Biochemical Studies of Toxic Agents

11. THE OCCURRENCE OF PREMERCAPTURIC ACIDS*

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Baumann & Preusse (1879) and Jaffé (1879) showed that the mercapturic acids which they isolated from the urine of dogs dosed with bromobenzene or chlorobenzene were formed by the decomposition of acid-labile compounds in the urine, and they believed that these precursors were derivatives of glucuronic acid. This work has recently been revised and extended as a result of the findings of Boyland, Sims & Solomon (1957) and Knight & Young (1957). From the urine of rabbits dosed with naphthalene, Boyland *et al.* (1957) and Boyland & Sims (1958) isolated a compound which on acidification yielded 1- and 2-naphthol as well as 1-naphthylmercapturic acid, and they suggested that the compound is *N*-acetyl-*S*-(2-hydroxy-1:2-dihydro-1-naphthyl)-L-cysteine. They also stated that anthracene and bromobenzene appear to give similar metabolites.

The present paper contains an account of chromatographic studies which have shown that the administration of benzene, naphthalene, anthracene, fluorobenzene, chlorobenzene, bromobenzene and iodobenzene to rabbits or rats is followed by the excretion of acid-labile precursors of the corresponding mercapturic acids. In a preliminary account of this work (Knight & Young, 1957) the name 'premercapturic acid' was proposed for such precursors. The radiochromatographic examination of urine containing ^{35}S-labelled 1-naphthylpremercapturic acid or *p*-bromophenylpremercapturic acid has indicated that acid decomposition of a premercapturic acid can lead to the formation of another sulphur-containing compound in addition to the mercapturic acid. Furthermore, by means of isotope-dilution techniques it has been possible to show that the amount of 1-naphthylmercapturic acid which can be liberated by mineral acid in the urine of rats dosed with naphthalene depends on the pH at which the 1-naphthylpremercapturic acid breaks down. Not all the mercapturic acids isolated from urine are derived from the decomposition of premercapturic acids, for chromatographic studies and tracer-isotope experiments have led to the conclusion that the urine of rats dosed with benzyl chloride does not contain an acid-labile precursor of benzylmercapturic acid.

* Part 10: Marsden & Young (1958).

CHROMATOGRAPHIC STUDIES

Reference compounds

Phenylmercapturic acid and 1-naphthylmercapturic acid. These non-radioactive compounds were obtained by the methods described by Marsden & Young (1958) for the ^{35}S-labelled compounds.

1-*Anthrylmercapturic acid.* The method of isolation of this compound was based on that used by Boyland & Levi (1936).

p-*Fluoro-*, p-*chloro-*, p-*bromo- and* p-*iodo-phenylmercapturic acid.* Methods similar to those described by Zbarsky & Young (1943) and Young & Zbarsky (1944) were used to isolate these compounds. In one experiment *p*-bromophenylmercapturic acid was extracted from acidified urine with 1:2-dichloroethane instead of with chloroform and this procedure had certain advantages. *p*-Fluorophenylmercapturic acid was isolated from the acidified urine of rats and rabbits dosed with fluorobenzene. The isolation of this compound from rabbit urine has not been reported hitherto.

Benzylmercapturic acid. S-Benzyl-L-cysteine was prepared by the reaction between benzyl chloride and L-cysteine in alkaline solution under conditions based on those used by Armstrong & Lewis (1951). The compound was acetylated with acetic anhydride in NaOH as described by Zbarsky & Young (1944) for the acetylation of *S*-(*p*-fluorophenyl)-L-cysteine.

Detection of premercapturic acids and mercapturic acids

Various methods have been used to detect mercapturic acids on paper chromatograms. Azouz, Parke & Williams (1955) employed a buffered acid–base indicator system for detecting certain of the mercapturic acids, a procedure described by Fewster & Hall (1951) for the detection of carboxylic acids. Boyland & Solomon (1956) detected 1-naphthylmercapturic acid on paper chromatograms by virtue of the fact that its fluorescence in ultraviolet light changed from pink to orange when the paper was sprayed with ammonia solution and heated. Bray, James & Thorpe (1956), in their studies of the mercapturic acids derived from the chloromononitrobenzenes, found that the ammonia in the solvent used for developing chromatograms of some of these mercapturic acids caused the acids to appear yellow on the paper.

A search for a general method of detecting premercapturic acids and mercapturic acids led us to the finding that quantities of the order of 5 μg. of these compounds can be located by spraying the dried chromatograms with 0·1 M-$K_2Cr_2O_7$–acetic acid (1:1) and then with 0·1 M-$AgNO_3$.

After this treatment all the premercapturic acids and mercapturic acids tested appear as orange spots against a red–brown background. Chloride, which gives rise to a colourless area, is also detected by this reagent. Once the chromatograms have been sprayed it is important to protect them from the action of light, otherwise metallic silver is formed and obscures the spots.

Thirty-two compounds containing bivalent sulphur were tested with the reagent and gave positive reactions. (We are grateful to Dr G. A. Maw and Mr A. E. R. Thomson for samples of certain of these compounds.) In addition to the premercapturic acids and the mercapturic acids used in the present investigation, compounds tested included L-cystine, L-cysteine, a number of *S*-alkyl- and *S*-aryl-L-cysteines and several *S*-substituted thioglycollates. Several compounds containing sulphur in a higher oxidation state, including methionine sulphoxide, taurine and L-cysteic acid, gave negative reactions, as did a number of compounds which contained no sulphur. It is known, however, that purines will react with this reagent, although in a different way (Reguera & Asimov, 1950).

