Title

A STUDY OF THE METABOLIC FATE OF THE RADIATION-PROTECTIVE COMPOUND, S, 2-AMINO-ETHYLISOTHIURONIUM BROMIDE (AET) IN THE RAT

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Radiation Laboratory

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(Ph. D. Thesis)

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Contents

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ABSTRACT

The compound S, 2-aminoethylisothiuronium bromide is representative of a class of radiation protective agents known as the aminoalkylisothiuronium salts. This study investigated the metabolic changes which this compound undergoes after its administration to a mammal using radioactively labeled AET.

Measurements of the disappearance from the circulation of labeled AET, combined with tissue analysis, showed that there is a rapid distribution of radioactivity throughout most of the body tissues. Elimination studies show that the major route of excretion is the urinary system.

The radioactivity found in the urine is in the main a mixture of chemical products. The data obtained by ion-exchange chromatography of urine indicated that approximately 18 to 19% of the injected dose of carbon-14-labeled AET appears as the following chemical structures: 2-mercaptoethylguanidine, guanidinoethyldisulfide, guanidinoethyldisulfoxide, and 2-guanidinoethylsulfonic acid.

The radioactivity excreted in the urine following injection of sulfur-35-labeled AET does not correspond to the pattern of products obtained with carbon-14-labeled AET. These data are interpreted as evidence that the sulfur atom of the injected thiol has exchanged with other compounds.

The data obtained from this study indicate that one path of metabolism that AET follows is dependent upon the oxidation of the mercapto group to a sulfonic acid group.

I. INTRODUCTION

The purpose of this thesis is to study the chemical changes that the compound S, 2-aminoethylisothiuronium bromide (AET) undergoes after its administration to a mammal. The compound S, 2-aminoethylisothiuronium bromide is representative of a class of radiation-protective agents known as the aminoalkylisothiuronium salts, although sometimes they are referred to as mercaptoalkylguanidines. The latter are directly derived from the parent aminoalkylisothiuronium salts by an intramolecular rearrangement.

It is hoped that the results obtained from this investigation will constitute information useful in the interpretation of the mechanism (s) underlying the interrelationship between the radiation sequelae and this radiation-protective agent during the process of irradiation.

II. HISTORICAL REVIEW

One of the ultimate goals of the radiobiologist whose interest lies within the general problem of protection against radiation injury is to obtain some logical rationale regarding the underlying mechanisms of the vast array of agents which, to various degrees, combat or ameliorate the radiation syndrome. The immediate difficulty that besets the investigator in this quest is the tremendous variety of compounds which have been reported as "protective" agents.[1] Recently it has been suggested that a subdivision be made of these "protective" agents based upon the time relationship between the administration of the compound and the time of irradiation exposure.[2] Thus, if the agent were administered prior to or during the radiation exposure it would be referred to as a protective agent, and those agents which are effective if given after the irradiation exposure would be termed recovery agents. This arbitrary subdivision seems both practical and logical in light of our present knowledge concerning a few of the agents reported to modify radiation effects. An example of a recovery agent is bone marrow transplantation. There is excellent evidence which establishes that this agent's action in combating the radiation syndrome is to replace a cell population destroyed in the irradiated animal.[3] Thus, we have some understanding of the basic mechanism exhibited by this agent which acts as a guide toward future attempts in cellular repopulation studies of the radiosensitive tissues affected by radiation.

However, many examples exist which fall into the category of protective agents and little, if any, conclusive evidence exists which clearly establishes their mode of action. Therefore it would be extremely difficult, if not impossible, with the present information to establish a basic underlying mechanism for all these diverse reagents. Thus, the complexity of the problem itself suggested that a reasonable approach would be to concentrate on one main class of compounds which has shown promising results as protective agents.

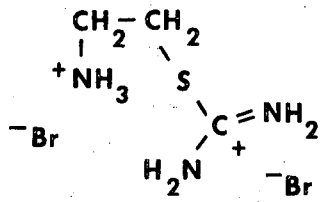
Early workers such as Barron[4] and Patt et al.[5,6,7] demonstrated that sulfhydryl (thiol) compounds were excellent protective agents. Among those tested, the amino acid cysteine showed very effective protective activity in the mammal.

Bacq and associates,[8,9] in the pursuit of new types of radiation-protective agents related to cysteine, found that a compound 2-mercaptoethylamine (MEA), which possessed only the amino and mercapto group, had a greater degree of protective ability than cysteine.[10]

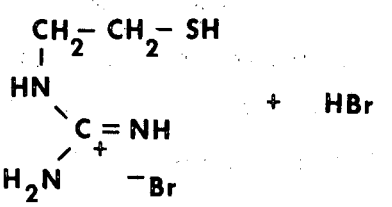
Later, Doherty and Burnett[11] reported that another type of compound, which will be shown later to be related to 2-mercaptoethylamine, possessed a higher degree of protection against radiation than the two aforementioned compounds. Thus, encouraged by their findings, Doherty and associates[12,13,14] made a series of aminoalkylisothiuronium compounds to see if any correlation could be ascertained between structure, chemical properties, and protective ability. The results of these studies showed that for maximum protective activity, a basic group such as a guanidino group ($-HN_2-\overset{NH}{\overset{\|}{C}}-$) or an amino group ($-NH_2$) must be present in conjunction with a free mercapto group (-SH). The maximum intramolecular separation of these two groups is by two or three methylene carbon atoms at most, with further separation diminishing the protective ability.

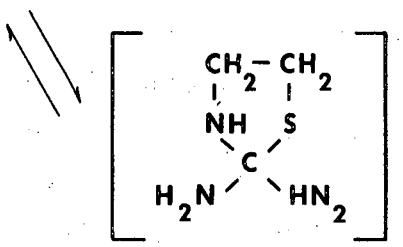
However, additional information was needed to account for the presence of the guanidino group and the free mercapto group, since the parent alkylisothiuronium salts possess neither. Khym et al. showed that an intra-transguanylation reaction takes place when the alkylisothiuronium compounds are placed in aqueous solution.[15] This sequence is shown as follows:

S,2-AMINOETHYL-ISOTHIURONIUM·Br·HBr



2-MERCAPTOETHYL-GUANIDINIUM·Br





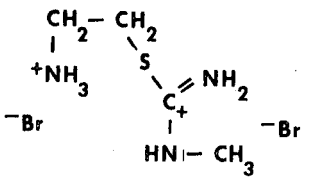
Hypothetical

Mu-21455

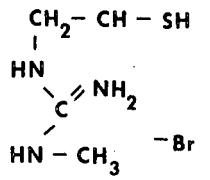
Further studies showed, in a comparison between alkylisothiuronium compounds and closely related mercaptoalkylamines (i.e., AET and MEA), that AET was less toxic and more protective than MEA on a molar basis.

Therefore, the presence of a guanidino group, which is a much stronger basic group than the amino group, has enhanced the radiobiological efficiency. Also, during the pursuit of these studies there was included a group of substituted aminoalkylisothioureas. It was found that the alkylaminoisothiouronium salts, which undergo intra-transguanylation, yielding N-substituted mercaptoalkylguanidines, all proved more toxic and less protective than the parent AET. This is shown as follows:

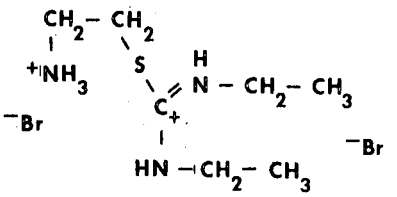
S,2–AMINOETHYL–N'–METHYL-ISOTHIURONIUM·Br·HBr



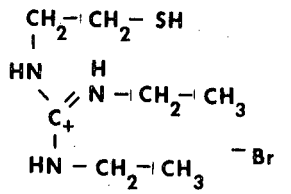
2–MERCAPTOETHYL–N'–METHYL-GUANIDINIUM·Br



S,2–AMINOETHYL–N',N''–DIETHYL ISOTHIURONIUM·Br·HBr



2–MERCAPTOETHYL–N',N''–DIETHYL GUANIDINIUM·Br



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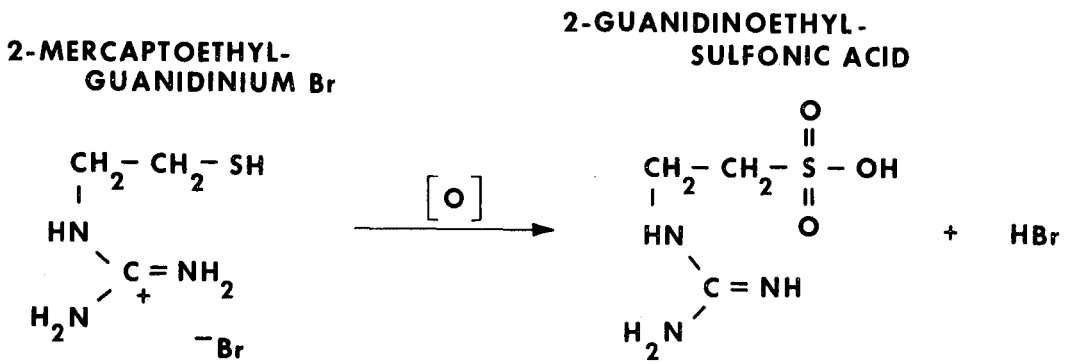
In addition, the acyl derivatives such as the N-acetyl-2-guanidino derivative, like the analogous acyl aminoalkylmercaptans, proved more toxic and less protective. Therefore, in the light of these observations it is indicative that to obtain maximum protective benefits, the presence of an unsubstituted guanidino group is essential.

Uniqueness of the unsubstituted guanidino group suggests that there may exist a biological route which can be taken by this foreign compound (AET) which aids in the efficiency of the compound as a protective agent. This is to say that the guanidine molecule is offered access into some radiation-sensitive cellular compartment in a higher degree than the amino homologue. Support for this hypothesis is derived from the findings of Bradford.[16]

Also, Thoai and associates have found the natural presence of taurocyamine (2-guanidinoethylsulfonic acid) in the urine of rats.[17] This compound is related to AET. It is one of the oxidized forms, theoretically at least, of the transguanylated structure 2-mercaptoethylguanidine, as shown in the following sequence:



Therefore it seemed logical that an investigation of the chemical changes of S, 2-aminoethylisothiuronium bromide, which is representative of a class of protective agents, would establish the existence or nonexistence of a naturally occurring biological route for these substituted guanidine compounds directly derived from them.

III. TEMPORAL RELATIONSHIPS FOLLOWING ADMINISTRATION OF AET

A. Circulatory Disappearance and Tissue Distribution of S, 2-Aminoethylisothiuronium Bromide as Measured with Radioactively Labeled S, 2-Aminoethylisothiuronium· Br· HBr

The fundamental tenet which underlies the basis of separation of radiation protective compounds from recovery agents is that protectiveness against radiation is afforded only when the agent is administered prior to or during exposure. The importance of the temporal relationships between administration and irradiation exposure is illustrated by the studies of Doherty and Burnett[11] and Melville and Leffingwell.[18] It was shown that administration of the compound intravenously was unsatisfactory. When the compound was given intraperitoneally, some 15 to 30 minutes prior to irradiation, it achieved maximal benefits, whereas if given 3 hours prior to irradiation, the compound was totally ineffective. By way of contrast, the compound administered orally some 3 to 4 hours prior to irradiation retained maximal effectiveness.

Therefore it was the purpose of these initial experiments to obtain data concerning the distribution of the radiation-protective compound AET within the tissues (including blood) of the rat, which would offer information on the following parameters:

(a) Effective time and route of administration of the compound prior to irradiation.

(b) Possible accumulation of the compound in radiosensitive tissues.

(c) Site of metabolism

(d) Route of elimination from the body.

Materials and Methods

1. Radioactive S, 2-Aminoethylisothiuronium· Br· HBr (AET)

a. Preparation of sulfur-35-labeled AET

The method of synthesis was described by Doherty.[12] Thiourea, S^{35}-labeled (obtained from Tracerlab), with specific activity of 7.0 mC per millimole, to which was added 103 mg of unlabeled thiourea for a total of 115 mg (1.5 millimoles) of thiourea, was placed along with 309 mg (1.5 millimoles) of recrystallized 2-bromoethylamine· HBr into a 3.0-ml reaction vessel. Then 1.0 ml of triple-distilled isopropyl alcohol was added. An air condenser was fitted to the reaction vessel and gentle heating initiated. All material dissolved immediately, and after 6 to 7 minutes of refluxing, recrystallization of a product began. Refluxing continued for a total of 15 to 20 minutes. The reaction was cooled and the contents poured into a glass-fritted funnel and filtered by suction. The product was washed repeatedly with isopropyl alcohol and ethyl acetate, and dried in vacuo. Yield=60.5%; mp 190—191°C.

b. Procedure

All animals used were male Sprague-Dawley rats weighing 300 to 400 grams, 3 to 4 months of age, and maintained in quarters with constant temperature (78 to 80°F) and humidity (55%). Purina laboratory chow and drinking water were given ad libitum.

All animals were anesthetized with Nembutal*-saline solution (1:10) by intraperitoneal (i. p.) injection. Each AET injection was 0.2 ml in volume and contained 0.2 mg of AET, except that oral injection (stomach tubing), which contained 1.0 mg AET. The AET was dissolved in saline with phosphate buffer (pH 7.0 to 7.2). The amount of total radioactivity injected was on the order of 2×10^6 counts per minute.

*Nembutal: Pentobarbital sodium, Abbott Laboratories, Chicago, Illinois.

The surgical technique employed in obtaining the disappearance data from the circulation consisted of cannulation of the common carotid artery. Instead of a regular cannula, a 1-inch 25-gauge needle attached to a three-way stopcock was placed into the artery and tied in place by a loop of thread around the artery. Blood samples were obtained simply by turning the valve and allowing the blood to flow through a polyethylene tube into a conical collection tube. The amount of blood removed during each sampling period was approximately 0.2 to 0.3 ml. After each blood sample, the valve and the polyethylene tube were flushed with 10 ml of saline and given a final rinse with a dilute heparin-saline solution to wash out any residual radioactivity. In order to maintain constant blood volume in the experimental animal, donor blood was administered in amounts equal to that removed during sampling.

