate groups; it was considered to be due to intact I. On the scans of ^{35}S-I after chromatographing solutions at pH 7.4 and 8.1, two radioactive peaks were found at R_f 0.59 and 0.79, with more of the radioactivity in the peak at R_f 0.79, particularly at pH 8.1. These peaks, on the basis of the UV absorption of the corresponding I solutions, represent the dithiocarbamate and intact I, respectively.

Synthesis of Dithiocarbamate—Attempted synthesis of the dithiocarbamate of cysteine led either to I or unreacted cysteine. Attempts to isolate a hydrolysis product of I containing the dithiocarbamate but not the trithiocarbonate group gave solutions from which sulfur was slowly deposited. Reaction of cystine with carbon disulfide, however, gave the bis-dithiocarbamate of cystine, which gave an R_f value (0.57) approximating both that of the hydrolysis product and one of the metabolic products. UV absorption at 270 nm. was characteristic of a dithiocarbamate, as was IR absorption at 1275, 1050, and 945 cm.$^{-1}$.

Distribution *In Vivo*—Thirty minutes after the injection of ^{35}S-I, 18% of the administered radioactivity was found in the liver (Table III). Scans of the chromatograms for radioactivity showed four peaks with R_f values of 0.79, 0.59, 0.15, and 0.0; the highest

Table IV—Radioactive Components in Mouse Liver: Percent Radioactivity after Injection of ^{35}S-I

Hours	Percent Radioactivity in Peaks—R_f 0.79	R_f 0.59	R_f 0.15	R_f 0.0
0.5	25.6 (6.6)[a]	39.7 (17.9)	10.2 (4.8)	26.1 (17.8)
1	32.6 (3.5)	33.9 (10.5)	5.1 (1.0)	29.8 (5.4)
2	— —	67.8 (3.0)	— —	26.4 (8.7)

[a] Numbers in parentheses are standard deviations.

Table V—Radioactive Components in Mouse Kidney: Percent Radioactivity after Injection of ^{35}S-I

Hours	Percent Radioactivity in Peaks—R_f 0.59	R_f 0.20	R_f 0.0
0.5	29.7 (5.5)[a]	36.2 (5.8)	30.3 (8.8)
1	30.8 (8.1)	45.6 (9.8)	23.8 (7.4)

[a] Numbers in parentheses are standard deviations.

level of radioactivity (40%) was associated with the peak at 0.59 (Table IV). The peak at 0.59 (measured at the center) corresponds quite well to the spots of R_f 0.59 for a dithiocarbamate for the *in vitro* experiments of both I and ^{35}S-I and to R_f 0.57 for the bis-dithiocarbamate of cystine.

The component at R_f 0.79 appears to be intact I, which showed the same R_f value *in vitro*. The substance of R_f 0.15 is most likely the disulfide cystine, since cystine had an R_f of 0.16 in the *in vitro* system. However, for cystine to show radioactivity, sulfur exchange between the ^{35}S-CS_2 and the thiol group of cysteine would be required.

Radioactivity was found remaining at the origin of the chromatograms of all tissue homogenates. Although it was not possible to determine the chemical nature of this component, it is most likely protein bound. Stromme (15), for instance, found diethyldithiocarbamate bound to plasma and liver proteins through mixed disulfide formation.

In regard to the possibility of conjugation products, Stromme (15) found that diethyldithiocarbamate is excreted to a small extent as the *S*-glucuronide in rats, along with sulfate, unchanged dithiocarbamate, and CS_2. However, Kaslander (16) found very little of this dithiocarbamate conjugated in humans (0.75%). Other conjugates of dimethyldithiocarbamate, including the alanine, α-aminobutyric acid, and β-glucoside derivatives, were found as metabolic products from plant tissues and microorganisms (17). These conjugates had low R_f values (in propanol–H_2O) over a range (0.26–0.39) in which no radioactivity was found in our determinations.

After 1 hr., the level of radioactivity in the liver had decreased by about 50%, and it remained the same at 2 hr. (Table III). Similar storage and excretion behavior has been noted for other radioprotective thiols. After 1 hr. (Table IV), the amount of intact I appeared to increase somewhat, but the amount, if considered within the limits of the standard deviation, probably was comparable to that at 0.5 hr. After 2 hr., no I was evident. The amount of disulfide also diminished, and at 1 hr. it was only about 5% of the measured amount. After 2 hr., only dithiocarbamate and the protein-bound component remained in the liver homogenate.