Animal experiments

Male rabbits and rats were used. The rabbits received a diet of rat cakes [J. Murray and Sons (London) Ltd.] and cabbage; the rats were given rat cakes only. The animals had access to water at all times and were housed in metabolism cages which permitted the collection of urine separate from faeces. Urine was collected daily and stored in the refrigerator.

Chromatograms of the urine of animals which had received benzene, naphthalene, anthracene, fluorobenzene, bromobenzene and benzyl chloride were developed by the descending method at 5°. Chromatograms of the urine of animals which had been dosed with chlorobenzene and iodobenzene were developed by the ascending method at room temperature. Whatman no. 1 paper was used throughout and chromatograms were developed in the upper phase of the system butan-1-ol–3N-$(NH_4)_2CO_3$–aq. 3N-NH_3 soln. (4:1:1). To see whether acid-labile premercapturic acids could be detected in the urine of the dosed animals a series of eight spots was applied to each 5 in. wide strip. These were: (i) and (ii), urine from dosed animals; (iii) and (iv), reference mercapturic acid; (v) and (vi), normal urine; (vii) and (viii), normal urine plus reference mercapturic acid. The second spot of each pair was treated with about 1·5 µl. of conc. HCl, the excess of which was removed in a current of warm air.

Results

Typical R_F values for the materials detected in the urines before and after acidification, together with the values for the mercapturic acids, are shown in Table 1. It will be seen that after the administration of every compound except benzyl chloride, the urine contained no compound with an R_F value equal to that of the corresponding mercapturic acid. A compound with a lower R_F value was present, however, and this was replaced on acidification by a compound with an R_F value equal to that of the corresponding mercapturic acid. The R_F value of the compound present in the urine of rabbits and rats dosed with benzyl chloride was similar to that of benzylmercapturic acid and was unchanged after acidification. Insofar as a urinary precursor of benzylmercapturic acid might have had the same R_F value as the mercapturic acid itself, these experiments were equivocal. Tracer-isotope experiments reported below, however, support the conclusion that no precursor of benzylmercapturic acid is present in the urine of rats which have been dosed with benzyl chloride.

Table 1. *R_F values of premercapturic acids and mercapturic acids*

Chromatograms of the unacidified urine and the acidified urine of the dosed animals were developed in the upper phase of the system butan-1-ol–3N-$(NH_4)_2CO_3$–aq. 3N-NH_3 soln. (4:1:1) by the descending method, except for those marked with an asterisk, for which the ascending method was used. Chromatograms were sprayed with 0·1M-$K_2Cr_2O_7$–acetic acid (1:1) followed by 0·1M-$AgNO_3$. Anthracene was administered by feeding the rats on a diet containing 4% (w/w) of the hydrocarbon (D). All other compounds were administered by stomach tube (ST) or by subcutaneous injection (SC).

Compound administered	Animal	Route	Dose (g./kg. body wt.)	Reference mercapturic acid	R_F values: Reference mercapturic acid	R_F values: Unacidified urine	R_F values: Acidified urine
Benzene	Rat	ST	1·0	Phenyl-	0·33	0·09	0·31
Naphthalene	Rat	SC	1·0	1-Naphthyl-	0·51	0·25	0·47
	Rabbit	ST	1·0		0·51	0·25	0·51
Anthracene	Rat	D	—	1-Anthryl-	0·54	0·39	0·55
Fluorobenzene	Rat	SC	1·0	*p*-Fluorophenyl-	0·41	0·19	0·38
	Rabbit	ST	1·0		0·41	0·20	0·42
Chlorobenzene	Rat	SC	1·0	*p*-Chlorophenyl-	0·51*	0·33*	0·50*
Bromobenzene	Rat	SC	1·0	*p*-Bromophenyl-	0·51	0·32	0·53
	Rabbit	ST	0·5		0·51	0·28	0·50
Iodobenzene	Rat	SC	1·0	*p*-Iodophenyl-	0·50*	0·29*	0·47*
Benzyl chloride	Rat	SC	1·0	Benzyl-	0·36	0·35	0·35
	Rabbit	SC	0·2		0·34	0·31	0·32

Evidence that 1-naphthylmercapturic acid was formed from the compound in the unacidified urine which gave an orange-coloured spot with the $K_2Cr_2O_7$–$AgNO_3$ reagent was obtained from the following experiment. Three spots, each containing 20 μl. of the urine of rats dosed with naphthalene, were applied to a length of Whatman no. 1 paper and development was carried out in butan-1-ol saturated with aq. 3N-NH_3 soln. Ammonium carbonate was not included in the solvent system in this part of the experiment, in order to exclude this salt from the material which was to be eluted subsequently. Its absence had only a slight effect on the R_F values of the compounds detected. The developed chromatogram was cut into three 1 in. wide strips, one of which was sprayed with the $K_2Cr_2O_7$–$AgNO_3$ reagent and the compound giving a positive reaction was thereby located. The corresponding areas on the other two strips were cut out and together were macerated with 1 ml. of water. The suspension of cellulose fibres was filtered on a sintered funnel and the cellulose was resuspended in 0·5 ml. of ethanol. The ethanol and aqueous extracts were dried together in a current of warm air and the residue was dissolved in a mixture of 0·1 ml. of water and 0·1 ml. of ethanol. The resulting solution was applied to a 3 in. wide strip of Whatman no. 1 chromatographic paper so as to form two spots, and to one of these was added about 1·5 μl. of conc. HCl. A third spot containing 15 μg. of 1-naphthylmercapturic acid was formed on the paper. The chromatogram was developed in the upper phase of the system butan-1-ol–3N-$(NH_4)_2CO_3$–aq. 3N-NH_3 soln. (4:1:1) and was sprayed with the $K_2Cr_2O_7$–$AgNO_3$ reagent. The spot which had been treated with HCl yielded a compound with the same R_F value as 1-naphthylmercapturic acid, whereas the untreated spot gave rise to material which corresponded to that eluted from the first chromatogram.

