Biochemical Studies of Toxic Agents

10. OBSERVATIONS ON THE METABOLISM OF ^{35}S-LABELLED MERCAPTURIC ACIDS*

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Gutmann & Wood (1950) observed that in the 24 hr. period after the simultaneous administration of L-[^{35}S]cystine and bromobenzene to rats, about 4% of the ^{35}S appeared in the chloroform-extractable fraction of the acidified urine. Moreover, when they added non-radioactive *p*-bromophenylmercapturic acid to the urine of the dosed animals, the mercapturic acid which they were then able to isolate from the urine was radioactive. From these and other experimental results they concluded that the administered L-[^{35}S]cystine had participated in mercapturic acid formation, but that only a small proportion of the sulphur in the *p*-bromophenylmercapturic acid had been derived from this source.

In the present investigation the work of Gutmann & Wood (1950) has been extended by the isolation, without the addition of a carrier, of *p*-bromophenyl[^{35}S]mercapturic acid and 1-naphthyl[^{35}S]mercapturic acid from the urine of rats dosed with L-[^{35}S]cystine and also given either bromobenzene or naphthalene. Our main purpose, however, has been to gain information about the metabolism of mercapturic acids after their administration to animals. Although this problem has been investigated by various workers (e.g. Shiple, Muldoon & Sherwin, 1924; Callow & Hele, 1927; Coombs & Hele, 1927; Lawrie, 1931; Parke & Williams, 1951), it does not appear to have been studied by the use of ^{35}S-labelled mercapturic acids. The present paper contains an account of our observations on the fate of 1-naphthyl[^{35}S]-mercapturic acid, *p*-bromophenyl[^{35}S]mercapturic acid and phenyl[^{35}S]mercapturic acid (prepared by the debromination of *p*-bromophenyl[^{35}S]mercapturic acid), after their administration to rats.

EXPERIMENTAL AND RESULTS

The animals used were male black-and-white rats, each weighing between 150 and 200 g., and they were housed in metabolism cages which permitted the collection of urine separate from faeces. Urine and faeces were collected daily and when they were not used immediately they were stored in the refrigerator. In experiments in which the partition of ^{35}S in urine was measured, the urine was made to a known volume, usually 25 or 50 ml., with water. Water was available to the rats at all times, and except where otherwise stated each animal was provided daily with 20 g. of a diet (Maw, 1953) with the following percentage composition (by wt.): casein 10, plain flour 25, starch 34·8, margarine 22, dried yeast 4, cod-liver oil 2, Marmite 2, choline 0·2. The rats were kept in their metabolism cages for an acclimatization period of 2–3 days before they were dosed. While being dosed they were anaesthetized lightly with ether.

Measurement of ^{35}S

In order to determine the ^{35}S content of organic compounds and biological material, the sulphur present was separated as benzidine sulphate, the radioactivity of which was then measured. Where it was necessary to convert sulphur into sulphuric acid, this was accomplished by means of the Carius oxidation procedure. The methods employed for the measurement of ^{35}S were based on those described by Young, Edson & McCarter (1949) and Hawkins & Young (1954).

Inorganic sulphate ^{35}S *in urine.* Urine (2 ml.) was placed in a lipped boiling tube together with 1·5 mg. of sulphate S, in the form of sodium sulphate, to serve as a carrier. Sufficient water to give a total volume of 6 ml. was added followed by 4 ml. of 95% (v/v) ethanol. The inorganic sulphate was then precipitated by the addition of 2 ml. of benzidine hydrochloride reagent (Young *et al.* 1949). After the mixture had been allowed to stand for 30 min. the precipitate was filtered off on a Whatman no. 30 paper, 2·3 cm. in diameter, by the use of a filtration apparatus of the type described by Henriques, Kistiakowsky, Margnetti & Schneider (1946). The precipitate was washed with three 5 ml. portions of 95% (v/v) ethanol and allowed to dry by suction on the filter. The filter paper bearing the precipitate was then mounted on a Perspex carrier of the type described by Hawkins & Young (1954) and left overnight in a desiccator containing anhydrous $CaCl_2$. The radioactivity of the precipitate was measured by means of counting equipment which employed a Geiger–Müller tube with a thin window (1·7 mg./cm.^{2}, EHM2S, General Electric Co. Ltd., London).

After the precipitate of benzidine sulphate had been counted it was transferred to a boiling tube and 5–10 ml. of water was added. The contents of the tube were then heated to boiling and titrated with 0·02 N-NaOH with phenol red as indicator. This titration value was used in the calculation of the correction for self-absorption which was applied to the radioactivity measurement.

Total sulphate ^{35}S *in urine.* Urine (2 ml.) in a 50 ml. beaker was made N with respect to its content of HCl, 1·5 mg. of sulphate S in the form of sodium sulphate was added and the solution evaporated almost to dryness on a water bath. Water (2 ml.) was then added, the solution was

* Part 9: Brooks & Young (1956).

evaporated to dryness and heating continued for a further 10 min. The residue was dissolved in 3 ml. of water and the solution was transferred to a lipped boiling tube. The beaker was washed with 3 ml. of water and with two 2 ml. portions of 95% (v/v) ethanol, and each washing in turn was transferred to the boiling tube. The sulphate was then precipitated as the benzidine salt and its radioactivity was measured by the method already described.