To each centrifuge tube, 0.05 ml of a 1% Sequestrene*-saline solution was added as an additional measure against clotting. The blood samples were spun at 2,500 rpm for 5 minutes. For the radioactive assay, plasma was pipetted on stainless steel planchettes in duplicate and counted in a gas-flow proportional counter.† All activity measurements were corrected for backscattering, coincidence losses, absorption, decay, and background. Total plasma activity was expressed as per cent of the injected dose. The volume of plasma was calculated on the assumptions.[19, 20]

(a) 9.5 cc/100 g B. W. = total blood volume of rat,

(b) 50% hematocrit value for male rats.

Tissue distribution of the S^{35} activity was determined by preparing tissue homogenates from organs removed after sacrificing the animals immediately after the last blood sample. All animals were exsanguinated by removal of the blood through the cannula, then a saline perfusion was performed through this cannula and out the inferior vena cava. Tissues were then removed and homogenized. All samples were

*Sequestrene: Ethylenediamine tetra-acetic acid, Fisher Scientific Co., St. Louis, Missouri.

†Nuclear Measurement., Decade Scaler Model DS-1, Proportional Counter Converter Model Pcc-L1.

plated in duplicate and dried to constant weight. Corrections were made for absorption, coincidence losses, backscattering, decay, and background, and all activities are expressed as counts per minute per milligram of dry tissue.

Results

Protocol I (Appendix) describes the change with time in the concentration of AET in the plasma after a single intraarterial injection. The loss is apparently exponential and approximately 2 to 3% of the injected dose remained after equilibration, which occurred at 18 to 20 minutes postinjection. Protocol II (Appendix) shows the time change in concentration of AET in plasma after a single intraperitoneal injection. The peak concentration appeared between 8 and 10 minutes following the injection. Protocol III (Appendix) shows the time change in concentration of AET in plasma after a single oral injection (stomach tubing). In Fig. 1, the magnitude of the injected dose in the plasma after equilibration is found to be less than 3.0%, regardless of route of administration. Plasma obtained from the animals sacrificed by exsanguination was dialyzed, and most of the activity retained in the plasma appeared to be protein-bound. Addition of a trace of potassium cyanide to the plasma solution liberated some of the S^{35} activity. This suggested that the S^{35} is bound to plasma protein through disulfide linkages. Paper electrophoretic separation of the plasma indicated that the activity is bound to the albumin.

The results for the distribution of S^{35} activity in the tissues by intra arterial, intraperitoneal, and oral routes of administration are shown in Table I. The data in Table II indicate that the largest percentages of sulfur-35 activity occur in the muscle, skin, and liver. The kidney, spleen, and bone marrow have relatively large amounts of activity; however, in the oral route there remains a considerable amount of activity throughout the length of the gastrointestinal tract.

Fig. 1. Time change in concentration of labeled S^{35} AET in plasma following a single injection administered by three different routes.

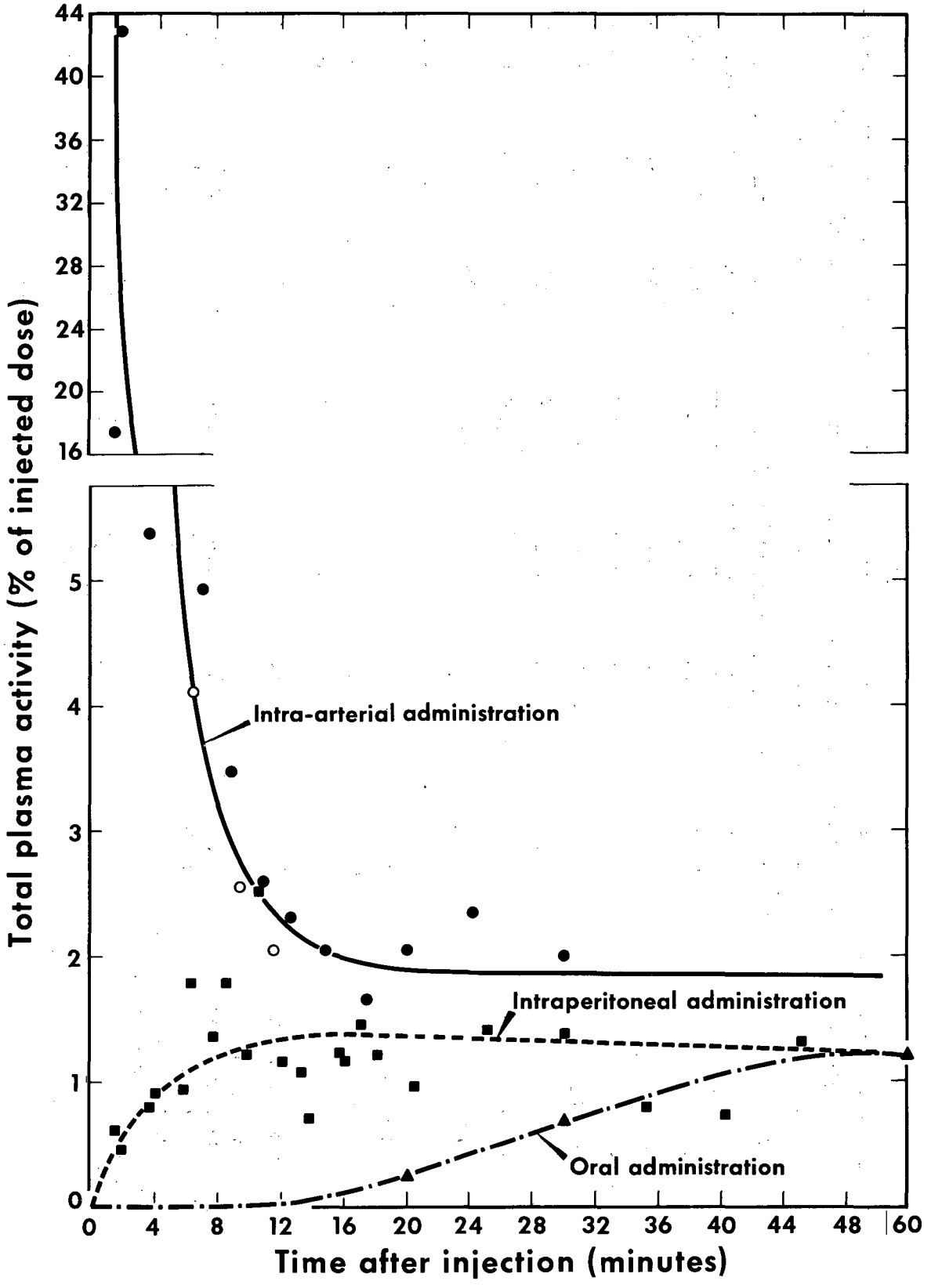

Total plasma activity (% of injected dose)
Time after injection (minutes)
Intra-arterial administration
Intraperitoneal administration
Oral administration
0
4
8
12
16
20
24
28
32
36
40
44
48
60
1
2
3
4
5
16
20
24
28
32
36
40
44

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Table I

Distribution of sulfur-35 activity in tissues after injection of sulfur-35 labeled AET

Tissue	Intra-arterial	Intraperitoneal			Oral		
	30 min	10 min	20 min	45 min	20 min	70 min	145 min
Heart	1.00	0.63	0.63	0.63	0.06	0.31	0.20
Lung	1.00	0.60	0.63	0.63	0.14	0.40	0.20
Skin	0.40	0.31	0.31	0.26	0.06	0.31	0.20
Abdominal muscle	0.63	0.59	0.69	0.89	0.03	0.07	0.11
Eye	0.17	0.14	0.14	0.14	0.03	0.14	0.06
Brain	0.11	0.03	0.06	0.09	0.03	0.03	0.03
Testis	0.11	0.11	0.14	0.20	0.03	0.11	0.20
Stomach	1.17	1.23	1.28	1.17	10.10	8.80	2.10
Duodenum	0.87	0.71	1.65	1.70	8.70	5.60	0.97
Jejunum	0.92	1.08	1.49	1.71	5.68	3.80	1.54
Cecum	0.60	0.23	0.40	0.43	1.90	0.74	0.31
Small intestine contents	0.06	0.11	0.34	0.91	4.57	1.51	3.72
Lymph nodes:							
Submaxillary	0.09	0.18	0.23	0.42	0.28	0.11	0.11
Deep cervical	0.69	1.11	1.94	1.31	0.28	0.42	0.14
Lumbar	0.66	0.83	0.43	0.28	0.09	0.31	0.11
Mesenteric	0.09	0.23	0.40	0.37	0.06	1.74	0.11
Liver	1.40	1.14	1.97	1.89	0.23	1.46	0.89
Spleen	0.97	1.14	3.00	1.06	0.14	0.37	0.20
Kidney	2.29	1.91	2.06	2.29	0.63	1.46	0.66
Bone marrow	1.06	0.09	0.89	0.37	0.06	0.11	0.09

Standard Unit = 1.00, which is equal to 35 cpm/mg dry tissue. Duplicates were made on all samples; errors in duplicates less than ±5%.

Table II

Percentage of injected dose found in various tissues following injection of sulfur-35-labeled AET

Route of Administration	Time (Min)	Liver	Kidney	Spleen	Muscle	Lymph nodes	Bone marrow	Eye	Skin	Stomach and intestine	Testis	Heart	Lung	Brain
Intra-arterial (2)	30	9.15	2.65	0.53	41.08	0.89	1.51	0.02	15.0	2.51	0.05	1.01	1.15	0.20
Intraperitoneal (6)	10	10.71	3.19	0.71	56.23	1.40	0.18	0.03	9.41	4.36	0.01	0.79	0.94	0.15
	20	18.15	3.36	1.84	63.15	1.40	1.81	0.03	16.45	6.33	0.01	0.67	1.01	0.15
	45	17.36	3.74	0.65	52.63	1.35	0.76	0.03	13.84	6.48	0.01	0.67	1.01	0.21
Oral (3)	20	1.63	0.75	0.07	2.05	0.23	0.09	0.01	2.39	26.45	0.01	0.05	0.18	0.05
	70	10.43	1.73	0.18	12.27	0.51	0.18	0.02	13.15	18.90	0.01	0.28	0.50	0.05
	145	6.32	0.78	0.10	8.20	0.18	0.14	0.01	8.37	4.93	0.01	0.18	0.25	0.05
Organ weight (50, 51, 52) (% of B. W.)		4.5	0.8	0.3	45.0	1.0	1.0	0.1	26.3	2.5	0.05	0.52	0.79	1.22

Total activity injected:
Intra-arterial = 1.85×10^6 counts/min
Intraperitoneal = 1.71×10^6 counts/min
Oral = 2.2×10^6 counts/min

( ) = total number of animals used per group.

Discussion

The rapid loss from the vascular compartment following the intra-arterial injection appears analogous to the disappearance of crystalloids.[21-24]

The comparison, at equilibrium, of AET in the plasma following a single intra-arterial, intraperitoneal, or oral injection indicates no difference in magnitude with approximately 2.0 to 3.0% of the injected dose remaining. Increasing the injected amount of the compound over 200-fold (from 0.2 mg to 55.0 mg) did not elevate the percent of injected dose found in the plasma to any significant degree.

Regardless of the route of administration, the distribution and circulation data indicate that the compound is absorbed throughout most of the body tissues. It will be noted that with time, changes in concentration do occur in the various tissues and therefore could influence the degree of protection.

Since the intraperitoneal injection reached a plateau between 10 and 20 minutes postinjection, it would be considered that this time would be the most favorable for irradiation to afford maximum protection. Also, if, at this time, one compares tissues believed to be extremely sensitive to radiation--spleen, lymph nodes, bone marrow-relatively large amounts of AET are found. Some tissues, such as the brain, eye, and testis, do not accumulate large concentrations of AET; therefore, failure to protect these organs might be explained on this basis. The high concentration in the kidney tissue suggests that the urine may be the major route of elimination. The liver accumulates a large amount of activity (15 to 20%) and presumably is the principal site of metabolism.

B. Appearance of Radioactive Products in the Urine Following Adminstration of Labeled AET

The observation of the disappearance from the circulation of labeled AET following its administration showed that very little of the unbound compound remained in the vascular system. In addition, distribution studies suggested that the kidneys played the major role in elimination of the compound from the body through the urine and that little of the compound appeared in the intestinal contents. To substantiate this further, experiments were conducted which attempted to quantify the amount of the compound appearing in the urine, as percent of the injected dose, with regard to route of administration. It was assumed that the radioactivity represented either the original compound or metabolic degradation products derived from it.

Materials and Methods

1. Radioactively Labeled S, 2-Aminoethylisothiuronium· Br· HBr (AET)

a. Preparation of sulfur-35-labeled AET

The method is described in the preceding section.

b. Procedure

All animals used were male Sprague-Dawley rats weighing 300 to 400 grams, 3 to 4 months of age, and maintained in quarters with constant temperature (78 to 80°F) and humidity (55%). Purina laboratory chow and drinking water were given ad libitum.

All animals were anesthetized with Nembutal-saline solution (1:10). Each injection was 0.2 ml in volume and contained 0.2 mg of AET except where otherwise noted. The AET was dissolved in phosphate-buffered saline (pH 7.0-7.2).

The surgical technique employed in obtaining either (a) serial urine sample or (b) total-time sample consisted of the following steps. First, to insure that no loss of urine would occur during the experiment, the penis of the animal was ligated prior to the injection of the radioactive material.

For serial sampling, a midline incision was made into the abdominal wall before injection of the compound and the bladder exposed. A 1-inch 18-gauge needle attached to a two-way stopcock was placed into the bladder through the prostate and tied in place. The body wall was sutured and then the skin was sutured with wound clips. Urine samples were obtained simply by attaching a 1.0 ml TB (tuberculin) syringe which contained 0.5 ml of saline and then turning the valve; the saline was injected into the bladder. After a few seconds, the saline-urine mixture was removed and the total volume of the sample measured. For the radioactive assay, an aliquot of the sample was pipetted on stainless steel planchets in duplicate and counted in a gas flow counter.

To obtain a total-time sample, a midline incision was made into the abdominal wall and the bladder exposed after the collection period had ended. A 1-inch 18-gauge needle attached to a 1.0-ml TB syringe was inserted into the bladder for the purpose of removing the collected urine. After the removal, two washings of 0.5 ml saline were inserted into the bladder and withdrawn for complete removal of any residual urine. The washings were added to the urine sample and the total volume of the sample was measured. Radioactive assay of an aliquot of urine sample was made in the same manner described in the preceding paragraph. Total activity contained in the urine samples was expressed as per cent of injected dose.