The amount of radioactivity in the kidneys (Table V) was much less than in the liver, less than 5% of the administered dose being present after 0.5 hr. This level declined to 1.4% after 2 hr. Kidney homogenates showed the presence of three components: dithiocarbamate, a substance of R_f 0.20 attributable to sulfate (inorganic sulfate showed an R_f 0.19 *in vitro*), and protein-bound compound. Here, the amount of radioactivity was fairly evenly distributed among the three compounds, that due to sulfate increasing with time. The amount of dithiocarbamate stayed the same during the time interval observed, while the amount of protein-bound component diminished.

Only 1% of the injected radioactivity was found in the spleen; this level diminished slightly with time (Table VI). Similarly to the kidney homogenate, no disulfide was visible in the spleen homogenate, but dithiocarbamate and the protein-bound component were found along with sulfate at R_f 0.20. The sulfate amount again increased with time; the amount of dithiocarbamate was relatively constant, and that of the protein-bound component diminished slightly.

Excretion—Compound I was metabolized and excreted rather rapidly. Over 50% of the administered radioactivity appeared in the urine at each time interval (Table III). Radioactivity was found in the feces after 2 hr.; no attempt was made to determine loss through respiration, where any carbon disulfide would be expected to appear. Carbon disulfide has been found in the expired air of rats after administration of diethyldithiocarbamate or disulfiram[4] (15, 18).

Four radioactive peaks were found in the urine: one corresponding to dithiocarbamate, one to sulfate, one to I, and one to a new component at R_f 0.92 (Table VII). The amount of the latter substance was relatively small, and its identity was not established.

In the first 0.5 hr. after injection, the radioactive component excreted in the largest amount was intact I, with dithiocarbamate and sulfate excreted in lesser amounts. The amount of I diminished rapidly, whereas the other two compounds increased in quantity. After 2 hr., sulfate accounted for 43% of the radioactivity excreted and dithiocarbamate 38%, while I had declined to 13%.

[4] Antabuse, Ayerst Labs.

Table VI—Radioactive Components in Mouse Spleen: Percent Radioactivity after Injection of ^{35}S-I

Hours	Percent Radioactivity in Peaks		
	R_f 0.59	R_f 0.20	R_f 0.0
0.5	29.4 (5.3)[a]	26.4 (4.6)	39.0 (8.9)
1	30.7 (5.2)	39.0 (11.9)	36.0 (10.7)

[a] Numbers in parentheses are standard deviations.

Table VII—Radioactive Components in Mouse Urine: Percent Radioactivity after Injection of ^{35}S-I

Hours	Percent Radioactivity in Peaks			
	R_f 0.92	R_f 0.80	R_f 0.59	R_f 0.20
0.5	—	36.2 (8.5)[a]	31.9 (6.9)	28.4 (2.4)
1	8.4 (4.0)	16.7 (12.9)	36.2 (4.9)	37.4 (5.4)
2	7.9 (3.5)	13.3 (6.5)	38.3 (3.6)	42.6 (5.8)

[a] Numbers in parentheses are standard deviations.

About 6% of the administered radioactivity was found in the fecal extract after 2 hr. Radioactive peaks corresponded to dithiocarbamate and sulfate, which were present in roughly equal amounts.

It can be seen that the most widely distributed metabolite of I in mice is the dithiocarbamate of cystine, found in the liver, kidney, and spleen. Protein-bound components were also present in these tissues; other metabolites found were unchanged I and possibly cystine in the liver and sulfate in the kidney and spleen. The metabolism and excretion of I were quite rapid, over 50% of the injected radioactivity appearing in the urine at each time interval measured. Radioactive compounds which appeared in the urine include the dithiocarbamate, intact I, sulfate, and a small amount of unidentified substance of a higher R_f value than any of the metabolic products and considerably higher than that of intact I.