TRACER–ISOTOPE STUDIES

Measurement of ^{35}S. Compounds containing radioactive sulphur were oxidized by the Carius procedure and ^{35}S was counted as benzidine sulphate as described previously (Hawkins & Young, 1954; Marsden & Young, 1958).

[^{35}S]*Yeast.* When yeast is grown in a medium containing inorganic [^{35}S]sulphate to incorporate labelled sulphur into sulphur-containing amino acids of the yeast (Williams & Dawson, 1952), a considerable proportion of the [^{35}S]-sulphate remains in the medium after the yeast has been harvested. It was found that by adding further quantities of the original constituents to the culture medium several crops of yeast could be grown and by this means the [^{35}S]-sulphate was more economically used.

The yeast was separated from the medium by centrifuging and was washed three times in the centrifuge bottles, twice with 95% ethanol and once with absolute ethanol. It was finally dispersed in ether and filtered on a Büchner funnel. After this treatment it was very powdery and was handled as far as possible in a glove box. The yeast preparation obtained in this way is described in the present paper as [^{35}S]yeast.

L-[^{35}S]*Cystine.* This was isolated from [^{35}S]yeast by the method of Williams & Dawson (1952) with the minor modifications introduced by Marsden & Young (1958).

^{35}S-*Labelled mercapturic acids*

Three methods were used to prepare mercapturic acids labelled with ^{35}S. Those required as reference compounds for radiochromatography were prepared from the urine of rats which had received the appropriate parent compound and [^{35}S]yeast. The 1-naphthylmercapturic acid which was used in the isotope-dilution experiments was prepared from the urine of rats which had received naphthalene and L-[^{35}S]cystine. The method used to prepare the [^{35}S]benzylmercapturic acid employed in the isotope-dilution experiments was based on the reaction between benzyl chloride and L-[^{35}S]cysteine in anhydrous NH_3.

[^{35}S]1-*Naphthylmercapturic acid.* This compound was obtained from the urine of rats dosed with naphthalene and L-[^{35}S]cystine by the method described by Marsden & Young (1958). It had a specific activity of 2·86 μc/m-mole.

[^{35}S]1-Naphthylmercapturic acid of much higher specific activity was obtained by giving the rats [^{35}S]yeast instead of L-[^{35}S]cystine. Two rats were fasted overnight and then each was given by stomach tube 2 g. of [^{35}S]yeast suspended in water. On the next day the rats were each given 1·5 g. of the [^{35}S]yeast and at the same time each was injected subcutaneously with 0·2 g. of naphthalene as a 20% (w/v) solution in arachis oil. In order to obtain the mercapturic acid and also a urine fraction containing the premercapturic acid, the following procedure was used. A volume (10 ml.) of the urine voided during the 24 hr. after the second dose of [^{35}S]yeast was treated with 10 ml. of ethanol. The mixture was centrifuged and the supernatant liquid, after evaporation under reduced pressure, yielded a gum. This was stirred with 10 ml. of ethanol to which was added the minimal amount of water to make solution of the gum almost complete. A large excess of ethanol was then added with stirring and, after the mixture had been chilled, the precipitate which had formed was separated by centrifuging in the refrigerator. The supernatant liquid was evaporated to a gum which was treated with butan-1-ol saturated with aq. 0·3N-NH_3 soln. and then with just sufficient ethanol and water to bring it into solution. This solution was chromatographed on a column (45 cm. × 3 cm.) containing cellulose powder. The column had been prepared from a slurry of cellulose in butan-1-ol saturated with aq. 0·3N-NH_3 soln. This solvent was also used for development and elution. The appearance of ^{35}S in the eluate was detected by allowing a drop of each fraction to dry on a filter paper and then examining the paper for the presence of radioactivity. Spots which contained ^{35}S also gave positive reactions with the $K_2Cr_2O_7$–$AgNO_3$ reagent. The fractions containing ^{35}S were pooled and evaporated under reduced pressure to a gum which was dissolved in 5 ml. of ethanol. A volume (1 ml.) of this solution was reserved for radiochromatographic examination. To the other 4 ml. was added 12 ml. of water and 1·5 ml. of conc. HCl; the resulting suspension was cooled for 2 days and yielded 0·016 g. of grey crystals. These were dissolved in ethanol and the solution was treated with activated charcoal and filtered. The addition of hot water to the filtrate caused the precipitation of a white crystalline material with m.p. 170° (m.p. values reported in this paper are uncorrected) unchanged by admixture with a sample of non-radioactive 1-naphthylmercapturic acid. The material had a specific activity of 254 μc/m-mole.

[^{35}S]p-*Bromophenylmercapturic acid.* Two rats were each given by stomach tube 1·4 g. of [^{35}S]yeast and at the same time each was injected subcutaneously with bromobenzene (1 g./kg. body wt.). The urine was collected for the next 24 hr. and was treated in a manner similar to that described for the urine of rats dosed with naphthalene, in order to obtain the mercapturic acid and also a fraction containing the premercapturic acid. After purification of the precipitate formed by acidification of part of the urine fraction containing the premercapturic acid, the product obtained had m.p. 154·5–155° and mixed m.p. 154·5–155·5° with a sample of non-radioactive *p*-bromophenylmercapturic acid.