Results

Table III shows the quantity of AET in the urine following a single injection either by intravenous, intraperitoneal, or oral route. The intravenous route gave the largest amount of activity within the shortest interval of time. Table IV describes the concentration of activity in the urine following serial sampling. Figure 2 describes an elimination pattern which closely parallels the concentration of activity in plasma during the same time and within the same animal.

Table III

The quantity of sulfur-35-labeled AET found in the urine

Route of administration	Time (min)	Total activity injected (cts/min$\times 10^6$)	Total activity in urine (cts/min)	Percent of injected dose
Intravenously (6)	0-10	1.41	2.50×10^5	17.5
	0-20	1.41	2.45×10^5	17.5
	0-45	1.41	5.65×10^5	39.8
Intraperitoneally (6)	0-10	1.71	3.60×10^4	3.0
	0-60	1.71	3.24×10^5	18.8
	0-180	44.2	2.18×10^6	49.2
Orally (4)	0-10	7.60	1.00×10^2	0.0
	0-60	7.60	6.52×10^5	8.6

Duplicates were made on all samples. Errors in duplicate less than ±5%.

( ) = total number of animals used per group.

Table IV

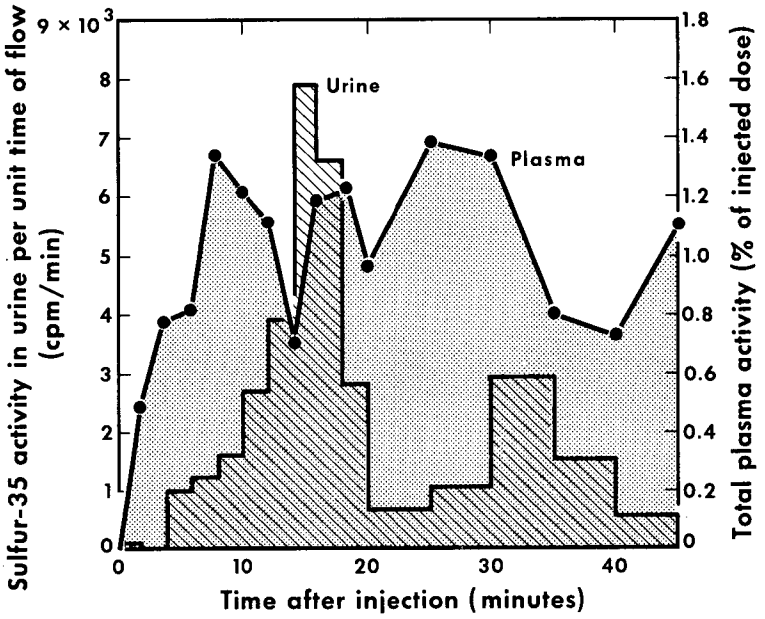
Time change in total activity of sulfur-35 in urine after a single intraperitoneal injection of labeled AET

Sample number	Time after injection (min. and sec)		Volume (Ml)	Total activity of S^{35} in urine sample (cts/min)	S^{35} activity in urine per unit time of flow (cts/min/min)
1	1	53	0.48	1.52×10^{2}	76
2	4	0	0.50	6.50×10^{1}	33
3	6	15	0.95	2.26×10^{3}	1130
4	8	10	0.50	2.53×10^{3}	1266
5	10	0	0.45	3.12×10^{3}	1600
6	12	5	0.55	5.40×10^{2}	2700
7	14	10	0.50	7.92×10^{5}	3957
8	16	0	0.47	1.59×10^{4}	7943
9	18	5	0.39	1.33×10^{4}	6659
10	20	5	0.42	5.61×10^{3}	2805
11	25	10	0.41	3.32×10^{3}	669
12	30	10	0.48	5.26×10^{3}	1051
13	35	30	0.70	1.47×10^{4}	2946
14	40	30	0.68	7.89×10^{4}	1577
15	45	30	0.48	3.09×10^{4}	618

Duplicates were made on all samples; errors in duplicate less than ±5%.

Total activity injected = 1.72×10^{6} cpm. Total activity excreted = 189×10^{5}cpm. Percent of injected dose = 11.1%.

Fig. 2. Comparison between the time change in concentration of activity found in the urine and the concentration of activity in plasma occurring within the same animal.


Sulfur-35 activity in urine per unit time of flow (cpm/min)
9 × 10^3
8
7
6
5
4
3
2
1
0
Urine
Plasma
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
Total plasma activity (% of injected dose)
0
10
20
30
40
Time after injection (minutes)

MU-21336

Discussion

In general, the ineffectiveness of the intravenous route as a method of administration of AET may be due in part to large losses of the compound into the urine. The high concentration of the activity in the kidney tissue as shown by the distribution studies can be explained on the basis of the urine contained therein. The data from the intraperitoneal route shows that an approximate time for excretion of 50% of the injected compound is 3 to 4 hours for the rat compared with 12 hours for the human as reported by Prickett.[25]

These data support the hypothesis that the kidney is the main route of elimination of this compound. Since a large portion of the activity appeared in the urine, the assumption that this represents either the original undegraded compound or metabolic products derived from it becomes quite important. If the activity is largely undegraded AET, then it would be beneficial to consider what modifications could be made to decrease its loss from the body. However, if the activity is mainly degradation products, then its metabolic pathway becomes increasingly important because of the rapidity with which the compound can be metabolized and excreted. In the next section, experiments which were performed to obtain answers to these questions by the identification of the radioactive components found in the urine, are described.

IV. IDENTIFICATION OF RADIOACTIVE PRODUCTS IN THE URINE FOLLOWING THE ADMINISTRATION OF RADIOACTIVELY LABELED AET

Observations on the urinary elimination of AET indicated that a large fraction of the injected activity appeared in the urine. It was assumed that identification of these radioactive products would give information regarding the path(s) that AET follows during its course through the animal.

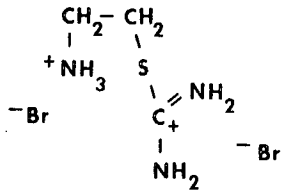
At this time, some consideration shall be given to a "hypothetical" situation in which one assumes the existence of certain chemical and biochemical reactions which AET may undergo. The products predicted from these reactions were synthesized and used as suspects in the identification study.

Four main types of chemical reactions are presented. These were based on the observations found in the literature for similar substances.

For instance, the findings of Khym clearly established that AET, when placed in aqueous solution at neutral pH or slightly alkaline, undergoes an intramolecular rearrangement to form substituted guanidines.[15] These compounds, which behave as strong cations in solution, are shown below.

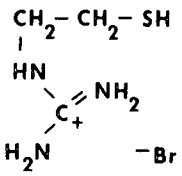
INTRA TRANSGUANYLATION REACTION

S,2—AMINOETHYLISO-THIURONIUM·Br·HBr

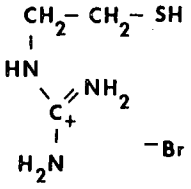


pH 7—7.5

2-MERCAPTOETHYL-GUANIDINIUM·Br

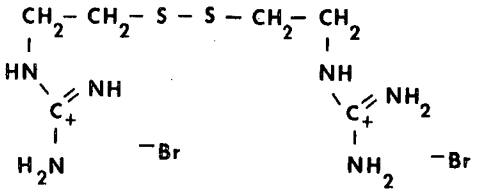


2-MERCAPTOETHYL-GUANIDINIUM·Br



pH 8—9

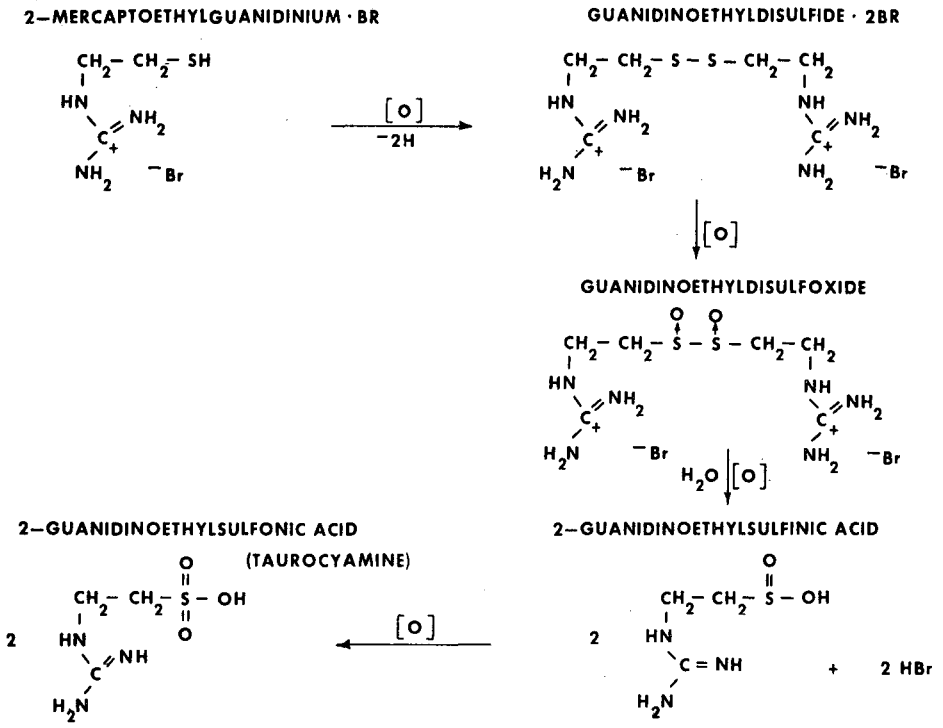
GUANIDOETHYLDISULFIDE·2Br



Mu-21450

Numerous studies have shown that thiol compounds, some of which are closely related to AET, undergo oxidative reactions in which the mercapto group is oxidized stepwise to either a sulfonic acid group or sulfate.[26-31] Thus, typical oxidative reactions of AET, following an internal rearrangement, would be as follows.

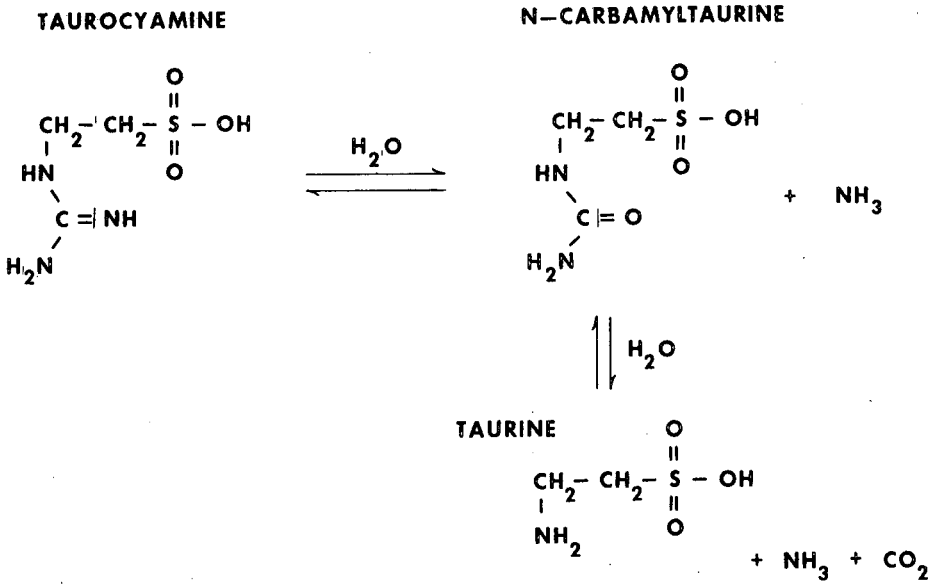
OXIDATION REACTIONS



Mu-21456

The existence of transamidination reactions in mammalian systems as a general type of biochemical reaction has had recent support.[32] Thoai, in his investigations of the biosynthesis of taurocyamine, has clearly demonstrated that a transamidination reaction of taurine to form taurocyamine exists in rats.[17, 33] Therefore, the reverse reaction being possible, the following reaction of taurocyamine could occur.

TRANSAMIDINATION REACTION



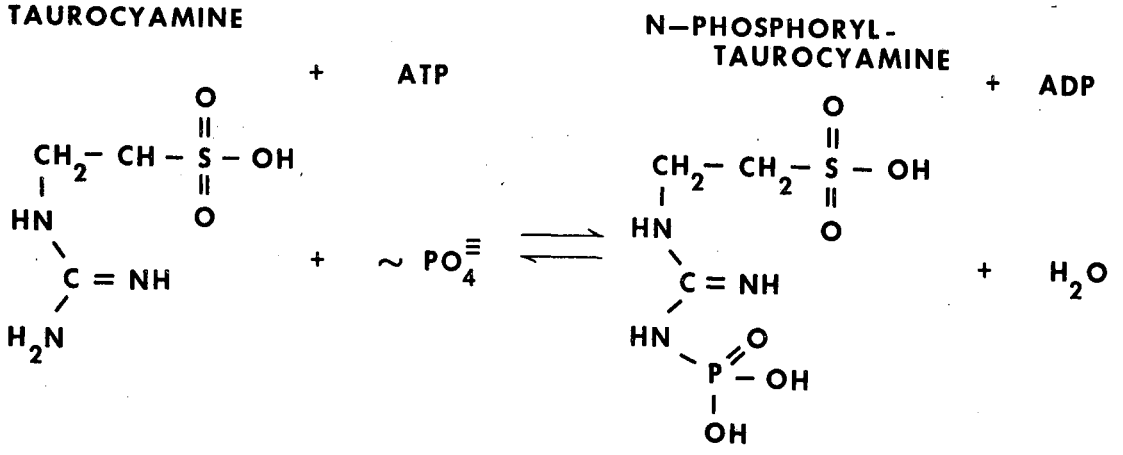
Mu-21452

However, it must be pointed out that the studies on the intracellular binding of AET by Bradford do not support the existence of the transamidination reaction.[16] These studies also concluded that it seemed unlikely that any large-scale cleavage of AET occurs. In addition, the investigations by Prickett, which showed no evidence of taurine formation, do not support the existence of a transamidination pathway.[25]

In a recent review on phosphagens, Ennor cites numerous examples of phosphorylation reactions involving substituted guanidines.[34]

One reaction in particular is of interest. This is the phosphorylation of taurocyamine to N-phosphoryltaurocyamine reported by Thoai and associates.[35] The evidence was obtained from an invertebrate system, and occurrence of this reaction in mammalian forms has not been reported. Thus, although the possibility is remote, a phosphorylation reaction of taurocyamine which would be derived from MEG oxidation would proceed as follows

PHOSPHORYLATION REACTION



Mu-21454

Therefore, from the theoretical considerations that AET may proceed through any, all, or none of the above reactions, it is abundantly clear that AET is capable of giving rise to many molecular structures. However, most of these structures possess electronic configurations which would be cationic, anionic, or neutral in character when in aqueous solution.