The ethereal sulphate ^{35}S content of the urine was calculated by subtracting the inorganic sulphate ^{35}S content from the total sulphate ^{35}S content.

Total sulphur ^{35}S in organic compounds and in biological material. The sulphur in the material being analysed was oxidized to sulphuric acid by the Carius procedure (Young *et al.* 1949). The sulphuric acid was precipitated as the benzidine salt, the radioactivity of which was measured by the method already described.

For the measurement of total sulphur ^{35}S in urine, 0·2 ml. portions of urine (or 0·3 ml. portions when the ^{35}S content was low) were oxidized in the presence of 1·5 mg. of added sulphate S. In this and all other measurements of total sulphur ^{35}S, where it was necessary to add carrier sulphate it was introduced into the bomb as solid sodium sulphate.

In most cases when tissues were analysed for total ^{35}S about 0·4 g. was used for each analysis. For whole blood about 0·3 ml. was measured into a small weighed tube and the tube and its contents were weighed and then introduced into the Carius bomb. Faeces were air-dried and ground in a mortar before analysis and samples of about 0·3 g. were used. For the measurement of the ^{35}S content of hair, samples weighing about 0·075 g. were oxidized and under these conditions it was unnecessary to add carrier sulphate.

Co-precipitation experiments. As the metabolic experiments described later in this paper involved the measurement of ^{35}S in the sulphur fractions of the urine excreted after the administration of radioactive mercapturic acids, it was necessary to determine whether the presence of a ^{35}S-labelled mercapturic acid in urine leads to errors in the measurement of sulphate ^{35}S. This was investigated as follows.

A series of aqueous solutions was prepared, each with a volume of 6 ml. and each containing 1·5 mg. of non-radioactive sulphate S (as sodium sulphate) together with a known amount of 1-naphthyl[^{35}S]mercapturic acid, phenyl-[^{35}S]mercapturic acid or *p*-bromophenyl[^{35}S]mercapturic acid. The amounts of mercapturic acid present ranged from 0·0012 to 0·0040 m-mole, and these corresponded in general to the amounts which would have been present in the urine sample if an animal had excreted from 30 to 100% of a dose of 0·1 m-mole of mercapturic acid in unchanged form. The sulphate was precipitated as the benzidine salt and its radioactivity was measured as already described. The results obtained are given in Table 1, and they show that under the conditions of the present investigation, the amount of mercapturic acid carried down with the precipitate of benzidine sulphate is unlikely to cause a serious error in the measurement of urinary sulphate ^{35}S.

Biosynthesis of L-[^{35}S]*cystine*

Williams & Dawson (1952) have described a method of preparing L-[^{35}S]cystine from baker's yeast that has been grown in a medium containing [^{35}S]sulphate. The yeast is dehydrated and defatted, and the residue is extracted with hot trichloroacetic acid to remove nucleic acids. The crude protein residue is then hydrolysed in the presence of added L-cystine, and the ^{35}S-labelled cystine which is obtained from the hydrolysate is purified by repeated crystallization at pH 4·5. By this means Williams & Dawson (1952) obtained L-[^{35}S]cystine of high specific activity and of a purity such that a series of tests failed to reveal the presence of either inactive or radioactive impurities.

With certain minor changes, the method of preparing L-[^{35}S]cystine described by Williams & Dawson (1952) was used in the present work. Among the changes introduced were an increase in the ^{35}S content of the yeast culture medium to about 5 mc/l. and an increase in the amount of carrier L-cystine added to 0·125 g./g. of yeast residue. Another change was the addition of sodium sulphate (1 mg. of SO_4^{2-} ion/ml.) to the solution before carrying out one of the precipitations of the cystine at pH 4·5.

In one experiment radioactive cystine was prepared from yeast grown in two 2 l. batches of culture medium each of which initially contained 9·5 mc of ^{35}S as $^{35}SO_4^{2-}$ ion. The weight of L-cystine added as carrier before the hydrolysis of the yeast protein was 2 g. After the radioactive cystine had been separated from the hydrolysate it was recrystallized to constant specific activity. The weight of product obtained was 1·093 g. and it had a specific activity of 157 μc/m-mole. This represented a recovery as cystine ^{35}S of 3·8% of the ^{35}S introduced as $^{35}SO_4^{2-}$ ion into the culture medium. No radioactive impurities were detected when the constant-solubility test of Gutmann & Wood (1949) was applied to the L-[^{35}S]cystine with water as the solvent.

Another preparation of radioactive cystine carried out under conditions almost identical with those used in the preparation just described yielded 1·151 g. of product with a specific activity of 153 μc/m-mole.

Table 1. *Precipitation of mercapturic acids under the conditions used to precipitate benzidine sulphate in the measurement of* ^{35}S

For experimental conditions see text.