[^{35}S]*Benzylmercapturic acid.* Two rats were dosed by stomach tube with an aqueous suspension of [^{35}S]yeast on 2 consecutive days, and on the second day each rat was injected subcutaneously with benzyl chloride (1 g./kg. body wt.). During the next 24 hr., 16 ml. of urine was collected. Some of this urine was used for radiochromatography and the rest (14 ml.) was treated with 1·4 ml. of conc. HCl and extracted by shaking three times with 15 ml. portions of ethyl acetate. The combined extracts were evaporated under reduced pressure and yielded a gum. When this was warmed with 2 ml. of water and stirred vigorously, most of it dissolved. The supernatant liquid was decanted from a small amount of undissolved material and was then cooled. The crystalline precipitate which formed was filtered off and treated in aqueous solution with charcoal. The suspension was filtered and the filtrate deposited white crystals (0·032 g.), m.p. 146–147°.

[^{35}S]Benzylmercapturic acid was synthesized by the following method. L-[^{35}S]Cystine (0·962 g.) was dissolved in about 100 ml. of anhydrous NH_3 contained in a flask surrounded by a mixture of solid carbon dioxide and ethanol. Small, freshly cut portions of sodium metal were added while the reaction mixture was vigorously stirred, until a total of 0·4 g. had been introduced. The reduction was taken as complete when a blue colour persisted in the reaction mixture for a short time. Benzyl chloride (1·1 ml.) was then added drop by drop until a nitroprusside test showed the absence of –SH groups. The mixture was stirred for 10 min. and the NH_3 was then allowed to evaporate. The ^{35}S-labelled *S*-benzyl-L-cysteine was acetylated with acetic anhydride in alkaline solution by the method used for the non-radioactive compound. After crystallization from water and treatment with charcoal the product had a specific activity of 11·9 μc/m-mole.

Radiochromatographic experiments

The observations made from paper-chromatographic studies were confirmed and extended by radiochromatographic experiments. This work included the examination not only of urine, but also of urine fractions prepared as described earlier in the paper. The object of the fractionation was to

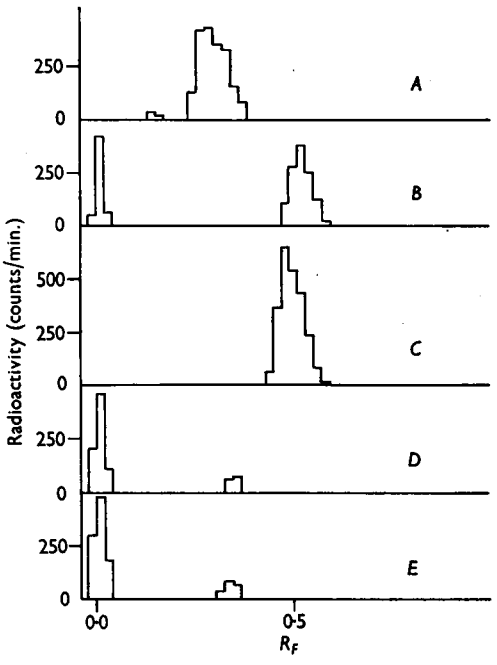


Fig. 1. Naphthalene metabolism. Radiochromatograms developed in the upper phase of the system butan-1-ol–3N-$(NH_4)_2CO_3$–aq. 3N-NH_3 soln. (4:1:1). *A*, Unacidified and *B*, acidified fraction from urine of rats dosed with naphthalene and [^{35}S]yeast; *C*, [^{35}S]1-naphthylmercapturic acid; *D*, unacidified and *E*, acidified urine of rats dosed with [^{35}S]yeast.

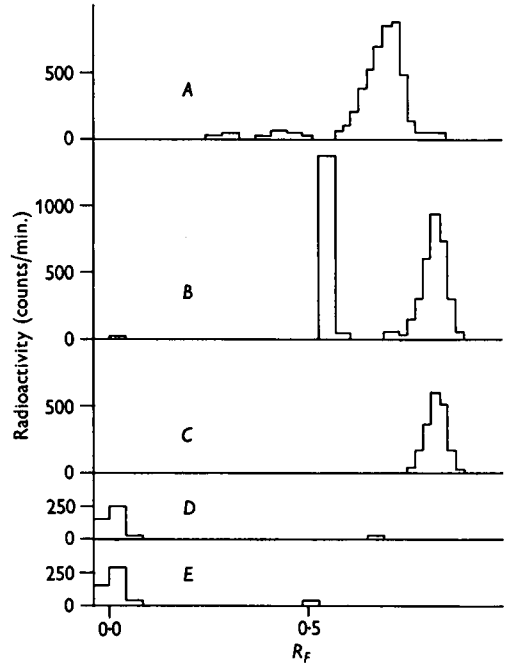


Fig. 2. Naphthalene metabolism. Radiochromatograms developed in the upper phase of the system butan-1-ol–saturated aq. sodium acetate–acetic acid–water (80:3:1:36). *A*, *B*, *C*, *D* and *E* as in Fig. 1. The sharpness of the peak with the lower R_F value in *B* arises from the fact that this material travels at the front of the area containing chloride.

free the premercapturic acids from other radioactive constituents, the presence of which complicated the interpretation of the radiochromatograms.