Thus, the radioactive products that appear in the urine could be a host of compounds which in the main possess differences in ionic charge. The application of ion-exchange chromatography to the solution of this problem has special merit, since resolution of chemical mixtures by ion exchange can be achieved through these differences.[36] The ion-exchange process becomes both a method of separation and a method of identification. The following schematic illustrates this point

Materials and Methods

Animals used were male rats weighing between 400 and 500 grams and 3 to 5 months of age. Animals were maintained in quarters with constant temperature (78 to 80°F) and humidity (55%). Purina laboratory chow and drinking water were given ad libitum. All animals were anesthetized with Nembutal-saline solution of a 1:10 (v/v) dilution. The volume of injection was approximately 1.0 ml and contained 50 to 55 mg of labeled AET.

Surgical technique for obtaining urine samples was the same as in the proceding section.

1. Radioactively Labeled S, 2-Aminoethylisothiuronium· Br· HBr (AET)

a. Preparation of sulfur-35-labeled AET

The method was as described in the preceding section.

b. Preparation of carbon-14-labeled AET

This was synthesized by the method of Doherty as described in an earlier section for the preparation of S^{35}-labeled AET.[12] Carbon-14-labeled thiourea was obtained from Tracerlab with specific activity of 2.84 mC per millimole.

2. Preparation of Synthetic Compounds Used for Identification Purposes in Quantitative Ion-Exchange Chromatography

a. 2-Mercaptoethylguanidine· HBr

Preparation was that described by Khym.[15] One gram of AET was dissolved in 20 ml of 0.18 N NaOH (final pH 7.0-7.5), which converts it quantitatively to 2-mercaptoethylguanidine.

In Fig. 3a is a standard quantitative chromatogram of MEG as obtained by cationic ion exchange.

b. Guanidinoethyldisulfide· 2HBr

4.05 grams (0.1 mole) of NaOH was placed in 20.0 ml of H_2O in a 250-ml Erlenmeyer flask. A teflon-coated magnetic stirrer was inserted into the flask to facilitate the solution of 28.1 grams (0.1 mole) of AET which was added. The pH was checked (7.5 to 8.0). After all the material had dissolved, 80.0 ml of acetone was added to the above solution. Then,

with the reaction flask placed in an ice bath but with stirring continued vigorously, 5.65 ml (0.05 mole) of 30% hydrogen peroxide was cautiously added dropwise. Frothing and a sudden increase in temperature due to this exothermic reaction cause bumping unless the reaction flask is kept cool (between 30 and 35°C). After addition of hydrogen peroxide, a white crystalline material began precipitating out of solution. The reaction mixture was stirred for 24 hours and at the end of this time the material was collected by suction filtration, and washed several times with aliquots of acetone, absolute ethanol, and ethyl acetate. The product was recrystallized from absolute ethanol and washed subsequently with ethyl acetate and dried in vacuo. Yield = 6.8 grams (24.2%), mp 189—190°C. This material is hygroscopic and was kept over P_2O_5 in vacuo.

Elemental Analysis (calculated for $C_6H_{18}N_6S_2Br_2$)

	Carbon (%)	Hydrogen (%)	Nitrogen (%)	Sulfur (%)
Calculated	18.1	4.5	21.1	16.1
Found	18.5	4.6	21.8	16.3

c. Preparation of picric acid derivative

The picrate derivative of guanidinoethyldisulfide was prepared by dissolving 1.2 grams (0.002 mole) of the disulfide into 10.0 ml of water in a 50-ml Erlenmeyer flask. Twenty ml of saturated solution of picric acid was added to this flask. Yellow, long, needlelike crystals began to precipitate. The flask was placed in the refrigerator overnight (12 hours). The crystals were collected by suction filtration and washed consecutively with water, 95% ethanol, and acetone, and then dried in vacuo. Yield = 0.93 gram (81.1%), mp 230—231°C.

Elemental Analysis (calculated for $C_{18}H_{22}N_{12}S_2O_{14}$)

	Carbon (%)	Hydrogen (%)	Nitrogen (%)	Sulfur (%)
Calculated	31.12	3.17	24.21	9.22
Found	31.24	3.31	24.32	9.04

Figure 3b is the quantitative chromatogram of guanidinoethyldisulfide (GED) as obtained by cationic ion exchange.

d. Guanidinoethyldisulfoxide·2HBr

4.05 grams (0.1 mole) of sodium hydroxide was placed in 20.0 ml of distilled water in a 250-ml Erlenmeyer flask. A teflon-coated magnetic stirrer was inserted into the flask to facilitate the solution of 28.1 grams (0.1 mole) of AET added. The pH was checked (7.5 to 8.0). After the material had dissolved, the flask was placed in an ice bath. Then, with vigorous stirring by the magnetic stirrer, 11.3 ml (0.1 mole) of 30% hydrogen peroxide was slowly added dropwise. Caution was taken to keep temperature of flask between 30 and 35°C. The pH of the reaction mixture after complete addition of hydrogen peroxide was checked (acidic, <1.0). The solution was heated to 50 to 60°C and maintained at this temperature for 24 hours. The reaction mixture was allowed to cool to room temperature. After several minutes' standing, crystallization within the solution began. The crystals were collected by suction filtration and washed with several aliquots of absolute ethanol and acetone. The product was recrystallized from absolute methanol, washed with ether, and dried in vacuo. Yield = 14.4 grams (33.4%), mp 179–182°C.

Elemental Analysis: Calculated for $C_6H_{18}N_6S_2O_2Br_2$

	Carbon (%)	Hydrogen (%)	Nitrogen (%)	Sulfur (%)
Calculated	16.74	4.18	19.53	14.89
Found	18.15	4.35	20.52	13.25

In Fig. 3c is the quantitative chromatogram of guanidinoethyldisulfoxide (GEDO) as obtained by cationic ion exchange.

e. Bis(2-guanidinoethyl)sulfone·2HBr

In a 250-ml round-bottom boiling flask was placed 1.2 grams (0.002 mole) of guanidinoethyldisulfide. To this flask was added 3.0 ml of glacial acetic acid followed by 45.0 ml of a 50% acetone-water mixture. To this mixture 0.32 ml (0.005 mole) of liquid bromine was added. A

reflux condenser was fitted to the flask and refluxing was commenced. The mixture was refluxed for 1 hour, during which time the bromine color disappeared completely. After refluxing, the mixture was allowed to come to room temperature and crystallization began. The flask was placed in the refrigerator overnight. The crystals were collected by suction filtration and washed in ether twice and dried in vacuo. Yield = 0.53 gram (84.4%); mp 167-168°C.

Elemental Analysis (calculated for $C_6H_{18}N_6S_1O_2Br_2$)

	Carbon (%)	Hydrogen (%)	Nitrogen (%)	Sulfur (%)
Calculated	18.09	4.52	21.11	8.04
Found	18.70	4.61	20.98	9.82

In Fig. 3d is the quantitative chromatogram of bis(2-guanidinoethyl)sulfone·2HBr (GEDU) as obtained by cationic ion exchange.

f. 2-guanidinoethylsulfonic acid (Taurocyamine)

This method is a modification of that proposed by Smith.[37] In a 100-ml Erlenmeyer flask is placed 9.3 grams (0.05 mole) of S-ethylthiourea hydrobromide. The flask is immersed in an ice bath and 20.0 ml of 10% sodium hydroxide is added, followed by the rapid addition of a hot solution (80°C) which contains 6.7 grams of taurine in 20.0 ml of water. When the temperature reaches 25°C, the flask is removed from the ice bath. Gaseous evolution denotes liberation of ethyl mercaptan.* After about a half-hour, crystallization begins. Approximately 20.0 ml of ether is added and the mixture is left in the hood overnight. The mixture is then chilled for two hours in an ice bath, the ether layer decanted, and the solid is filtered by suction. The crystals are washed on the funnel successively with aliquots of ice water, 95% ethanol and ether. The product is then air dried. Yield = 4.7 grams (56.3%), mp 267-268°C.

A mixed melting point sample was prepared from a sample of taurocyamine generously supplied to the author by Professor A. H. Ennor. This mixed sample showed no depression of the melting point.

*Note: All reactions should be carried out in a hood.

Elemental Analysis (Calculated for $C_3H_9N3SO_3$)

	Carbon (%)	Hydrogen (%)	Nitrogen (%)	Sulfur (%)
Calculated	21.56	5.88	25.15	19.16
Found	21.81	5.77	25.12	19.35

g. N-phosphoryl-2-guanidinoethylsulfonic acid (N-phosphoryltaurocyamine)

50.0 mg of the ammonium salt of this acid was obtained as a gift from Professor A. H. Ennor, Department of Biochemistry, John Curtin School of Medical Research, Australian National University, Canberra, ACT, Australia.

h. N-cholyl-2-aminoethylsulfonic acid (Taurocholic acid)

Taurocholic acid was prepared by the method of Norman,[38] which is an adaptation of the mixed-anhydride synthesis of peptides.

i. 2-aminoethylsulfonic acid (Taurine)

This was obtained from California Foundation of Biochemical Research, 1408 Fowler Street, Los Angeles, California.

3. Procedure Used in Ion-Exchange Chromatography

a. All separations of the cationic species were made with 200 to 400 mesh strong-acid cation exchanger (Dowex AG-50X12, hydrogen form, which was converted to Na^+ form with an equivalent amount of NaOH). Duplicate columns 1 cm^2 in cross section and 5 cm high were prepared in the conventional manner.[39] Samples were absorbed on the resin column from 0.002 M HCl (5-10 ml) at a pH of 2.8 to 3.0. Elution was began with distilled water, which was followed by a succession of increasing concentrations of HCl. Fractions were collected with an automatic sample changer.

b. All separations of the anionic species were made with 200 to 400 mesh strong-acid anion exchanger (Dowex AG1X2, chloride form). Duplicate columns 1 cm^2 in cross section and 5 cm high were prepared as described in the preceding paragraph. Samples were absorbed on the

resin column from 1×10^{-4} M NaOH (5 to 10 ml) at a pH of 9.0 to 9.5. Elution was begun with this solvent, which was followed by a succession of solvents of increasing concentrations of NaCl (pH 9.0 to 9.5). Fractions (20 ml) were collected with an automatic sample changer.

c. All guanidine compounds were assayed quantitatively by the colorimetric method of Rosenberg.[40] Standard curves were made for each compound, and in each case followed the Beer-Lambert law.

d. Nindydrin reaction

Taurine was assayed by the colorimetric method described by Moore and Stein,[41]

e. Bile-acid assay

Mylius's modification of Pettenkofer's test.[42]

f. For the radioactive assay, a 1.0-ml sample from each fraction collected was plated on a polyethylene planchet and counted in a gas-flow proportional counter. All activity measurements were corrected for backscattering, coincidence losses, absorption, decay, and background. Activities of samples are expressed as corrected counts per minute per ml of eluent.

g. The mixture of radioactive products from the urine was separated on identical cation-exchange and anion-exchange columns using the same amounts of eluting agents as was used in the standard columns. The position of each radioactive peak was then compared to a standard column made on known amounts of the synthetic compounds. Furthermore, several different qualitative chemical test (Sakaguchi, Nitroprusside, etc.) were used as an aid to identification.

Results

In Fig. 3a through 3f are shown the standard chromatograms obtained by cation exchange for the compounds prepared by methods described in the preceding section.. All these compounds possess at least one guanidino group as a functional end group with the exception of taurine (2-aminoethylsulfonic acid). Thus, each of these should possess cationic properties. One should note that the position of the elution peak, which is a measure of the affinity of the compound for the resin, is markedly influenced by the type and presence of the other functional group. For example, if this group is strongly anionic in character, then the entire molecule behaves as a neutral molecule (Fig. 3e). This is analogous to the zwitterion of amino acids, in which both functional groups, amino and carboxyl, are ionized at the isoelectric point. However, if the group is strongly cationic in character (say another guanidino group), it reinforces the cationic properties, and the molecule behaves as a much stronger cation (Fig. 3b).

Thus the products derived from the stepwise oxidation of MEG arrange themselves into a spectrum of cations from the very skrongest (GED) to the weakest (GT), as shown in the composite chromatographic profile (Fig. 4a).

In Fig. 4b is a typical chromatogram obtained by the cation exchange of the radioactive products found in the urine of a rat 3 hours after intraperitoneal injection of carbon-14-labeled AET. It should be noted that AET was converted into MEG in the process of preparing the injection solution. This was confirmed by chemical tests for functional groups (-SH and $-NH-\overset{NH}{C}-NH_2$) and by chromatography. Thus, the resulting radioactive labeling of the molecular structure of the injected compound is as shown below

2-Mercaptoethyl-C^{14}-guanidine

$$\begin{array}{l} HN-CH_2-CH_2-SH \\ \quad | \\ \quad *C=NH \\ \quad | \\ \quad H_2N \end{array}$$

Fig. 3. Standard chromatograms obtained by cation exchange of synthetic compounds prepared for identification of metabolic products of AET on Dowex AG50 x 12, Na^+ form.

a. Cation exchange of 2-mercaptoethylguanidine (MEG). Units on column: 28 mg. Assay: Colorimetric method of Rosenberg. Flow rate: 1.0 ml/min. Elution peak range: 1000±50 ml of 0.2 M HCl. Av. recovery: 89%.

b. Cation exchange of guanidinoethyldisulfide (GED). Units on column: 50 mg. Assay: Colorimetric method of Rosenberg. Flow rate: 0.9 ml/min. Elution peak range: 425±50 ml of 3.0 M HCl. Av. recovery: 91%.