Mercapturic acid present (m-mole)	Percentage of mercapturic acid precipitated		
	1-Naphthyl-mercapturic acid	Phenyl-mercapturic acid	*p*-Bromophenyl-mercapturic acid
0·0012	0·20	—	—
0·0016	—	0·32	0·05
0·0020	0·13	—	—
0·0024	—	0·37	0·03
0·0032	0·17	0·41	0·11
0·0040	0·22	0·22	0·04

Table 2. *Influence of administration of naphthalene on the urinary excretion of ^{35}S as inorganic sulphate, ethereal sulphate and neutral sulphur by rats dosed with* L-[^{35}S]*cystine*

Rat no. 1 was injected subcutaneously with 1 ml. of arachis oil and rat no. 2 was similarly injected with 1 ml. of a 20% (w/v) solution of naphthalene in arachis oil. Immediately after the injection each rat was given by stomach tube an aqueous suspension containing 0·1 m-mole of L-[^{35}S]cystine.

		^{35}S in urine (percentage of ^{35}S administered)			
Day	Rat no.	Inorganic $^{35}SO_4$	Ethereal $^{35}SO_4$	Neutral ^{35}S	Total ^{35}S
1	1	31·92	1·15	8·70	41·77
	2	18·15	5·45	28·52	52·12
2	1	6·38	0·74	2·11	9·23
	2	4·13	1·23	5·29	10·65
3	1	2·59	0·16	1·14	3·89
	2	1·79	0·23	1·70	3·72
4	1	1·48	0·21	1·40	3·09
	2	1·05	0·18	0·72	1·95
5	1	0·94	0·14	0·87	1·95
	2	0·97	0·13	0·61	1·71

Preparation of ^{35}S-labelled mercapturic acids

In experiments in which they studied the effect of administration of bromobenzene on the metabolism of ^{35}S-labelled L-cystine in rats, Gutmann & Wood (1950) gave the bromobenzene by stomach tube and the radioactive cystine by intraperitoneal injection, and they reported that in the next 48 hr. about 16% of the administered ^{35}S was excreted in the urine. The greater part of this ^{35}S was present in the form of inorganic sulphate. They concluded, however, that some ^{35}S was present in the form of mercapturic acid, for when they added an ammoniacal solution of unlabelled *p*-bromophenylmercapturic acid to the urine of the dosed animals the *p*-bromophenylmercapturic acid which they isolated from the acidified urine was radioactive.

In the present investigation 1-naphthylmercapturic acid and *p*-bromophenylmercapturic acid labelled with ^{35}S were isolated from the acidified urine of rats which had been dosed subcutaneously with naphthalene or bromobenzene and immediately afterwards had been given L-[^{35}S]cystine either by intraperitoneal injection or by stomach tube. No carrier mercapturic acid was added to the urine in these experiments.

As a preliminary to this work some experiments were performed in which the partition of ^{35}S in the urine of rats dosed with L-[^{35}S]cystine was studied. The results of one such experiment are shown in Table 2. In this experiment a pair of rats were fasted for 24 hr., one (no. 1) was then given a subcutaneous injection of 0·5 ml. of arachis oil at each of two sites on the back, and the other (no. 2) was injected similarly with arachis oil containing in solution 0·2 g. of naphthalene. Each rat was then given by stomach tube 0·5 ml. of an aqueous suspension of 0·1 m-mole of L-[^{35}S]cystine. The rats were fasted for another 24 hr. and were then provided with food for the remainder of the experiment. The urine was collected daily for 5 days and analysed. As in other experiments in which L-[^{35}S]cystine alone was given to rats by stomach tube, it was found that about one-half of the administered ^{35}S appeared in the urine within 48 hr. after dosing. Most of the ^{35}S was present in the form of inorganic sulphate and little was excreted in the neutral sulphur fraction. The administration of naphthalene was attended by a greater excretion of ^{35}S as neutral sulphur and a lower excretion of ^{35}S as inorganic sulphate, which suggests that some cystine which would otherwise have undergone oxidation to sulphate underwent conjugation with naphthalene. More ^{35}S was present in the ethereal sulphate fraction of the urine of the animal which received naphthalene than in that of the rat which received only radioactive cystine.

These observations emphasized the fact that in order to prepare from urine ^{35}S-labelled mercapturic acid with a high degree of radioactive purity, it would be necessary to take into account the presence in the urine of comparatively large amounts of ^{35}S in other forms. The chief of these was inorganic sulphate. The presence of SO_4^{2-} ions in urine was found to have no effect on the purity of 1-naphthylmercapturic acid isolated by the procedure used in the present work (described later), for when carrier-free [^{35}S]-sulphate (104 μc) was added to the urine collected from four rats in the 4 days after each had received 0·2 g. of naphthalene (20%, w/v, in arachis oil) by subcutaneous injection, the mercapturic acid which was isolated contained no detectable amount of ^{35}S. The isolation procedure was also tested in an experiment in which four rats were each dosed subcutaneously with 0·2 g. of naphthalene (20%, w/v, in arachis oil) and the urine was collected for 4 days. At the same time four other rats each received by intraperitoneal injection approx. 0·1 m-mole of L-[^{35}S]cystine (16·7 μc) in aqueous suspension, and the urine was collected for 4 days. The urines from the two groups of animals were then mixed, the 1-naphthylmercapturic acid was isolated and was found to contain no ^{35}S. As a result of these observations it appeared that 1-naphthyl[^{35}S]mercapturic acid isolated by the method about to be described was unlikely to be contaminated by ^{35}S-containing compounds derived from L-[^{35}S]cystine by normal metabolic processes.