Methods. Radiochromatograms were developed at room temperature by the ascending method on 3 in. wide Whatman no. 1 paper. The two solvent systems used were the upper phases of the systems butan-1-ol–3 N-$(NH_4)_2CO_3$–aq. 3 N-NH_3 soln. (4:1:1) and butan-1-ol–saturated aq. sodium acetate soln.–acetic acid–water (80:3:1:36). Two spots of material to be chromatographed were applied to each paper, each spot being 1 in. from the edge. After development the papers were dried in the air and sprayed with the $K_2Cr_2O_7$–$AgNO_3$ reagent. Strips 1 in. wide were then cut from the chromatograms so that each included all the material derived from a single spot. Each strip was then marked in intervals of 0·5 cm. along its length and scanned for the presence of radioactivity with an apparatus described by Lowenstein (1953; see also Corner & Young, 1955). In the present work the radioactivity of each 0·5 cm. section was counted for at least 1 min. Although the background count varied somewhat when measured over 1 min. only, it was generally about 25 counts/min. It did not exceed 40 counts/min. and the graphical representation of the data has been simplified by excluding activities below this figure.

Experiments with naphthalene. Because of the higher activity of the sulphur-containing compounds present, it was found that the urine excreted after giving [^{35}S]yeast and naphthalene to rats was more suitable for radiochromatographic examination than that obtained after giving L-[^{35}S]cystine with the naphthalene. The urine of rats which had received [^{35}S]yeast alone gave rise to no areas of radioactivity on the chromatograms which might have been confused with the mercapturic acid or its precursor. Diagrammatic representations of the radiochromatograms developed in two solvent systems from the urine fraction from rats dosed with naphthalene, before and after acidification, are shown in Figs. 1 and 2. These diagrams show that a radioactive component which is present in the urine is replaced on acidification by two components, one with a higher R_F value and the other with a lower R_F value than the original component. There seems little doubt that the faster-moving component is 1-naphthylmercapturic acid, not only on account of its R_F value, but also because this compound was isolated from the urine fraction used in the radiochromatographic experiments.

Experiments with bromobenzene. The diagrams in Fig. 3 are of the radiochromatograms developed from the urine fraction before and after acidification. The results obtained are similar to those just described for experiments with naphthalene in that a radioactive compound gives rise to two radioactive components on acidification, one of which is the mercapturic acid.

Experiments with benzyl chloride. Fig. 4 shows the radiochromatograms obtained from the urine

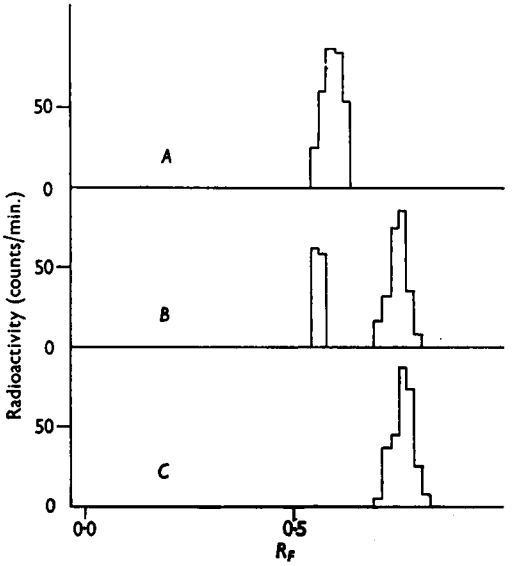


Fig. 3. Bromobenzene metabolism. Radiochromatograms developed in the upper phase of the system butan-1-ol–saturated aq. sodium acetate–acetic acid–water (80:3:1:36). *A*, Unacidified, and *B*, acidified fraction from urine of rats dosed with bromobenzene and [^{35}S]-yeast; *C*, [^{35}S]*p*-bromophenylmercapturic acid. The sharpness of the peak with the lower R_F value in *B* arises from the fact that this material travels at the front of the area containing chloride.

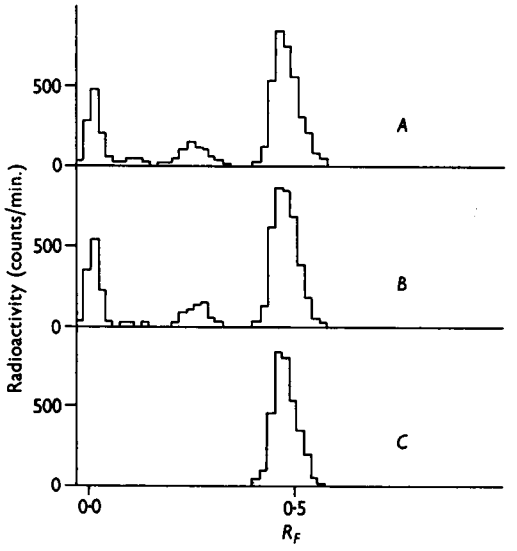


Fig. 4. Benzyl chloride metabolism. Radiochromatograms developed in the upper phase of the system butan-1-ol–3 N-$(NH_4)_2CO_3$–aq. 3 N-NH_3 soln. (4:1:1). *A*, Unacidified, and *B*, acidified urine of rats dosed with benzyl chloride and [^{35}S]yeast; *C*, [^{35}S]benzylmercapturic acid.

before and after acidification. These gave no evidence for the presence of a precursor of benzylmercapturic acid in the urine.

Isotope-dilution experiments

The availability of ^{35}S-labelled mercapturic acids made possible the determination of the unlabelled compounds by an isotope-dilution technique. This was used in experiments in which portions of the urine of rats which had been dosed with naphthalene or benzyl chloride were kept under different conditions of acidity for known lengths of time. A known amount of the ^{35}S-labelled mercapturic acid in the form of its ammonium salt was added to the portion of urine after adjustment to pH 8·0, and the radioactive mercapturic acid together with any non-radioactive mercapturic acid which was present in the urine was then extracted at pH 5·0. From a comparison of the specific activity of the added mercapturic acid and that isolated it was possible to calculate the amount of mercapturic acid present in the treated urine.