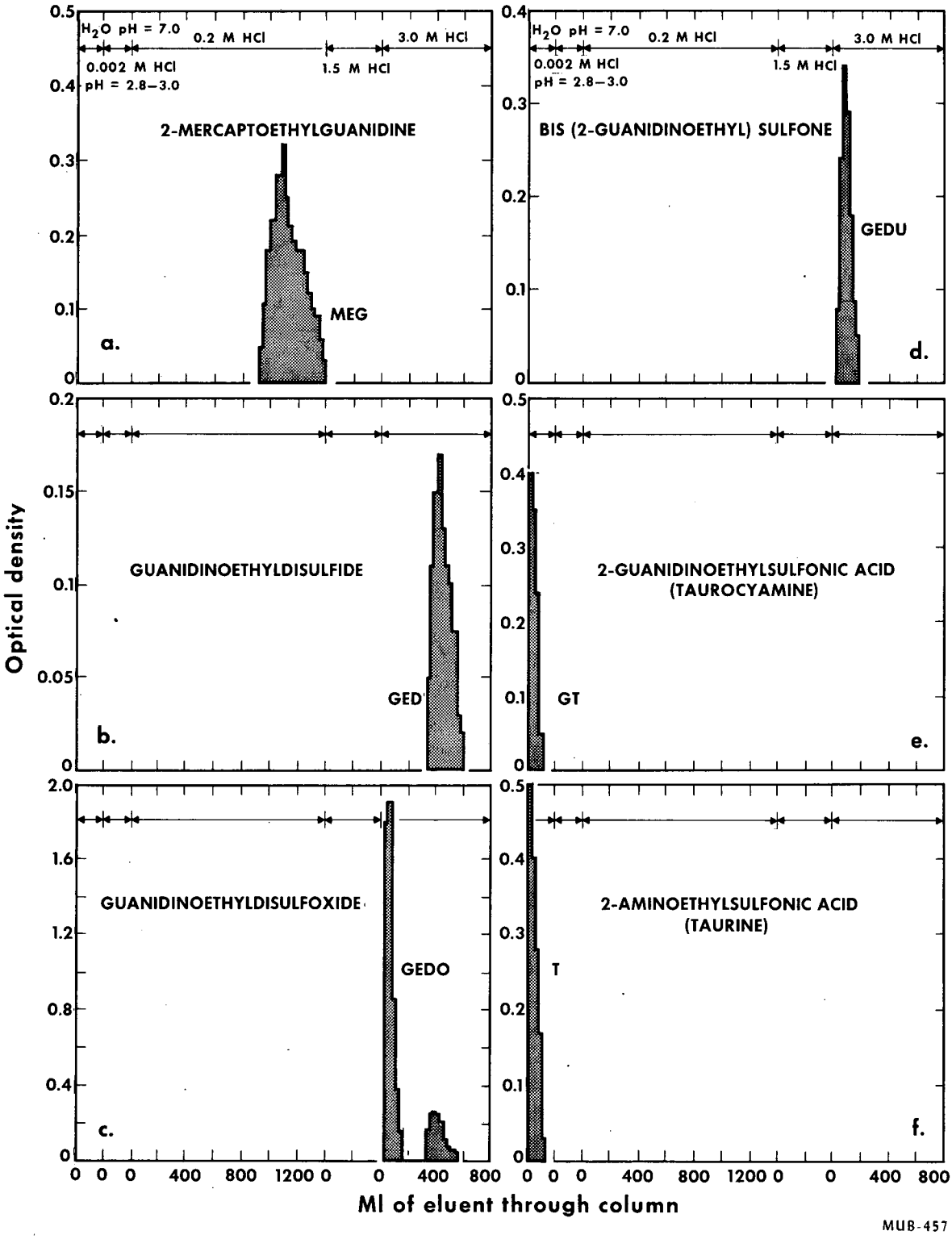
c. Cation exchange of guanidinoethyldisulfoxide (GEDO). Units on column: 86 mg. Assay: Colorimetric method of Rosenberg. Flow rate: 1.0 ml/min. Elution peak range: 50±25 ml of 3.0 M HCl. Av. recovery: 97%. Note the small peak of GED appearing as a contaminant in this preparation.

d. Cation exchange of bis(2-guanidinoethyl) sulfone (GEDU). Units on column: 46 mg. Assay: Colorimetric method of Rosenberg. Flow rate: 1.0 ml/min. Elution peak range: 75±15 ml of 3.0 M HCl. Av. recovery: 70%.

e. Cation exchange of 2-guanidinoethylsulfonic acid (GT). Units on column: 100 mg. Assay: Colorimetric method of Rosenberg. Flow rate: 1.0 ml/min. Elution peak range: 50±10 ml of H_2O(pH = 7.0). Av. recovery: 98%.

f. Cation exchange of 2-aminoethylsulfonic acid (T). Units on column: 30 mg. Assay: Colorimetric method of Moore and Stein. Flow rate: 1.0 ml/min. Elution peak range: 40±15 ml of H_2O (pH = 7.0). Av. recovery: 97%.

f. Cation exchange of 2-aminoethylsulfonic acid (T). Units on column: 30 mg. Assay: Colorimetric method of Moore and Stein. Flow rate: 1.0 ml/min. Elution peak range: 40±15 ml of H_2) (pH = 7.0). Av. recovery: 97%.

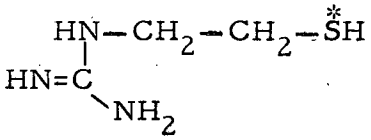

H_2O pH = 7.0
0.2 M HCl
3.0 M HCl
0.002 M HCl
pH = 2.8–3.0
1.5 M HCl
2-MERCAPTOETHYLGUANIDINE
MEG
a.
BIS (2-GUANIDINOETHYL) SULFONE
GEDU
d.
GUANIDINOETHYLDISULFIDE
GED
b.
2-GUANIDINOETHYLSULFONIC ACID
(TAUROCYAMINE)
GT
e.
GUANIDINOETHYLDISULFOXIDE
GEDO
c.
2-AMINOETHYLSULFONIC ACID
(TAURINE)
T
f.
Optical density
Ml of eluent through column
MUB-457

Comparison of this chromatogram to the standard cation-exchange chromatographic profile shows the presence of a large quantity of the oxidized product taurocyamine (GT). Presence of peaks for MEG substantiates that the injection compound was MEG. Activity was also present for GED, which is the first oxidative step. No appreciable activity was present for the disulfoxide or sulfone peaks of MEG. A small peak appeared which is tentatively thought to be the sulfonic acid. There appeared two peaks, one at 825 ml of 0.2 M HCl, and the other at approximately 50 ml of 1.5 M HCl. These remain unidentified.

To confirm that major cleavage of the molecule has not taken place, another position in the molecular structure of the compound was radioactively labeled. The radioactive label (sulfur-35) was placed at the mercapto group. Therefore, if the previous studies are correct and one does not see cleavage of the molecule, then comparison between the chromatographic analysis of the urine containing sulfur-35 products to that which contained carbon-14 products should yield chromatograms identical in pattern. Of course, the quantity of each component as represented by each elution peak(s) may vary because of its dependence on concentration of injected dose.

In Fig. 4c are typical chromatograms obtained by the cation ion exchange of the radioactive products found in the urine of a rat 3 hours after intraperitoneal injection of 55.3 mg of sulfur-35-labeled AET. Again, prior to injection the compound was converted into MEG as in the preceding paragraph. The labeling of the molecular structure of the injected compound now appears in a position as shown below.

S^{35}, 2-mercaptoethylguanidine



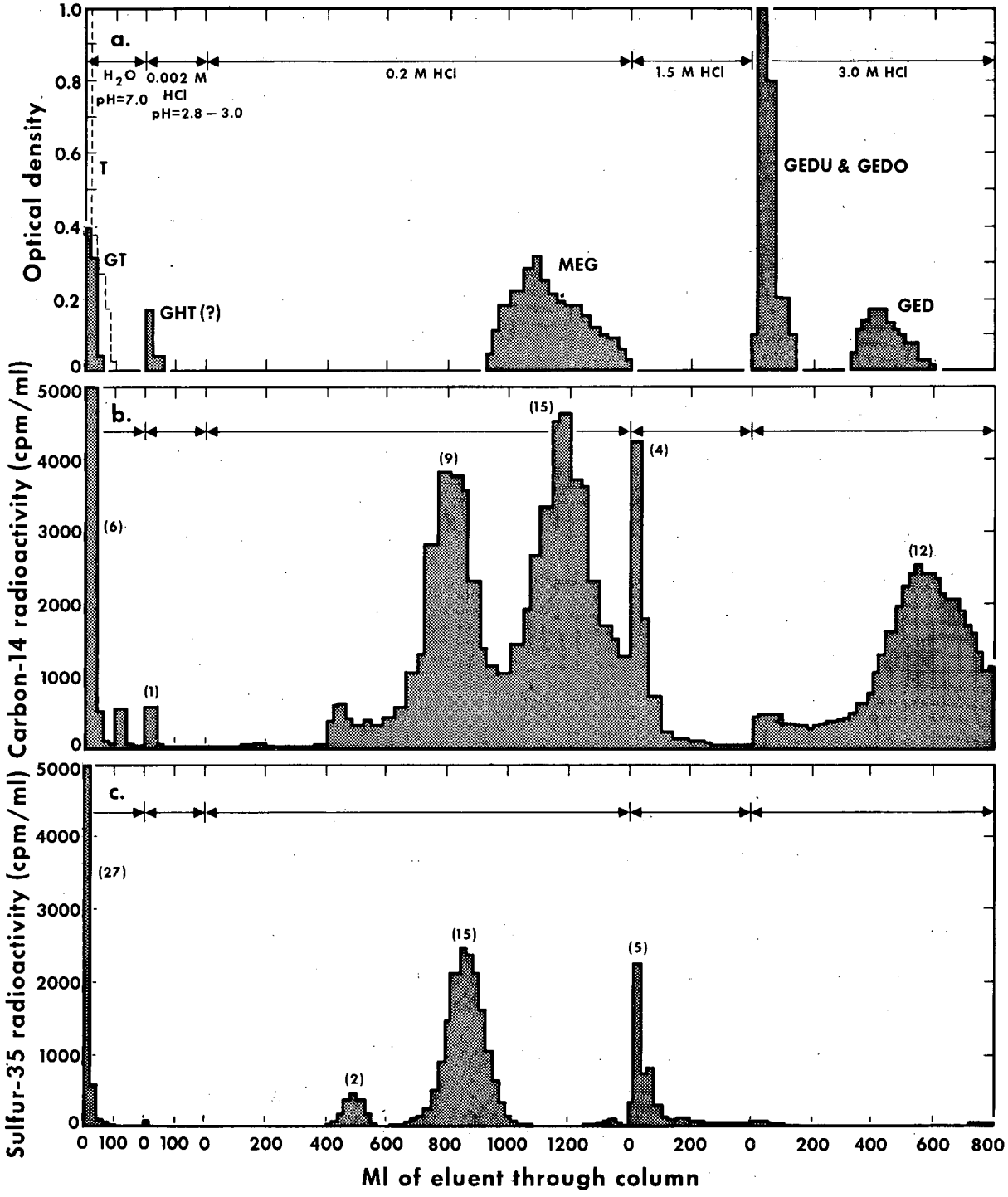
The comparison clearly shows that the two chromatographic patterns are not identical. Note the absence of peaks for MEG and GED

Fig. 4. Typical chromatograms obtained by cation exchange of radioactive products found in urine on Dowex AG50 x 12, Na^+ form.

a. Standard chromatographic elution curves of synthetic compounds which were described in Fig. 3.

b. Cation exchange of radioactive products obtained from a rat injected with 55.5 mg of carbon-14-labeled AET intraperitoneally. Units on column: 6.8×10^6 cpm. Assay: Radioactivity as measured on a gas-flow counter. Flow rate: 1.0 ml/min. Av. recovery: 82%.

c. Cation exchange of radioactive products obtained from a rat injected with 55.3 mg of sulfur-35-labeled AET intraperitoneally. Units on column: 1.2×10^6 cpm. Assay: Radioactivity as measured on a gas-flow counter. Flow rate: 1.0 ml/min. Av. recovery: 79%.


a.
b.
c.
Optical density
Carbon-14 radioactivity (cpm/ml)
Sulfur-35 radioactivity (cpm/ml)
Ml of eluent through column
H2O pH=7.0
0.002 M HCl pH=2.8 – 3.0
0.2 M HCl
1.5 M HCl
3.0 M HCl
T
GT
GHT (?)
MEG
GEDU & GEDO
GED
(1)
(2)
(4)
(5)
(6)
(9)
(12)
(15)
(27)

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in the sulfur-35 chromatogram. The importance of this will be emphasized in the discussion. One major point is that the two peaks at 825 ml of 0.2 M HCl and 50.0 ml of 1.5 M HCl also appear in both chromatograms. Therefore, it must be concluded that for these two peaks, the molecular structure remains intact. The presence of a large fraction of activity again in the taurocyamine (GT) peak proves that this thiol compound, like others, undergoes an oxidative reaction of the mercapto group.

Further proof is necessary to establish that the activity appearing in this fraction is taurocyamine because, as was shown in the urine fractionation scheme, this could very well represent a mixture of compounds having neutral or anionic properties. Therefore, this fraction obtained in each cation-exchange run was subjected to anion-exchange analysis.

In Fig. 5a and 3c are the typical standard chromatograms obtained by anion exchange of two compounds, taurine and taurocyamine. These two compounds possess a sulfonic acid group as one functional group. Again, one notes that the affinity of the compound for the resin is markedly influenced by the character of the other functional group on the opposite end of the molecule. If the group is cationic in character (such as amino or guanidino), the molecule possesses zwitterion characteristics. However, if this group is modified, such as by acylation or phosphorylation, then the molecule behaves over all as an anion because the cationic properties of the amino or guanidino group are being masked. The standard chromatogram of N-phosphoryltaurocyamine (Fig. 5b) illustrates this to be the case for taurocyamine (2-guanidinoethylsulfonic acid), a neutral molecule, which is converted to a weak anion by phosphorylation. Another example is the acylation of taurine by cholic acid to form taurocholic acid (Fig. 5d).