Isolation of 1-naphthyl[^{35}S]mercapturic acid. In all experiments male rats weighing 150–200 g. were used and each received 0·2 g. of naphthalene as a 20% (w/v) solution in arachis oil by the subcutaneous injection of 0·5 ml. of the solution into each of two dorsal sites, one to the left and the other to the right of the mid-line. In all experiments these injections were followed immediately by the

administration of 0·5 ml. of an aqueous suspension of about 0·1 m-mole of L-[^{35}S]cystine to each rat, either by intraperitoneal injection or by stomach tube. The urine of the dosed rats was collected for 3 days and 1-naphthyl[^{35}S]mercapturic acid was isolated by a method similar to that described by Young (1947) for the isolation of the non-radioactive compound. The following is an account of a typical experiment.

Twelve rats were injected subcutaneously with naphthalene and were given L-[^{35}S]cystine by stomach tube as already described. Each rat received approx. 12 μC of ^{35}S in the form of L-cystine. The animals were fasted for 24 hr. before and after being dosed. The volume of the urine excreted in the 3 days after dosing was 360 ml. It was made just acid to Congo red by the addition of conc. HCl, and this was followed by the addition of 36 ml. of conc. HCl. The acidified urine was left overnight and was then shaken with three 400 ml. portions of chloroform. The emulsions which formed were broken by centrifuging. The clear chloroform extracts were separated, combined and concentrated on a water bath. The concentrate (40 ml.) was shaken first with 30 ml. of M-$NaHCO_3$ and then with 20 ml. of M-$NaHCO_3$. The bicarbonate extracts were combined and shaken with an equal volume of ether. The ether extract was discarded. The aqueous layer was warmed to remove ether and was then cooled. It was next made just acid to Congo red by the addition of conc. HCl. This operation was conducted carefully in a fume cupboard, for the evolution of carbon dioxide which occurred gave rise to a radioactive spray. Crude mercapturic acid separated as a tar which solidified on standing overnight in the refrigerator. The product was separated by filtration, and after being dried over P_2O_5 it weighed 0·806 g. In order to purify the mercapturic acid use was made of the salting-out process described by Bourne & Young (1934). The crude acid was dissolved in 8 ml. of 2N-NaOH, and when 1·6 ml. of 10N-NaOH was added, the sodium salt of the mercapturic acid separated. It was washed on to a sintered-glass filter with the aid of a few millilitres of 2N-NaOH and the liquid was removed by suction. The sodium 1-naphthyl-[^{35}S]mercapturate was dissolved in water and the free acid was precipitated by the addition of conc. HCl until the solution became acid to Congo red. The crystalline precipitate was allowed to separate overnight in the refrigerator. After being collected by filtration the crystals were dissolved in warm ethanol, and the solution was treated with charcoal and filtered. The filtrate was concentrated by evaporation in an air current and the acid was precipitated by the addition of warm water. After being left overnight in the refrigerator the precipitate was collected on a sintered-glass funnel, dried by suction and then stirred on the funnel with 2 ml. of ether. The ether was drawn off and the residue on the funnel was recrystallized from aqueous ethanol to constant specific activity. The final product melted at 170·5–171° (m.p. values reported in this paper are uncorrected) and when it was mixed with 1-naphthylmercapturic acid the melting point was not depressed. The weight of 1-naphthyl[^{35}S]mercapturic acid obtained was 0·54 g., and this corresponded to 9·9% of the naphthalene administered. The specific activity of the mercapturic acid was 5·4 μC/m-mole, whereas that of the L-[^{35}S]cystine administered was 107 μC/m-mole. The ^{35}S which was separated as mercapturic acid represented 7% of that given as cystine.

The data just given are summarized in Table 3, as are those of other experiments in which 1-naphthyl[^{35}S]-mercapturic acid was isolated from the urine of rats dosed with naphthalene and L-[^{35}S]cystine. The conditions in Expt. 2 closely resembled those in Expt. 1, and the results obtained were similar. In Expt. 3 two groups of six rats were treated in the same way except that one group was fasted for 24 hr. before and after being dosed (as in Expts. 1 and 2), whereas the other group was given food throughout the experiment. As might be expected, the specific activity of the mercapturic acid isolated from the urine of the group which was fasted was somewhat higher than that obtained from the urine of the group which was given food throughout the experiment. The data from Expt. 4 show that the specific activity of the mercapturic acid obtained from rats which had been given L-[^{35}S]cystine by intraperitoneal injection was much lower than that obtained under comparable conditions when the cystine was administered by stomach tube.