The dithiocarbamate, because of its more general distribution and the fact that a variety of dithiocarbamates have been found radioprotective (19), may contribute significantly to the radiation protection exerted by I. Unchanged I could very well contribute to the protection, since it has thiol groups capable of hydrogen atom-exchange reactions (20) as well as does the dithiocarbamate. Since irradiation of the animals is generally done 15–30 min. after administration of the candidate protecvtie agent in antiradiation screening, it appears from the results here that the dithiocarbamate is already well distributed in radiosensitive organs by this time, and intact I is present in some tissues. Although cysteine itself is radiation protective (21), it shows a much lower order of protection than does I; and since none or practically none of it was found in the mouse experiments, it can be concluded that cysteine is not contributing to the radioprotection.

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Pharmacological Properties of Hypobranchial Gland of *Thais haemastoma* (Clench)

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Abstract □ The aqueous extract of the hypobranchial glands of *Thais haemastoma*, administered intravenously to anesthetized cats at doses equivalent to 60–90 mg. of fresh gland/kg. body weight, produced an increase in the blood pressure accompanied by tachycardia. These effects were partially blocked by hexamethonium bromide but were not modified by atropine or adrenergic blockade. In the isolated rabbit heart preparation, the extract caused an increase in the heart rate, coronary outflow, and amplitude of cardiac contractions. The extract produced marked contractions in the guinea pig ileum, rabbit duodenum, and rat uterus, which were blocked by atropine and hexamethonium bromide, and induced contractions in the frog *rectus abdominis* muscle, which were abolished by *d*-tubocurarine. It also blocked the conductions in the frog nerve–muscle preparations. It appeared that two active components were present in the hypobranchial gland extract; one produced a direct stimulatory effect on the blood pressure and heart actions and the other acted as a neuromuscular blocking agent of depolarizing type. The LD_{50} in mice was found to be 215 mg./kg.

Keyphrases □ *Thais haemastoma*—pharmacological properties of hypobranchial gland, toxicity □ Hypobranchial glands, *Thais haemastoma*—pharmacology, toxicity □ Toxicity—hypobranchial gland of *Thais haemastoma*

Thais haemastoma is a sea snail which belongs to a family of *Muricidae*. *Thais* usually feeds on oysters, and with its sharp rasplike device called the radula, it can drill through an oystershell in a few hours. Because of its carnivorous eating habit, *Thais* often presents a considerable economic threat to oyster farming. The drilling seems to be aided by acids or enzymes secreted from its salivary gland or tongue, but these secretions are not necessary for drilling. Apparently, *Thais* injects a poison into a molluscan prey at the moment it opens the shell. The poison is considered to be located in the hypobranchial gland or purple gland, which is part of the skin and appears as a conspicuous, folded glandular structure on the roof of the mantle cavity. The gland appears as three narrow, elongated parallel bands or zones oriented in an anteroposterior direction (1). Fisher (2) claimed that it functions as a genital organ, a viewpoint also held by Jullien (3, 4). Erspamer and Glassar (5) suggested that a neurotoxin, murexine, is used by the gastropods for food procurement and as a defense mechanism; however, no further information supports this speculation.

A review of the literature revealed no pharmacological study on this marine animal. Therefore, this study was undertaken to investigate the toxicity and pharmacological properties of the hypobranchial gland of *Thais haemastoma* (Clench), obtained from the Gulf of Mexico.

EXPERIMENTAL

Materials—One hundred and twenty specimens were collected near the Biloxi bridge, Biloxi, Miss., and the surrounding areas where the oysterbeds exist. Most *Thais* were taken by dredges and some in trawls. The salinity and temperature varied from 24.9 to 30.0 p.p.t. and from 15.0 to 24.9°, respectively.

The hypobranchial glands from 30 animals were weighed (10 g.) and homogenized in a blender (Waring) for 5 min., using distilled water as a solvent. The homogenate was centrifuged at 9000 r.p.m. for 5 min., and the yellowish supernatant liquid was used for pharmacological investigations. The doses used were expressed in terms of the fresh gland weight.

Toxicity Studies—General acute toxicity and various toxic manifestations were studied in mice. Male albino mice, weighing 15–22 g., were randomly grouped with five animals in each group. They were administered with the aqueous extract of hypobranchial