During the process of acidification and also when the urine was subsequently being made alkaline, great care was taken to reduce to a minimum the occurrence of local concentrations of acid or alkali. The urine was adjusted to pH 8·0 for the addition of the ^{35}S-labelled mercapturic acid in order to ensure the complete mixing of this with the non-radioactive mercapturic acid. All the extractions of the mercapturic acids were carried out under the same conditions of acidity and each portion was adjusted to pH 5·0 and buffered at this pH immediately before extraction. The samples of mercapturic acid isolated from the urine were purified to constant specific activity.

Occurrence of 1-naphthylpremercapturic acid in the urine of rats dosed with naphthalene. While lightly anaesthetized with ether, 12 rats were each dosed by subcutaneous injection with 0·5 ml. of 20% (w/v) naphthalene in arachis oil at each of two dorsal sites. The 24 hr. urine (110 ml., pH 6·4) was diluted with water to 250 ml. and four 50 ml. portions were treated as follows.

Portion A. This was further diluted with 20 ml. of water and stirred vigorously while it was adjusted to pH 5·0 by the slow addition of 0·25 ml. of 2N-HCl. Water (4·75 ml.) was then added to bring the total volume to 75 ml. The solution was adjusted to pH 8·0 by the addition of 0·8 ml. of 2N-NaOH, and 0·0925 g. of [^{35}S]1-naphthylmercapturic acid which had been dissolved in a small amount of aq. 2N-NH_3 soln. was added. The solution was again stirred vigorously while it was adjusted to pH 5·0 by the slow addition of 1·2 ml. of 2N-HCl. After the addition of 20 ml. of phosphate–citrate buffer at pH 5·0 (McIlvaine series; Vogel, 1951) the volume of the solution was adjusted to 110 ml. with water. The solution was next extracted by shaking for 15 min. with each of four 110 ml. portions of chloroform. The combined chloroform extracts were evaporated to a small volume, which was transferred to a 30 ml. beaker in which evaporation was completed in a current of air. The residue (0·062 g.) was stirred with 2 ml. of chloroform, cooled in ice and filtered off, by which process most of the colouring material was removed. The residue was dissolved in 6 ml. of ethanol and warmed with charcoal. The suspension was filtered and the filter paper was washed with 2 ml. of ethanol. The filtrate and washings were combined and evaporated in a current of air to 4 ml. The solution was warmed, 16 ml. of hot water was added, and a crystalline precipitate separated. After the mixture had been cooled overnight in the refrigerator it was filtered on a sintered funnel and the residue was suspended in 2 ml. of ether and then sucked dry. This process yielded 0·044 g. of material, which was purified to constant specific activity by three crystallizations from ethanol–water (20:80, v/v). The weight of non-radioactive 1-naphthylmercapturic acid present in the portion of urine was calculated from the difference in specific activity of the [^{35}S]1-naphthylmercapturic acid added to the urine and that of the mercapturic acid isolated from the urine.

Portion B. To this was added 5 ml. of 2N-HCl in order to bring it to pH 1·0, after which its volume was adjusted to 75 ml. by the addition of water. After 5 min. the solution was adjusted to pH 8·0 by the addition of 6·7 ml. of 2N-NaOH. [^{35}S]1-Naphthylmercapturic acid (0·1088 g.) was dissolved in a small volume of aq. 2N-NH_3 soln. and added to the urine, followed by 1·4 ml. of 2N-HCl which brought the solution to pH 5·0. Phosphate–citrate buffer, pH 5·0 (20 ml.), was next added and the volume of the solution was adjusted to 110 ml. with water. The resulting solution was extracted with chloroform and the extract was treated in a similar manner to the extract of portion A.

Portion C. This was treated in the same way as portion B except that the solution was kept at room temperature at pH 1·0 for 24 hr. instead of for 5 min.

Portion D. This was treated with 7·5 ml. of conc. HCl followed by 17·5 ml. of water and left at room temperature for 24 hr. It was then adjusted to pH 8·1 by the addition of 9 ml. of 40% (w/v) NaOH solution, and 0·1075 g. of [^{35}S]1-naphthylmercapturic acid which had been dissolved in a small volume of aq. 2N-NH_3 soln. was added. The addition of 1·5 ml. of 2N-HCl brought the solution to pH 5·0 and 20 ml. of phosphate–citrate buffer, pH 5·0, was then added. The extraction with chloroform and the purification of the extract were carried out as described for portion A.

The results of this experiment (Expt. 1) and of another (Expt. 2) carried out in the same way are shown in Table 2. It will be seen that when the acidity of a portion of the urine of rats dosed with naphthalene was not greater than pH 5·0, the amount of 1-naphthylmercapturic acid detected was less than 2% of that found in another portion which had been treated with one-tenth of its volume of conc. HCl. While a small amount of the mercapturic acid may have been formed after the urine had been excreted, the urine kept at pH 5·0 may, nevertheless, have contained no free mercapturic acid, for the small quantity found lies within the limits of experimental error.

The data in Table 2 suggested that the method of breakdown of 1-naphthylpremercapturic acid might

Table 2. *Occurrence of* 1-*naphthylpremercapturic acid in the urine of rats dosed with naphthalene*

Twelve rats each received 0·2 g. of naphthalene and the urine excreted in the following 24 hr. was used. Results are shown for two experiments in which an isotope-dilution technique was used to determine the amounts of 1-naphthylmercapturic acid present in portions of the urine which had been kept at room temperature under different conditions of acidity for known lengths of time. Each portion represented one-fifth of the 24 hr. urine. For further experimental details see text.