Thus the product taurocyamine derived from complete oxidation of MEG, which possesses neither cationic or anionic properties, has the possibility of undergoing the biochemical reactions which could transform this neutral molecule into a molecule possessing anion properties.

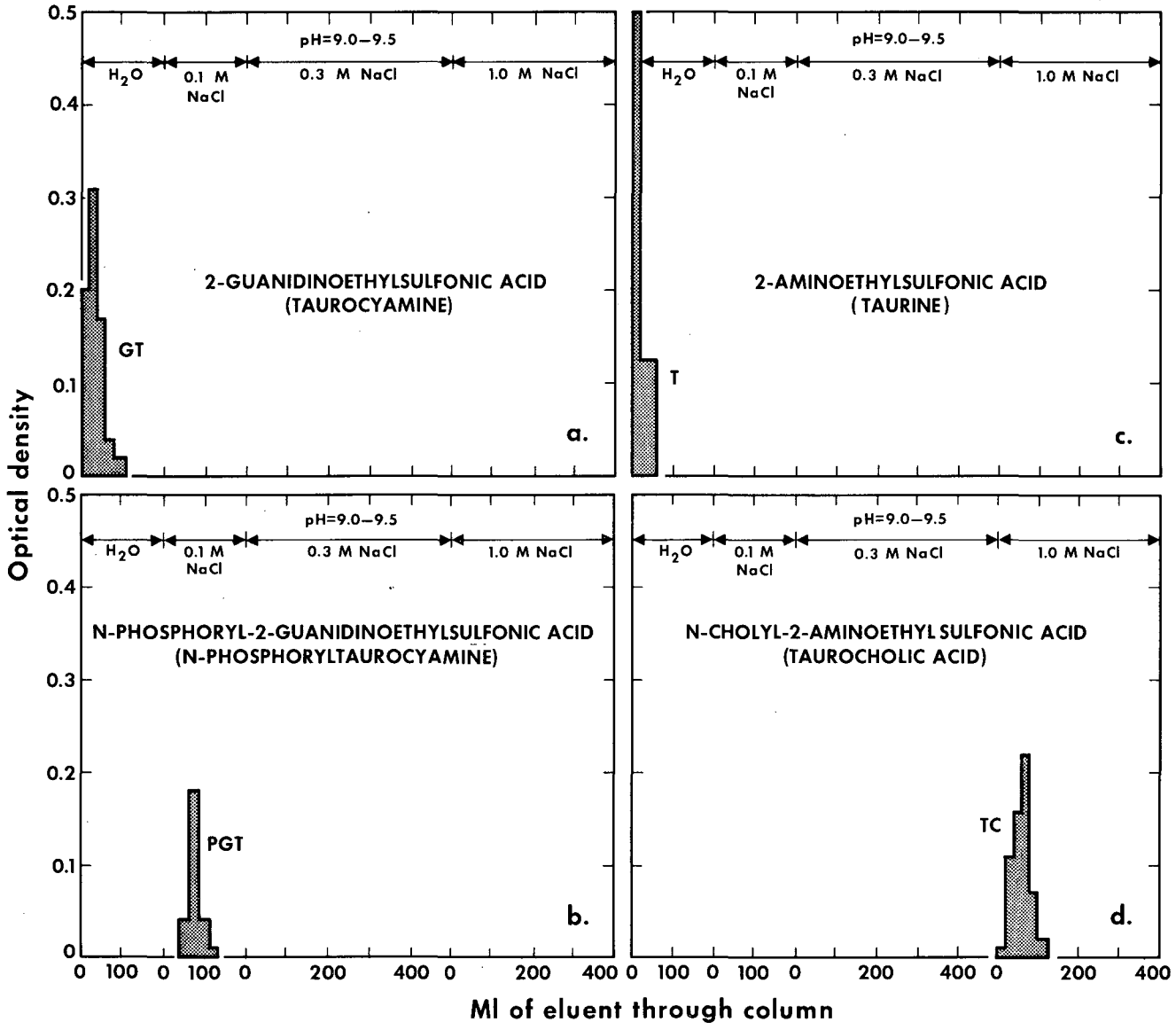
Fig. 5. Standard chromatograms obtained by anion exchange of synthetic compounds prepared for identification of metabolic products of AET on Dowex AG1 x 2, Cl form.

a. Anion exchange of 2-guanidinoethylsulfonic acid (GT). Units on column: 25 mg. Assay: Colorimetric method of Rosenberg. Flow rate: 1.0 ml/min. Elution peak range: 50±20 ml of H_2O (pH 9.0-9.5). Av. recovery: 95%.

b. Anion exchange of N-phosphoryl-2-guanidinoethylsulfonic acid (PGT). Units on column: 30 mg. Assay: Colorimetric method of Rosenberg. Flow rate: 1.0 ml/min. Elution peak range: 75±10 ml of 0.1 M NaCl. Av. recovery: 79%.

c. Anion exchange of 2-aminoethylsulfonic acid (T). Units on column: 30 mg. Assay: Colorimetric method of Moore and Stein. Flow rate: 1.0 ml/min. Elution peak range: 25±5 ml of H_2O (pH 9.0-9.5). Av. recovery: 91%.

d. Anion exchange of N-cholyl-2-aminoethylsulfonic acid (TC). Units on column: 50 mg. Assay: Colorimetric method of Mylius. Flow rate: 1.0 ml/min. Elution peak range: 75±10 ml of 1.0 M NaCl. Av. recovery: 83%.


Optical density
0.5
0.4
0.3
0.2
0.1
0
pH=9.0–9.5
H2O
0.1 M NaCl
0.3 M NaCl
1.0 M NaCl
2-GUANIDINOETHYLSULFONIC ACID
(TAUROCYAMINE)
GT
a.
2-AMINOETHYLSULFONIC ACID
(TAURINE)
T
c.
N-PHOSPHORYL-2-GUANIDINOETHYLSULFONIC ACID
(N-PHOSPHORYLTAUROCYAMINE)
PGT
b.
N-CHOLYL-2-AMINOETHYL SULFONIC ACID
(TAUROCHOLIC ACID)
TC
d.
0 100 0 100 0 200 400 0 200 400
0 100 0 100 0 200 400 0 200 400
Ml of eluent through column

MUB-454

In Fig. 6b is a typical chromatogram obtained by the anion exchange of the urine fraction containing the carbon-14 products which have passed through the cation-exchange columns and appeared as taurocyamine (GT).

In Fig. 6c is another typical chromatogram obtained by the anion exchange of the urine fraction containing the sulfur-35 products treated in the above manner.

Comparison of these two chromatograms to the standard anion-exchange chromatographic profile (Fig. 6a) confirms the presence of taurocyamine but also shows that the fraction was a mixture of compounds. The presence of a sizeable amount of activity in the elution peak at 50 ml of 0.1 M NaCl in each chromatogram suggests that modification of the guanidino group has taken place. However, to determine what this modification is requires further investigation.

In conclusion, the results of the these studies clearly show that both functional groups of the molecule have undergone chemical attack. The free mercapto group of the substituted guanidine is transformed into the sulfonic acid derivative (taurocyamine). Then the guanidino group of this sulfonic acid derivative is further modified into an as yet unidentified structure. Possibly, it has undergone phosphorylation or transamidination.

Discussion

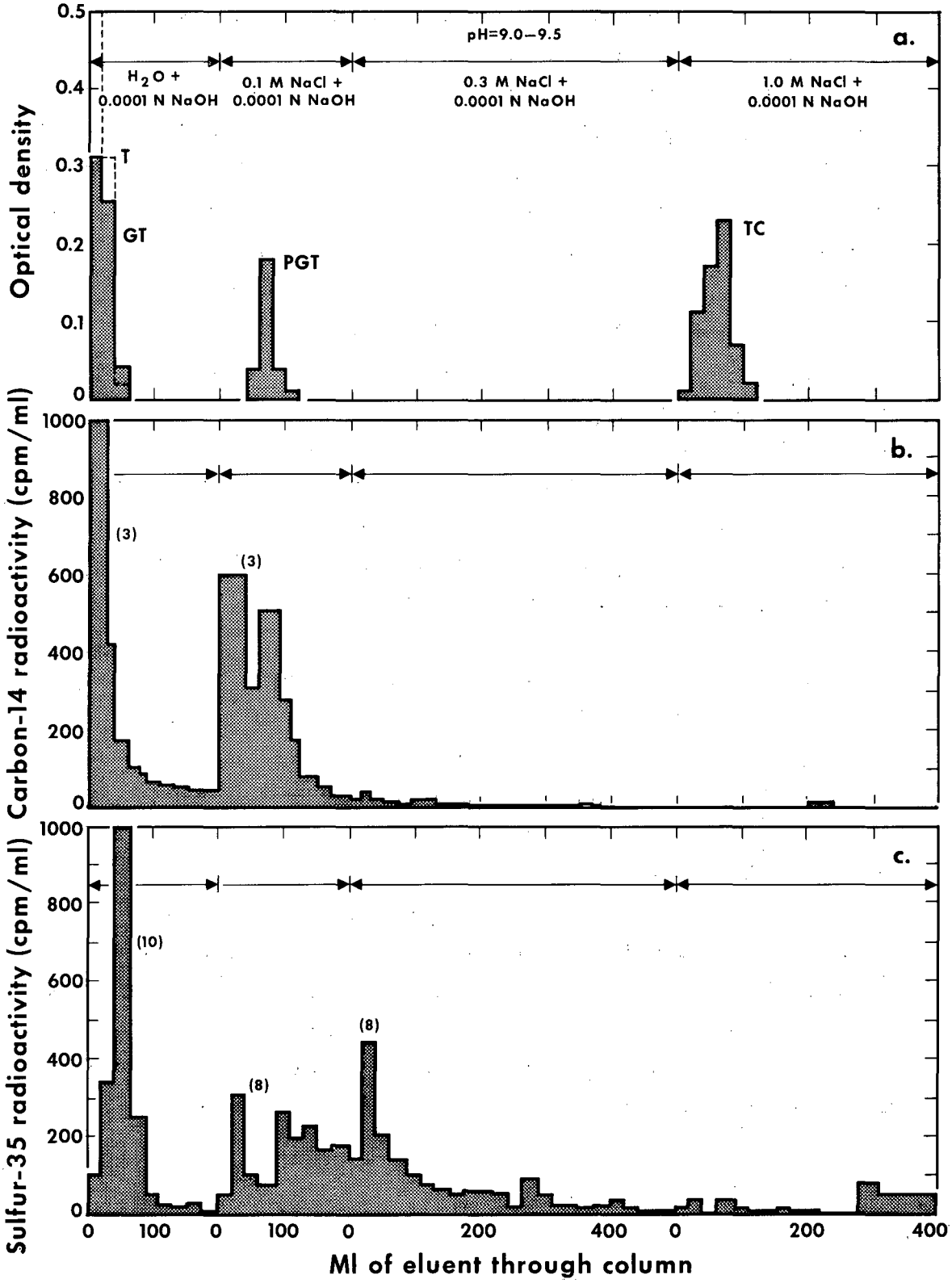
The chromatographic patterns obtained from the cationic- and anionic-exchange analysis in this study do not show identical components. The absence of the MEG and GED components in the cation exchange of the urine products from animals injected with sulfur-35 AET is in accord with the findings of Pritchett, who found no evidence for the presence of the MEG in the urine of humans given sulfur-35-labeled AET.[25] However, the recent report of Shapiro shows a guanidine compound to be present in the urine of mice, tentatively identified as MEG.[43] Thus these findings for the sulfur-labeled AET seem

Fig. 6. Typical chromatograms obtained by anion exchange of radioactive products found in urine on Dowex AG1 x 2, Cl^- form.

a. Standard chromatographic elution curves of synthetic compounds which were described in Fig. 5.

b. Anion exchange of the urine fraction containing the radioactive products which have passed through the cation-exchange column. Rat injected with 55.5 mg of carbon-14-labeled AET intraperitoneally. Units on column: 3.6×10^5 cpm. Assay: Radioactivity as measured on a gas-flow counter. Flow rate: 1.0 ml/min. Av. recovery: 78%.

c. Anion exchange of the urine fraction containing the radioactive products which have passed through the cation-exchange column. Rat injected with 55.3 mg of sulfur-35-labeled AET intraperitoneally. Units on column: 3.1×10^5 cpm. Radioactivity as measured on a gas-flow counter. Flow rate: 1.0 ml/min. Av. recovery: 81%.


a.
pH=9.0—9.5
H_2O + 0.0001 N NaOH
0.1 M NaCl + 0.0001 N NaOH
0.3 M NaCl + 0.0001 N NaOH
1.0 M NaCl + 0.0001 N NaOH
Optical density
T
GT
PGT
TC
b.
Carbon-14 radioactivity (cpm/ml)
(3)
(3)
c.
Sulfur-35 radioactivity (cpm/ml)
(10)
(8)
(8)
Ml of eluent through column

MUB-456

to be in conflict which should cast doubt on the assumption that this molecule does not undergo cleavage. The data in this study constitute strong evidence that the molecule is indeed split.

Also, this study reports the definite existence of taurocyamine along with other products found in the urine following AET administration. This finding is substantiated by the recent results of Shapiro and thus supports the view that one possible metabolic route for AET is through the oxidation of the mercapto group of MEG to the sulfonic form, taurocyamine (GT).

However, the results obtained by anion exchange of this fraction (taurocyamine) are revealing in that the assumption that the guanidino group was chemically "inert" was erroneous. That the guanidino group plays a functional part in the metabolic degradation is evidenced by the fact that 50% of the peak appearing as taurocyamine is identified tentatively as phosphotaurocyamine. This identification awaits further confirmation. However, what is definite is that the basic property of the guanidino group, in some unknown manner, has been changed. Thus, the two unidentified peaks (Fig. 4) in the cation exchange could represent some change in the guanidino group instead of the mercapto group.