Examination of the 1-naphthyl[^{35}S]mercapturic acid by means of the constant solubility test (Gutmann & Wood, 1949), with water as the solvent, gave no evidence of the presence of radioactive impurities.

Isolation of p-*bromophenyl*[^{35}S]*mercapturic acid.* Twelve rats were each given 0·2 g. of bromobenzene as a 20% (w/v) solution in liquid paraffin by subcutaneous injection

Table 3. *Isolation of* 1-*naphthyl*[^{35}S]*mercapturic acid from the urine of rats dosed subcutaneously with naphthalene and given* L-[^{35}S]*cystine by stomach tube or by intraperitoneal injection*

Each rat received by subcutaneous injection 0·2 g. of naphthalene as a 20% solution (w/v) in arachis oil, followed by an aqueous suspension of 0·1 m-mole of L-[^{35}S]cystine by stomach tube or by intraperitoneal injection. In some experiments (fasted) the rats were fasted for 24 hr. before and after dosing, whereas in others (fed) they were provided with food throughout the experiment.

			L-[^{35}S]Cystine			1-Naphthyl[^{35}S]mercapturic acid		
Expt. no.	No. of rats	Feeding conditions	Mode of administration	Specific activity (μC/m-mole)	Total ^{35}S administered (μC)	Amount isolated (g.)	Specific activity (μC/m-mole)	^{35}S recovered (% of ^{35}S administered)
1	12	Fasted	Stomach tube	107	144	0·540	5·4	7·0
2	12	Fasted	Stomach tube	142	192	0·437	7·8	6·2
3	6	Fed	Stomach tube	120	72	0·392	5·0	9·5
	6	Fasted	Stomach tube	120	74	0·363	5·8	9·8
4	12	Fed	Intraperitoneal injection	120	136	0·712	1·9	3·5

at two sites on the back. Each rat was then given by stomach tube approx. 0·1 m-mole of L-[^{35}S]cystine suspended in 0·5 ml. of water. The animals were provided with food and water throughout the experiment, and their urine was collected daily for 3 days. The urine was then acidified and extracted with chloroform, and the *p*-bromophenyl-[^{35}S]mercapturic acid was separated from the extracts by the procedure already described for the isolation of 1-naphthyl[^{35}S]mercapturic acid, with the omission of the step in which the compound was separated as the sodium salt. The mercapturic acid was recrystallized from aqueous ethanol to constant specific activity. The final product melted at 153·5–154° alone and when mixed with *p*-bromophenylmercapturic acid. The yield was 0·71 g., and this corresponded to 14·6 % of the bromobenzene administered. The specific activity of the L-[^{35}S]cystine was 94 μc/m-mole, whereas that of the mercapturic acid was 4·7 μc/m-mole. The total activity of the radioactive cystine given to the animal was 114 μc, and 9·2 % of the ^{35}S was recovered as mercapturic acid.

Preparation of phenyl[^{35}S]*mercapturic acid.* This compound was prepared by the debromination of *p*-bromophenyl[^{35}S]mercapturic acid with sodium amalgam in a manner similar to that described by Baumann & Preusse (1881) and Zbarsky & Young (1943). Sodium amalgam was freshly prepared by the method of Fieser (1941). It contained 1·2 % of sodium (by wt.) and was semi-solid at room temperature. It was kept in an atmosphere of nitrogen until required. A solution of 0·2 g. of *p*-bromophenyl-[^{35}S]mercapturic acid in 10 ml. of 0·2 M-$NaHCO_3$ was left in contact with 50 g. of the amalgam in a lightly corked conical flask. The mixture was shaken occasionally and the reaction was allowed to proceed for 24 hr. at room temperature. The supernatant liquid was then decanted, filtered, made acid to Congo red by the addition of conc. HCl, and left overnight in the cold store. The crystalline precipitate which formed was recrystallized from aqueous ethanol to constant specific activity. The total amount of phenyl-[^{35}S]mercapturic acid obtained from two preparations each employing 0·2 g. of *p*-bromophenyl[^{35}S]mercapturic acid was 0·198 g., a yield of 66 %. The product melted at 142·5°, alone and when mixed with phenylmercapturic acid. Its specific activity was 4·65 μc/m-mole, whereas that of the *p*-bromophenyl[^{35}S]mercapturic acid from which it was prepared was 4·66 μc/m-mole.

The ^{35}S-labelled mercapturic acids prepared by the methods just described were used in the following metabolic experiments.