Portion	Acidity	Duration	1-Naphthylmercapturic acid found (mg.)	
			Expt. 1	Expt. 2
A	pH 5·0	0	1	1
B	pH 1·0	5 min.	9	6
C	pH 1·0	24 hr.	20	38
D	Normal to HCl	24 hr.	59	78

Table 3. *Influence of* pH *on the breakdown of* 1-*naphthylpremercapturic acid*

Twenty-four rats each received 0·2 g. of naphthalene and the urine excreted in the following 24 hr. was used. Results are shown for experiments in which an isotope-dilution technique was used to determine the amounts of 1-naphthylmercapturic acid present in portions of the urine which had been kept at 30° for known lengths of time at pH 1·0 or normal to HCl. Each portion represented one-tenth of the 24 hr. urine. For further experimental details see text.

Duration (hr.)	1-Naphthylmercapturic acid found (mg.)	
	pH 1·0	Normal to HCl
0	2	—
2	18	58
6	18	—
24	19	67
72	19	62

be dependent on the pH at which the decomposition occurs, and this possibility was investigated in the following experiment.

Influence of pH *on the breakdown of* 1-*naphthylpremercapturic acid.* This experiment was designed to enable an investigation to be made of the maximal amounts of 1-naphthylmercapturic acid which could be formed under two conditions of acidity in the urine of rats dosed with naphthalene. To this end twenty-four rats were injected with naphthalene as previously described. The 24 hr. urine was collected and its volume adjusted to 500 ml. by the addition of water. Eight 50 ml. portions of the diluted urine were treated as follows. Four were adjusted to pH 1·0 and kept at 30° for periods of 2, 6, 24 and 72 hr., and three other portions were made normal to HCl and kept at 30° for periods of 2, 24 and 72 hr. At the end of these periods they were brought to pH 5·0 and a solution of 0·1 g. of [^{35}S]1-naphthylmercapturic acid as its ammonium salt was added to each. Each solution was cooled in ice, adjusted to pH 1·0 and was shaken for 10 min. with twice its volume of chloroform. The 1-naphthylmercapturic acid was then isolated from the chloroform extract and its specific activity measured as already described. The eighth portion of the urine was brought to pH 5·0, 0·1 g. of [^{35}S]1-naphthylmercapturic acid as the ammonium salt was added and the solution was cooled in ice. It was then adjusted to pH 1·0 and immediately extracted with chloroform and the chloroform extract was treated as already described. The amounts of 1-naphthylmercapturic acid found in the various portions of urine are shown in Table 3.

As it seemed likely that some breakdown of the mercapturic acid itself occurred under the conditions of the experiments just described, further experiments were carried out to determine the extent to which this might have taken place. Urine from normal rats was collected, a solution of 1-naphthylmercapturic acid was added to it and four portions were taken. Two were made to pH 1·0 and the other two were made normal to HCl. All four were incubated at 30° and the isotope-dilution method already described was used to determine the amount of 1-naphthylmercapturic acid present in each after 24 and 72 hr. The results showed that even after 72 hr. in N-HCl less than 13 % of the mercapturic acid originally present had been broken down.

Absence of a urinary precursor of benzylmercapturic acid in the urine of rats dosed with benzyl chloride. An experiment similar to that carried out with the urine of rats dosed with naphthalene was undertaken with the urine of rats dosed with benzyl chloride to examine the possibility, suggested by the chromatographic and radiochromatographic studies, that benzylmercapturic acid is excreted as such and not as an acid-labile precursor.

Each of eighteen rats was lightly anaesthetized with ether and injected subcutaneously with 0·075 ml. of benzyl chloride in each of two dorsal sites. During the subsequent 24 hr. the animals were allowed water but not food. The 24 hr. urine (206 ml., pH 6·4) was collected and four 50 ml. portions were treated as follows.

Portion A. This was treated with 2N-HCl, which was added slowly and with continuous stirring until the solution was at pH 5·0. In a similar manner 1·3 ml. of 2N-NaOH was added to adjust the solution to pH 8·0, and then 0·1270 g. of [^{35}S]benzylmercapturic acid which had been dissolved in 1 ml. of aq. 2N-NH_3 soln. was added. The solution was brought back to pH 5·0 by the addition of 1·7 ml. of 2N-HCl and water was added to make a total volume of 100 ml. After the addition of 20 ml. of phosphate–citrate buffer, pH 5·0, the urine was extracted four times by shaking each time for 15 min. with 120 ml. of ethyl acetate. The combined extracts were evaporated to a gum which was dissolved in 5 ml. of warm water. The crystalline precipitate which formed on leaving this solution overnight in the refrigerator was filtered off, dissolved in warm water and treated with charcoal, after which the suspension was filtered and the filtrate was again cooled in the refrigerator. The cold filtrate was filtered on a sintered funnel, and the residue was suspended in ether on the funnel and sucked dry. The residue was purified to constant specific activity by three further crystallizations from water. The amount of benzylmercapturic acid present in the urine was calculated from the difference in specific activity of the [^{35}S]benzylmercapturic acid added to the urine and that isolated.

Portion B. This was adjusted to pH 1·0 by the addition of 20 ml. of 2N-HCl and retained at this pH for 5 min. The addition of 24·2 ml. of 2N-NaOH brought the solution back to pH 8·0 and 0·1284 g. of [^{35}S]benzylmercapturic acid, as a solution of its ammonium salt, was added. The solution was then adjusted to pH 5·0 and adjusted to 100 ml. with water. Phosphate–citrate buffer, pH 5·0 (20 ml.), was added and the solution was then extracted with ethyl acetate. The extract was then purified in the manner described for portion A.