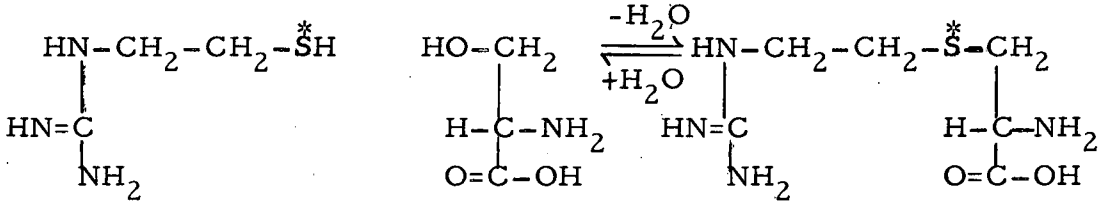
Comparisons of the results of the anion-exchange chromatograms reveal that there is a larger number of labeled compounds found for the sulfur-35 AET than for the carbon-14 AET. This could be interpreted that the S^{35} atom of the mercapto group enters into more metabolic paths than the C^{14} atom of the guanidino group. Thus, one explanation for the absence of MEG and GED components in the sulfur-35-injected animal might be the dilution of the injected thiol, which equilibrates and exchanges its sulfur atom with other compounds, thus making it seem to have disappeared, whereas the labeled guanidino group, being unaffected by this sulfur exchange, correctly identifies the intermediate steps in the degradative pathway.

Therefore, the question posed by this hypothesis is: Through what possible mechanism could this exchange take place?

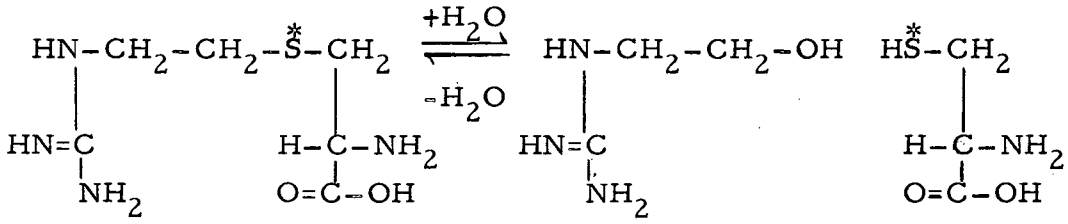
Tabachnick and Tarver presented evidence for a transthiolation reaction which results in the formation of a mixed sulfide.[44] Their studies on rats fed DL methionine and L serine showed a conversion of the sulfur-35-labeled DL methionine to labeled taurine, glutathione, cystine, and cysteic acid, which took place through the mixed sulfide, cystathionine.

Therefore one could speculate that MEG behaves in a somewhat similar manner, forming a mixed sulfide in which the sulfur exchange takes place. Thus the process would be a transulfuration reaction:

(1) 2-mercaptoethylguanidine + serine ⟶ mixed sulfide



(2) Mixed sulfide ⟶ 2-hydroxyethylguanidine + cysteine



Consideration of the number of charges that the mixed sulfide molecule could possess suggests that it would enter into the chromatographic pattern represented by the peak at 50 ml of 1.5 M HCl of the cation exchange.

However, Eldjarn favors a different hypothesis in which the main reaction between the injected thiol and other body constituents, mainly disulfides, is in the formation of mixed disulfides.[45] Also it is assumed that this mixed-disulfide reaction plays the important role in effecting protection against radiation injury. There is abundant evidence in the

literature which substantiates that exchange reactions between thiol and disulfide compounds result both in vivo and in vitro in the formation of mixed disulfide.[46, 47, 48] However, in these reactions, the evidence favors the splitting of the disulfide -S-S- between the sulfur atoms rather than between -C-S- bond. Therefore, no exchange of sulfur atoms between the injected thiol and other potential thiols can occur unless there is exchange of the sulfur atoms within the mixed-disulfide linkage. Thus, if exchange did occur, it would constitute a breakage of a -C-S- bond within the disulfide linkage. If the assumption that the molecular structure of AET remains intact is incorrect then the hypothesis that this thiol may undergo transulfuration reaction, which results in the formation of a mixed sulfide in addition to the mixed disulfide formation, becomes significant. However, the radiobiological significance that can be attached to this biochemical reaction as regards the radiation sequelae is another problem.

Furthermore, the chemical modification of the guanidino group justifies that further inquiry should be made so as to ascertain what biochemical role this group plays in the metabolism of AET.

V. IDENTIFICATION OF RADIOACTIVE PRODUCTS IN THE BILE FOLLOWING THE ADMINISTRATION OF RADIOACTIVE LABELED AET

The observation in the tissue distribution studies indicated that the probable site of metabolism of AET was the liver. In addition, the results obtained from the analysis of the urine products in the preceding section showed the formation of a large quantity of taurocyamine. Furthermore, anion analysis indicated that the guanidino moiety of this molecule had undergone a modification which changed the charge of this group. It was thought that a first step in the attempt to elucidate the nature of this chemical reaction was to consider what type of reaction might occur for the guanidino group.

Thoai in his studies on the biosynthetic pathway of taurocyamine reported that taurine could be converted to taurocyamine by a transamidination reaction.[17] Thus, if this reaction is reversible, one should find, following taurocyamine administration, the presence of taurine.

In the study on taurine metabolism by Kay, it was shown that a large fraction of taurine excretion occurs through the urine.[49] Analysis of the urine products in the present study could not resolve taurine and taurocyamine adequately because of the overlap of the elution peaks for these two compounds in the cation- and anion-exchange procedure. However, in the study by Eldjarn, evidence is presented which shows that taurine, in addition to being excreted through the urinary route, can also be converted to taurocholic acid and excreted into the intestines as such via the bile.[26] Thus, an analysis made of the bile obtained from sulfur-35-AET- and carbon-14-AET-injected animals for the presence of labeled taurocholic acid would be a means to obtain evidence either in favor of or against the presence of a transamidination reaction.

Materials and Methods

Animals used were male rats weighing between 300 and 400 grams, 3 to 4 months of age, maintained in quarters with constant temperature (78 to 80°F) and humidity (55%). Purina laboratory chow and drinking water were given ad libitum. All animals were anesthetized with Membutal-saline solution (1:10). Each injection for the serial uptake of radioactivity in the bile was 0.2 ml in volume and contained 0.2 mg of AET, except where the dose was specified as being otherwise.

The surgical technique employed in obtaining the serial uptake of radioactivity via the bile consisted of cannulation of the bile duct with a 1-mm o.d. polyethylene tubing that was beveled at a 45-degree angle at one end. Entrance into the peritoneal cavity was made via a midline incision. Ligation of the duct proximal to the intestine was followed by partial sectioning of the duct wall into which the cannula was inserted and then tied in place. A 6-inch piece of the cannula was brought out through the body wall, after which the body wall was sutured the skin was closed with wound clips. The polyethylene tubing was placed in a position on animal board in order that the bile could be collected by allowing it to drop either onto tared stainless steel planchets for the serial-uptake study or into a collection flask for the chemical separation on ion-exchange columns. After each sample was taken for the serial uptake, the planchets were immediately weighed on an analytical balance, then dried, and counted in a gas-flow proportional counter. All activity measurements were corrected for backscattering, coincidence losses, absorption, decay, and background.

Radiactive labeled S, 2-aminoethylisothiuronium· Br· HBr(AET)

a. Preparation of sulfur-35-labeled AET

The method was as described in the preceding section.

b. Carbon-14-labeled AET.

This was synthesized by the method of Doherty as described in an earlier section.[12]

The chemical separation of the bile was made by ion-exchange chromatography procedure as described in the preceding section.

Radioactive products from the bile were identified as described in preceding section.

Results

The serial uptake studies of the bile (Protocol IV, V, VI Appendix) indicate that, in addition to the urinary route of excretion, radioactively labeled AET or metabolic products derived from it can be eliminated through the biliary system. The total amount of radioactivity excreted within the bile for a 60-minute period was calculated as approximately 1.0% of the injected dose (55.0 mg) for the intraperitoneal route, and 0.1% for the intravenous and oral routes, respectively. In contrast to urinary excretion, this constitutes a very small fraction.

The identification of the radioactivity found in the bile was then undertaken. In Fig. 7 is the chromatographic separation of the bile using cation exchanger. The results show that activity represents a mixture of compounds, and comparison of the carbon-14-injected animal with the sulfur-35-injected animal indicates a completely different spectrum of compounds for the two differently labeled AET. However, the largest fraction of activity appeared in the taurocyamine fraction.

In Fig. 8 is the chromatographic separation of the taurocyamine fraction on anionic exchanger. These findings, in which there is absence of the taurocholic peak in both the sulfur-35-injected animals and the carbon-14-injected animals, support the view that transamidination is not a reaction undergone by AET or products derived from it.

Fig. 7. Typical chromatograms obtained by cation exchange of radioactive products found in bile on Dowex AG50 x 12, Na^+ form.

a. Standard chromatographic elution curves of synthetic compounds which were described in Fig. 3.

b. Cation exchange of radioactive products obtained from a rat injected with 49.5 mg of carbon-14-labeled AET intraperitoneally. Units on column: 4.1×10^5 cpm. Assay: Radioactivity. Flow rate: 1.0 ml/min. Av. recovery: 79%.

c. Cation exchange of radioactive products obtained from a rat injected with 55.4 mg of sulfur-35-labeled AET intraperitoneally. Units on column: 3.5×10^5 cpm. Assay: Radioactivity. Flow rate: 1.0 m/min. Av. recovery: 75%.

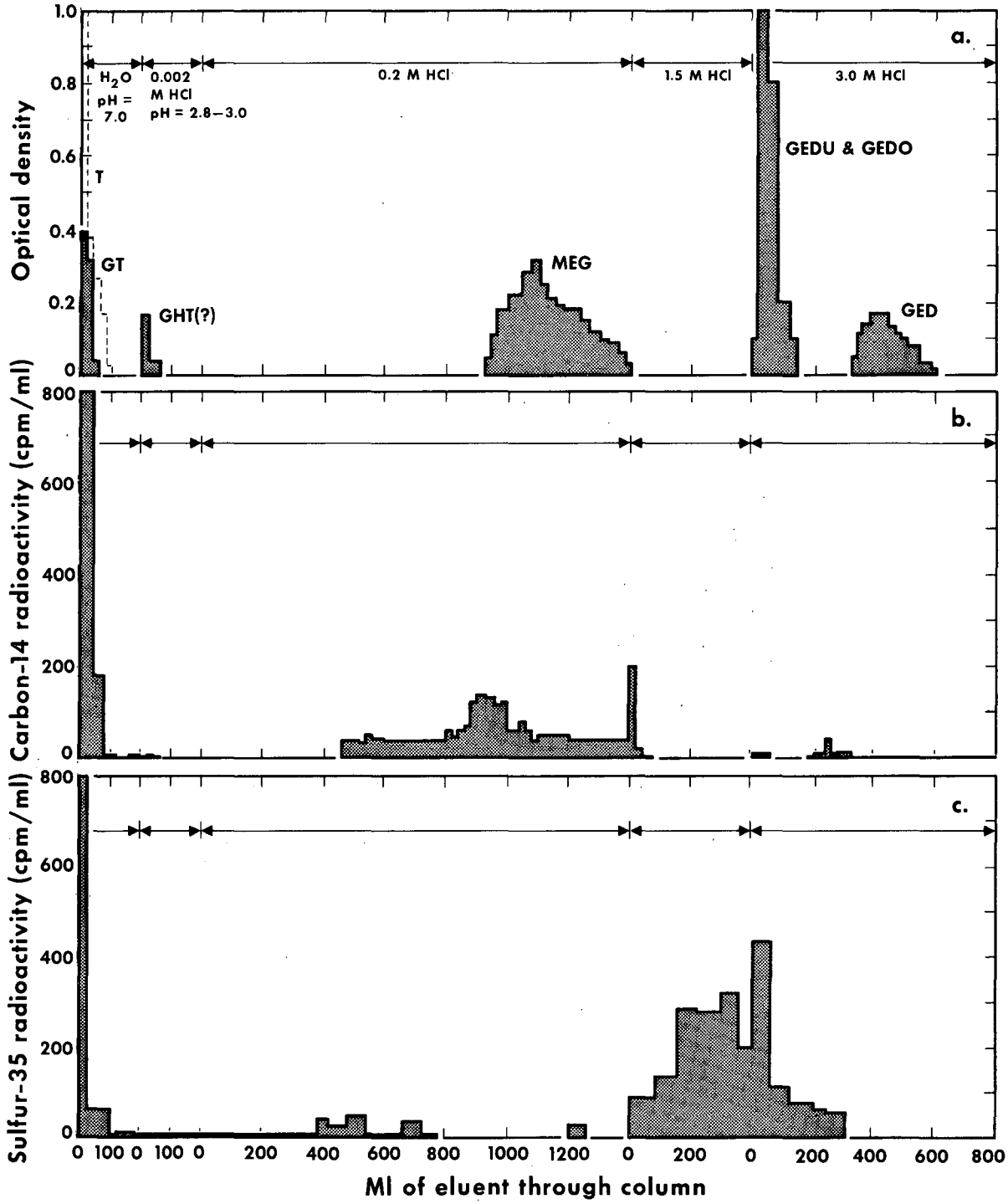
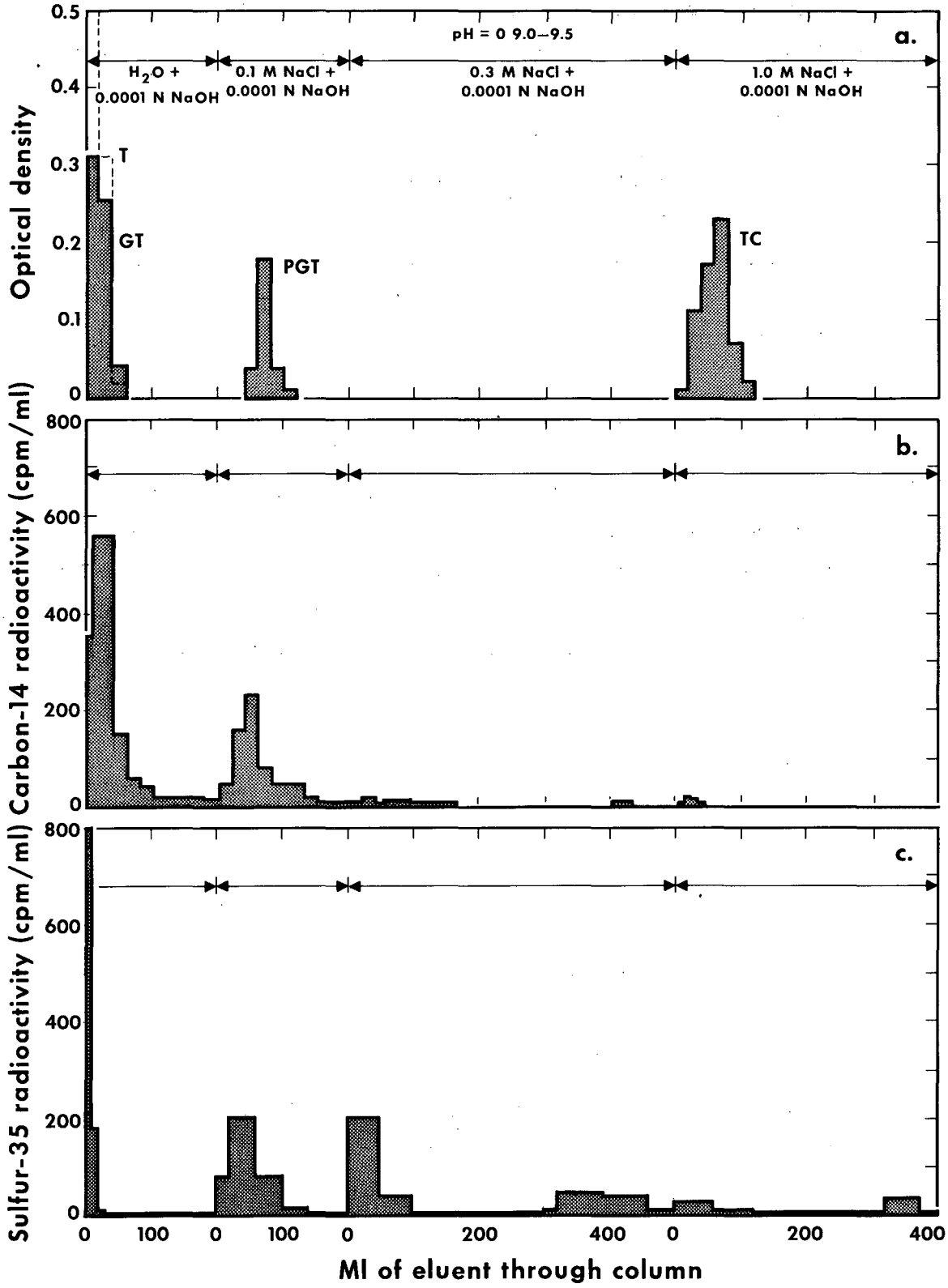