Fate of 1-*naphthyl*[^{35}S]*mercapturic acid when administered to the rat*

A study was made of the excretion of ^{35}S by rats after they had been dosed with 1-naphthyl[^{35}S]-mercapturic acid by various routes and at two different dose levels. In the first group of experiments each rat received by stomach tube, by intraperitoneal injection or by subcutaneous injection, the solution obtained by dissolving 0·1 m-mole of 1-naphthyl[^{35}S]mercapturic acid in 0·5 ml. of dilute $NaHCO_3$ solution. The urine was collected daily for 4 days after dosing and each day's collection was analysed for the ^{35}S content of the inorganic sulphate, total sulphate and total sulphur fractions. The faeces were collected for the 4-day period and their total ^{35}S content was measured. The results obtained for the analysis of the excreta are shown in Table 4. At the end of the experimental period the rats were killed and samples of blood, liver, kidney, stomach, intestine, muscle and hair were analysed for their content of ^{35}S. These samples showed either no activity or gave counting rates very slightly above that of background. As the results in Table 4 show, irrespective of the route by which the radioactive mercapturic acid was introduced into the animal, most of the ^{35}S was excreted in the urine within 24 hr. of dosing. Some mercapturic acid sulphur was oxidized to inorganic sulphate and almost none was excreted as ethereal sulphate. By far the greatest part of the urinary ^{35}S was present in the form of neutral sulphur.

In order to test whether the pattern of excretion of ^{35}S was influenced markedly by the size of the dose of radioactive 1-naphthylmercapturic acid administered, a second group of experiments was carried out. These experiments were similar to those of the first group, but they were more limited in scope. Each rat received by stomach tube, by intraperitoneal injection or by subcutaneous injection, the solution obtained by dissolving 0·025 m-mole of 1-naphthyl[^{35}S]mercapturic acid in 0·5 ml. of dilute $NaHCO_3$ solution. The urine was collected for 24 hr. after dosing, and the distribution of ^{35}S in the urinary sulphur fractions was determined. The results are shown in Table 5. It is clear that when the results are expressed as percentages of the ^{35}S administered as mercapturic acid, they are not greatly different from those obtained (Table 4) when each animal received four times as much mercapturic acid.

Presence of 1-*naphthyl*[^{35}S]*mercapturic acid in the urine of rats dosed with this compound.* From the results given in Tables 4 and 5 it is apparent that the administration of 1-naphthyl[^{35}S]mercapturic acid to rats was followed by the excretion of about 80 % of the ^{35}S in the neutral sulphur fraction of the urine, and the question arises of whether most of the ^{35}S was present in the urine as mercapturic acid. This was investigated by means of the isotope-dilution technique. 1-Naphthyl[^{35}S]mercapturic acid of known specific activity was added to the urine of rats which had been dosed with non-radioactive 1-naphthylmercapturic acid, and after the urine had been acidified a sample of pure mercapturic acid was isolated from it. The specific activity of the isolated compound was measured and from the dilution of the ^{35}S which had taken place the amount of 1-naphthylmercapturic acid in the acidified urine was calculated. The following is an account of an experiment of this type.

Six male rats were each given by intraperitoneal injection the solution obtained by dissolving 0·1 m-mole of non-radioactive 1-naphthylmercapturic acid in 0·5 ml. of dilute $NaHCO_3$ solution. The urine was collected for 3 days after dosing. The animals were given food and water throughout the experiment. To the urine was added the solution obtained by dissolving 0·0998 g. of 1-naphthyl-[^{35}S]mercapturic acid (specific activity, 0·850 μc/m-mole) in 10 ml. of dilute $NaHCO_3$ solution. The urine was acidified with HCl as already described and left at room temperature for 24 hr. A sample of 1-naphthylmercapturic acid was then isolated from the urine by a procedure which differed somewhat from that described earlier. The acidified urine was extracted twice with an equal volume of chloroform, and the combined extracts were evaporated on a water bath to a small volume. The concentrate was then evaporated to dryness at room temperature in a current of air, and the dark residue thus obtained was treated with 1·5 ml. of ice-cold chloroform. The mixture was stirred well and was kept in an ice bath for 15 min. The chloroform dissolved most of the coloured impurities and left undissolved material which consisted of almost colourless crystals. These were separated by filtration and washed with two 1 ml. portions of ice-cold chloroform. The crystals were dissolved in about 8 ml. of ethanol, the solution was treated with charcoal and filtered. The filtrate was concentrated in an air current to 4 ml., and hot water was then added with stirring until the total volume was 20 ml. The mixture was left in the refrigerator overnight and the crystalline precipitate was dried on the filter by suction and then washed with two 1 ml. portions of ether. The 1-naphthylmercapturic acid thus obtained was recrystallized from aqueous ethanol until it showed a constant melting point and constant specific activity.

The specific activity was found to be 0·533 μc/m-mole, from which it was calculated that the radioactive mercapturic acid added to the urine had been diluted with 0·0594 g. of non-radioactive mercapturic acid. This amount represented 34·2 % of the administered 1-naphthylmercapturic acid.

Table 4. *Excretion of* ^{35}S *in the inorganic sulphate, ethereal sulphate and neutral sulphur fractions of urine, and in the faeces of rats each dosed with* 0·1 *m-mole of* 1-*naphthyl*[^{35}S]*mercapturic acid*

Each rat received by stomach tube, by intraperitoneal injection or by subcutaneous injection the solution obtained by dissolving 0·1 m-mole of 1-naphthyl[^{35}S]mercapturic acid in 0·5 ml. of dilute $NaHCO_3$ solution. The data given are mean values for pairs of rats.