Portion C. This was treated in the same way as portion B except that it was kept at pH 1·0 for 24 hr.

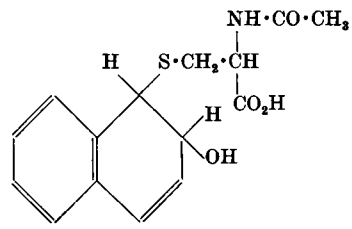
Portion D. This was treated with 7 ml. of conc. HCl and 13 ml. of water and kept at room temperature for 24 hr. It was then adjusted to pH 8·0 by the addition of 9·2 ml. of 40% (w/v) NaOH and this was followed by the addition of 0·1278 g. of [^{35}S]benzylmercapturic acid, as a solution of its ammonium salt. The solution was adjusted to pH 5·0 with 3 ml. of 2N-HCl and its volume was made to 100 ml. by the addition of water. After the addition of 20 ml. of phosphate–citrate buffer, pH 5·0, the solution was extracted and the extract was purified as described for portion A.

The amounts of benzylmercapturic acid found (expressed as percentages of benzyl chloride converted into the mercapturic acid) were as follows: A, 14; B, 14; C, 13; D, 14. These findings are quite different from those obtained in similar experiments with naphthalene (see Table 2) in that they indicate no significant increase in the amount of mercapturic acid in the urine after acidification.

DISCUSSION

The present work has provided evidence that the monohalogenobenzenes and certain cyclic hydrocarbons are converted in the animal body into pre-mercapturic acids, that is, into compounds which are broken down on acidification to yield mercapturic acids. The question arises of whether compounds which give rise to premercapturic acids are excreted in part as mercapturic acids. As chromatographic experiments did not indicate the presence of mercapturic acids together with premercapturic acids in unacidified urine, this matter was examined further by making use of an isotope-dilution technique. In these experiments the unacidified urine of rats dosed with naphthalene appeared to contain a small amount of 1-naphthylmercapturic acid, but this did not correspond to more than 0·1% of the naphthalene administered. While this matter requires further investigation, the lability of the premercapturic acids is such that this small amount of mercapturic acid may have been formed by breakdown of the precursor in the urine.

The labile nature of 1-naphthylpremercapturic acid immediately brings to mind similar characteristics shown by certain other metabolites of naphthalene. When Bourne & Young (1934) observed that the urine of rabbits which had received naphthalene yielded the hydrocarbon on acidification, they pointed out that this might be accounted for by the presence in the urine of a compound such as 1:2-dihydro-2-naphthol. The naphthalene precursor has since been shown to be 1:2-dihydro-1-naphthyl glucosiduronic acid (Boyland & Solomon, 1955). Two other derivatives of dihydronaphthalene have also been shown to be metabolites of naphthalene, namely 1:2-dihydronaphthalene-1:2-diol (Young, 1947; Booth & Boyland, 1949) and 1:2-dihydro-2-hydroxy-1-naphthyl glucosiduronic acid (Corner, Billett & Young, 1954). From both of these compounds the elements of water are eliminated on acidification. Under these circumstances it seems reasonable to suppose that the premercapturic acid might also have a dihydronaphthalene structure with a hydroxy group on C-2, as shown below. This structure has been suggested by Boyland & Sims (1958) on the basis of their study of the isolated compound.



Boyland & Sims (1958) have shown that the breakdown of 1-naphthylpremercapturic acid under acid conditions gives rise to 1- and 2-naphthol as well as the mercapturic acid. In the present work

it has been shown that the amount of 1-naphthylmercapturic acid formed is dependent on the pH at which the breakdown occurs. This raises the interesting possibility that there may be some premercapturic acids which on acidification yield mainly hydroxy compounds and little or no mercapturic acid. For example, although studies of the influence of phenanthrene on sulphur metabolism (Elson, Goulden & Warren, 1945; Crabtree, 1945, 1946) have yielded results consistent with the formation of an acetylcysteine conjugate of this hydrocarbon, there have been no reports of the isolation of such a compound from the urine of the dosed animals. Although mercapturic acids have not been isolated from the urine of animals dosed with carcinogenic hydrocarbons, the isolation of hydroxy derivatives has been reported. Under these circumstances, the possibility that the hydroxy compounds are derived, in part at least, from the breakdown of premercapturic acids would seem to merit consideration.

An interesting aspect of the present work is that benzyl chloride appears to be converted directly *in vivo* into a mercapturic acid. This was first suggested by the results of chromatographic and radiochromatographic studies and was subsequently supported by evidence obtained from isotope-dilution experiments. It would seem therefore that there are some compounds which are metabolized to mercapturic acids and do not give rise to premercapturic acids. On the other hand, there are others which form premercapturic acids and in all probability are not metabolized to mercapturic acids.

SUMMARY

1. Paper-chromatographic methods and tracer-isotope techniques with ^{35}S-labelled compounds have been used in a study of the occurrence of premercapturic acids, i.e. acid-labile precursors of mercapturic acids.

2. It has been shown that the administration to animals of benzene, naphthalene, anthracene and the monohalogenobenzenes is followed by the excretion of the corresponding premercapturic acids.

3. The amount of 1-naphthylmercapturic acid which can be obtained by the breakdown of 1-naphthylpremercapturic acid under acid conditions has been shown to be dependent on the pH at which decomposition occurs.

4. Evidence has been obtained that in the animal body benzyl chloride is converted directly into a mercapturic acid and does not give rise to a premercapturic acid.

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