a.
b.
c.
Optical density
Carbon-14 radioactivity (cpm/ml)
Sulfur-35 radioactivity (cpm/ml)
H2O pH = 7.0
0.002 M HCl pH = 2.8–3.0
0.2 M HCl
1.5 M HCl
3.0 M HCl
T
GT
GHT(?)
MEG
GEDU & GEDO
GED
Ml of eluent through column
MUB-459

Fig. 8. Typical chromatograms obtained by anion exchange of radioactive products found in bile on Dowex AG1 x 2, Cl^- form.

a. Standard chromatographic elution curves of synthetic compounds which were described in Fig. 5.

b. Anion exchange of the bile fraction containing the radioactive products which have passed through the cation-exchange column. Rat injected with 49.5 mg of carbon-14-labeled AET intraperitoneally. Units on column: 2.0×10^5 cpm. Assay: Radioactivity. Flow rate: 1.0 ml/min. Av. recovery: 85%.

c. Anion exchange of the bile fraction containing the radioactive products which have passed through the cation-exchange column. Rat injected with 55.4 mg of sulfur-35-labeled AET intraperitoneally. Units on column: 9.0×10^4 cpm. Assay: Radioactivity. Flow rate: 1.0 ml/min. Av. recovery: 86%.


pH = 0 9.0–9.5
a.
H_2O + 0.0001 N NaOH
0.1 M NaCl + 0.0001 N NaOH
0.3 M NaCl + 0.0001 N NaOH
1.0 M NaCl + 0.0001 N NaOH
Optical density
T
GT
PGT
TC
b.
c.
Sulfur-35 radioactivity (cpm/ml) Carbon-14 radioactivity (cpm/ml)
Ml of eluent through column

MUB-458

However, the presence of a substantial amount of activity at the peak occurring at 50.0 ml of 0.1 M NaCl reaffirms the suggestion that some type of modification of the guanidino has taken place.

Discussion

As to the nature of the chemical modification of the guanidino group, the results of this study show that it is unlikely to be a transamidination reaction. Moreover, the data suggest the possibility that this modification may represent a phosphorylation reaction. The presence of a large peak of activity eluting off the column at the position corresponding to N-phosphoryltaurocyamine lends support to this suggestion. Further experimentation is needed to substantiate this hypothesis.

The large number and differences in properties of products noted in the cation-exchange chromatograms support the earlier findings concerning products obtained from the urine. That is, the comparison clearly shows that the two chromatographic patterns are not identical. The absence of peaks for MEG and GED in the sulfur-35 chromatogram is again indicative that the mercapto group is very labile.

VI. GENERAL SUMMARY

The results obtained from the measurement of the disappearance of labeled AET from the circulation showed that following the administration of the compound, there is rapid distribution throughout most tissues. However, the tissue distribution studies show that it is not a simple distribution, because of the dilution of the compound by the total body water. Instead, the nonuniformity of the distribution lends indirect support to the hypothesis that there is unique selection in the binding of the compound within the cellular components. Those radiosensitive tissues which do not accumulate large amounts of compound are not protected during the radiation exposure. The best routes of administration appear to be the oral and intraperitoneal, while the large urinary loss of AET immediately following intravenous administration may account for the unsatisfactory results obtained by this route of entry.

The results from the elimination studies show that the major route of excretion is the urinary system. Estimation of the half-time of excretion for the rat is from 3 to 4 hours, in contrast to 12 hours reported for the human.

The form in which the compound is excreted is in the main a mixture of products. The carbon-14 data indicate that approximately 8 to 9% of the injected dose appears in the urine as free 2-mercaptoethylguanidine. Another 10% of the injected dose excreted is made up of various metabolic products identified as the oxidative forms, GED, GEDO, GT, and PGT respectively. The remaining 30% of the injected dose, of which the peak at 825 ml of 0.2 M HCl and 50 ml of 1.5 M HCl constitute one-half, remains unidentified as to its exact chemical structure. Therefore, the stepwise oxidation of the mercapto group of MEG to a sulfonic acid group represents one major route of metabolism fof AET. The two unidentified peaks found in both carbon- and sulfur-injected animals suggest that they represents a common intermediate which can give rise to other forms of degradation.

Comparison of chromatograms obtained from animals injected with carbon-14 AET with those for sulfur-35 AET do not show identical patterns. The absence of the MEG and GED in the urine of the

sulfur-35-AET-injected animals is interpreted as being due to the ability of the sulfur atom of the injected thiol to exchange with other sulfur compounds. It may be speculated that the mechanism by which this sulfur exchange occurs may be a mixed-sulfide reaction rather than a mixed-disulfide reaction.

Additional results from the identification studies show that the cationic properties of the guanidino group of taurocyamine have been changed. This modification of basicity was postulated to be due to a transamidination reaction in which taurocyamine is converted to taurine. Resolution of taurocyamine and taurine could not be achieved by the methods employed in this study. Assuming that taurine would also form taurocholic acid in appreciable amounts, an investigation of the bile for evidence to support this hypothesis was undertaken.

Serial uptakes of the bile showed that a small amount of radioactivity was eliminated by the biliary system. Chemical analysis of the biliary products failed to show any evidence for the labeling of taurocholic acid. Thus, transamidination does not appear to be a biochemical reaction that modifies the guanidino group of taurocyamine.

The results of the chemical analysis of both the urine and bile show radioactivity appearing tentatively as N-phosphoryltaurocyamine. A further study of definitely identify this compound is to be pursued.

The importance of these biochemical reactions reported in this study, which modify the guanidino and mercapto groups of the mercaptoalkylguanidines, justifies that further inquiry should be made to ascertain their role in the events occurring during the process of irradiation.

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To Dr. John C. Schooley, my thanks for the help in the surgical techniques used in this problem.

Lastly, to my wife, whose critical judgment of the literary aspect of the manuscript was greatly appreciated and to whom this work is dedicated.

VII. APPENDIX

Protocol I

Time change in concentration of AET in plasma after a single intra-arterial injection

Sample number	Time after injection (min and sec)		Concentration of AET in plasma (cts/min/ml plasma)	Total plasma activity (% of injected dose)
SD Rat No. 3				
1	1	58	47,060	42.99
2	3	40	5,780	5.28
3	6	55	5,320	4.86
4	8	45	3,830	3.49
5	10	50	2,850	2.60
6	12	38	2,530	2.31
7	14	50	2,260	2.06
8	17	21	1.810	1.65
9	19	58	2,280	2.08
10	24	15	2,620	2.39
11	30	0	2,190	2.00
SD Rat No. 2				
1	1	30	22,650	17.51
2	5	0	10,880	8.41
3	6	30	5,320	4.11
4	9	20	3,320	2.56
5	11	30	2,650	2.05

Duplicates were made when the volumes of plasma permitted. Errors in duplicate less than ±5%. Total activity of injected dose for each animal = 1.85×10^6 cpm.

Protocol II

Time change in concentration of AET in plasma after a single intraperitoneal injection

Sample number	Time after injection (min and sec)		Concentration of AET in plasma (cts/min/ml plasma)	Total plasma activity (% of injected dose)
SD Rat No. 5				
1	1	21	710	0.60
2	4	0	1,076	0.91
3	6	15	2,117	1.79
4	8	25	2,110	1.78
5	10	33	3,001	2.54
6	13	18	1.266	1.07
7	15	37	1,460	1.23
8	17	4	1,710	1.45
SD Rat No. 7				
1	1	45	376	0.46
2	3	40	634	0.77
3	5	45	664	0.81
4	7	45	1,104	1.34
5	9	50	994	1.21
6	11	55	950	1.15
7	13	50	568	0.69
8	16	0	970	1.18
9	18	0	1,006	1.22
10	20	20	786	0.96
11	25	0	1,134	1.38
12	29	55	1,102	1.34
13	35	15	666	0.80
14	40	15	598	0.73
15	45	0	1,066	1.29

Duplicates were made when the volumes of plasma permitted. Errors in duplicate less than ±5%. Total activity of injected dose for SD No. 7 = 1.72×10^6 cpm; for SD No. 5 = 2.2×10^6 cpm.

Protocol III

Time change in concentration of AET in plasma after a single oral injection

Sample number	Time after injection (min and sec)		Concentration of AET in plasma (cts/min/ml plasma)	Total plasma activity (% of injected dose)
SD Rat No. 8				
1	20	0	1,062	0.25
SD Rat No. 11				
2	30	0	2,614	0.69
SD Rat No. 9				
3	60	0	4,011	1.18
SD Rat No. 10				
4	145	0	3,951	1.01
SD Rat No. 12				
5	240	0	3,841	1.00

Total activity of injected dose for each animal = 7.6×10^6 cpm.

Protocol IV

Time change in concentration of sulfur-35 activity in bile after a single intravenous injection

Sample number	Time after injection (min)	Total volume of bile (mg)	Concentration of sulfur-35 activity in bile (cts/min/mg×100)
1	3	39.6	12.5
2	6	37.1	286.5
3	9	39.5	186.0
4	12	43.1	210.0
5	15	40.1	241.0
6	18	34.4	252.0
7	21	35.3	236.0
8	24	41.3	214.0
9	27	35.1	281.0
10	30	36.2	242.0
11	33	42.4	194.0
12	36	40.7	179.5
13	39	43.5	183.0
14	42	40.0	200.0
15	45	46.9	190.0
16	48	46.0	181.0
17	51	41.9	214.0
18	54	40.3	221.0
19	57	42.1	190.5
20	60	42.7	198.5

Total volume of bile collected = 852.8 mg.

Average bile flow = 14.2 mg/min.

Total activity of injected dose = 1.51×10^6 cpm.

Amount of sulfur-35 labeled AET injected = 0.2 mg.

Protocol V

Time change in concentration of sulfur-35 activity in bile after a single intraperitoneal injection

Sample number	Time after injection (min)	Total volume of bile (mg)	Concentration of sulfur-35 activity in bile (cts/min/mg ×100)
1	3	16.6	0.0
2	6	16.9	0.0
3	9	16.8	0.0
4	12	18.8	72.0
5	15	17.5	262.2
6	18	17.7	374.6
7	21	23.3	374.6
8	24	11.2	334.3
9	27	12.5	964.3
10	30	12.9	1536.0
11	33	14.0	1536.0
13	39	12.8	1969.0
14	42	12.7	2016.0
15	45	12.8	1912.0
16	48	13.0	2150.0
17	51	12.2	2245.0
18	54	14.5	1958.0
19	57	13.2	2046.0
20	60	12.7	2150.0

Total volume of bile collected = 299.4 mg.

Average bile flow = 5.0 mg/min.

Total activity of injected dose = 1.34×10^6 cpm.

Amount of sulfur-35 labeled AET injected = 0.2 mg.

Protocol VI

Time change in concentration of sulfur-35 activity in bile after a single oral injection

Sample number	Time after injection (min)	Total volume of bile (mg)	Concentration of sulfur-35 activity in bile (cts/min/mg×100)
1	3	30.9	0.0
2	6	30.6	0.0
3	9	30.8	17.9
4	12	30.6	37.6
5	15	41.6	36.4
6	18	32.3	21.4
7	21	30.7	31.6
8	24	29.7	46.1
9	27	29.4	7.8
10	30	29.4	35.8
11	33	27.9	42.3
12	36	28.0	20.0
13	39	28.3	42.8
14	42	26.1	60.0
15	45	27.1	50.8
16	48	27.5	44.0
17	51	26.6	70.5
18	54	6.0	100.0
19	57	26.0	81.5
20	60	27.0	62.0

Total volume of bile collected = 675.1 mg.

Average bile flow = 9.0 mg/min.

Total activity of injected dose = 0.81×10^6 cpm.

Amount of sulfur-35 labeled AET injected = 0.2 mg.

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