	Excretion of ^{35}S (percentage of ^{35}S administered)				
Day	Inorganic $^{35}SO_4$	Ethereal $^{35}SO_4$	Neutral ^{35}S	Faecal ^{35}S	Total ^{35}S
	Administration by stomach tube				
1	0·73	0·18	78·21	—	—
2	0·36	0·02	1·93	—	—
3	0·06	0·01	0·97	—	—
4	—	0·02	0·21	—	—
1–4	1·15	0·23	81·32	5·98	88·68
	Administration by intraperitoneal injection				
1	1·76	0·55	76·24	—	—
2	1·50	0·06	5·11	—	—
3	0·35	0·01	2·83	—	—
4	0·12	0·05	1·44	—	—
1–4	3·73	0·67	85·62	3·44	93·46
	Administration by subcutaneous injection				
1	0·96	0·10	83·88	—	—
2	0·54	0·19	1·68	—	—
3	0·27	0·11	2·52	—	—
4	0·16	—	0·72	—	—
1–4	1·93	0·40	88·80	4·05	95·18

Table 5. *Excretion of* ^{35}S *in the inorganic sulphate, ethereal sulphate and neutral sulphur fractions of the urine excreted by rats in the* 24 *hr. after each was dosed with* 0·025 *m-mole of* 1-*naphthyl*[^{35}S]*mercapturic acid*

Each rat received by stomach tube, by intraperitoneal injection or by subcutaneous injection the solution obtained by dissolving 0·025 m-mole of 1-naphthyl[^{35}S]mercapturic acid in 0·5 ml. of dilute $NaHCO_3$ solution.

	^{35}S in urine (percentage of ^{35}S administered)			
Mode of administration	Inorganic $^{35}SO_4$	Ethereal $^{35}SO_4$	Neutral ^{35}S	Total ^{35}S
Stomach tube	1·17	—	73·35	74·52
Intraperitoneal injection	0·22	0·68	69·40	70·30
Subcutaneous injection	1·60	0·43	88·04	90·07

In an experiment similar to that just described four rats were each dosed by intraperitoneal injection with the solution obtained by dissolving 0·2 m-mole of 1-naphthylmercapturic acid in 0·5 ml. of $NaHCO_3$ solution, and analysis of the urine by the isotope-dilution technique showed the presence of 32·3 % of the mercapturic acid.

Urinary excretion of ^{35}S *by rats dosed with phenyl-*[^{35}S]*mercapturic acid and* p*-bromophenyl*[^{35}S]*-mercapturic acid*

Experiments were conducted in which phenylmercapturic acid and *p*-bromophenylmercapturic acid labelled with ^{35}S were administered to rats and the distribution of ^{35}S in the urine was determined. In these experiments each rat was given the solution obtained by dissolving 0·1 m-mole of mercapturic acid in 0·5 ml. of dilute $NaHCO_3$ solution. The phenyl[^{35}S]mercapturic acid was given to the animals by one of three routes, by stomach tube, by intraperitoneal injection or by subcutaneous injection. *p*-Bromophenyl[^{35}S]mercapturic acid was administered only by stomach tube. The general conditions of the experiments resembled closely those employed in studying the fate of 1-naphthyl-[^{35}S]mercapturic acid. The results obtained are shown in Table 6, and it will be seen that apart from a somewhat more rapid excretion of ^{35}S and less oxidation to inorganic sulphate, the results are similar to those obtained for 1-naphthyl[^{35}S]-mercapturic acid.

DISCUSSION

Early workers (e.g. Thomas & Straczewski, 1919; Kapfhammer, 1921) were of the opinion that dietary cystine is the chief source of the sulphur of the *p*-bromophenylmercapturic acid isolated from the urine of animals dosed with bromobenzene. This was not supported by the findings of later workers (e.g. Nishimura, 1929–30; Stekol, 1935–6), and it was eventually concluded that mercapturic acid sulphur is derived mostly from the non-dietary sources. This conclusion has been supported by the findings of Gutmann & Wood (1950), as well as those obtained in the present investigation. The immediate source of the cysteine portion of the mercapturic acid molecule has yet to be established. Various workers (e.g. Smith, Spencer & Williams, 1950; Mills & Wood, 1956) have suggested that mercapturic acids may arise from the interaction of certain halogenobenzenes and aromatic hydrocarbons with tissue proteins. On the other hand, Barnes & James (1957) have pointed out that the large amounts of mercapturic acid which can be isolated from the urine of animals dosed with some of the compounds they have investigated (cf. Bray, James & Thorpe, 1957) make it unlikely that the cysteine moiety of these mercapturic acids is derived solely from tissue proteins, and the suggestion has been made (Barnes & James, 1957; Bray & Franklin, 1957) that glutathione may serve as its source. Although the fact that only a small proportion of the mercapturic acid isolated in our experiments was labelled with ^{35}S derived from the administered L-[^{35}S]cystine is consistent with the possibility that the cystine must be incorporated into tissue components (e.g. proteins, glutathione) before conjugation, there are other ways in which our findings can be explained.

Until recently little was known about the fate of mercapturic acids after their administration to animals. Sherwin and his co-workers (Rose, Shiple & Sherwin, 1924; Muldoon, Shiple & Sherwin, 1924; Shiple *et al.* 1924) considered that mercapturic acids can be oxidized in the animal body to ethereal sulphates. No evidence to support this belief was obtained, however, by Callow & Hele (1927), Coombs & Hele (1927) or Lawrie (1931), and Coombs & Hele (1927) pointed out that the oxidation of a mercapturic acid would be more likely to lead to formation of a sulphonic acid. Considerable light was thrown on the metabolism of phenylmercapturic acid by the work of Parke & Williams (1951), who found that when this compound was administered to rabbits 45 % of it was excreted in

Table 6. *Excretion of* ^{35}S *in the inorganic sulphate, ethereal sulphate and neutral sulphur fractions of the urine excreted by rats in the* 24 *hr. after each was dosed with* 0·1 *m-mole of phenyl*[^{35}S]*mercapturic acid or* p*-bromophenyl*[^{35}S]*mercapturic acid*

Each rat received by the route indicated the solution obtained by dissolving 0·1 m-mole of the ^{35}S-labelled mercapturic acid in 0·5 ml. of dilute $NaHCO_3$ solution.

[^{35}S]Mercapturic acid	Mode of administration	^{35}S in urine (percentage of ^{35}S administered) Inorganic $^{35}SO_4$	Ethereal $^{35}SO_4$	Neutral ^{35}S	Total ^{35}S
Phenyl[^{35}S]mercapturic acid	Stomach tube	0·13	0·04	97·23	97·40
	Intraperitoneal injection	0·12	0·04	91·94	92·10
	Subcutaneous injection	0·12	0·09	97·49	97·70
p-Bromophenyl[^{35}S]mercapturic acid	Stomach tube	0·48	0·11	97·21	97·80
	Stomach tube	0·65	0·17	95·68	96·50

the urine unchanged, whereas 14% underwent deacetylation and was excreted as *S*-phenylcysteine. They also observed that there was an increased excretion of ethereal sulphate corresponding to 10% of the mercapturic acid administered. They expressed the view that this was due to the excretion of phenylsulphuric acid formed from phenol derived from the phenylmercapturic acid, but they stated that their experiments did not indicate whether the sulphate sulphur came from the same source. The data in Tables 4–6 of the present paper show that some breakdown of the ^{35}S-labelled mercapturic acids occurred in the rat with the formation of inorganic [^{35}S]sulphate in amounts which never exceeded 4% of the ^{35}S administered as mercapturic acid. Less than 1% of the administered ^{35}S appeared in the urine as ethereal sulphate, and it seems likely that this radioactive ethereal sulphate was formed from inorganic [^{35}S]sulphate derived from the mercapturic acid. In two experiments in which rats were dosed with 1-naphthyl[^{35}S]mercapturic acid it was found that 32·3 and 34·2% of the administered compound were excreted unchanged. This accounted for less than half the ^{35}S present in the neutral sulphur fraction of the urine, and in view of the findings of Parke & Williams (1951) with phenylmercapturic acid it seems possible that the urine contained some *S*-(1-naphthyl)-L-cysteine formed by metabolic deacetylation of the mercapturic acid.

It has long been customary to refer to the metabolic conversion of naphthalene and bromobenzene into 1-naphthylmercapturic acid and *p*-bromophenylmercapturic acid. It must be pointed out, however, that these mercapturic acids may not be true excretory products, for naphthalene and bromobenzene are known to be metabolized to derivatives which are excreted in the urine and are readily broken down by strong acids to yield mercapturic acids (Boyland, Sims & Solomon, 1957; Knight & Young, 1957). These acid-labile compounds have been named 'premercapturic acids' (Knight & Young, 1957) and that formed from naphthalene has been isolated by Boyland *et al.* (1957). It appears to be an *N*-acetyl-*S*-(hydroxy-1:2-dihydronaphthyl)cysteine and it is converted into 1-naphthylmercapturic acid under acidic conditions by the loss of the elements of water. Although the problem is still under investigation it may well be that the urine of animals dosed with naphthalene or bromobenzene contains no mercapturic acid until the premercapturic acid has been decomposed. While these considerations do not affect the findings reported in the present paper, they make it clear that the status of mercapturic acids in the metabolism of foreign organic compounds must be reconsidered.

SUMMARY

1. 1-Naphthyl[^{35}S]mercapturic acid and *p*-bromophenyl[^{35}S]mercapturic acid have been isolated from the urine of rats dosed with naphthalene and bromobenzene respectively and given L-[^{35}S]-cystine immediately afterwards by another route.

2. Phenyl[^{35}S]mercapturic acid has been prepared by the debromination of *p*-bromophenyl-[^{35}S]mercapturic acid.

3. Observations have been made on the influence of administration of naphthalene on the excretion of ^{35}S by rats dosed with L-[^{35}S]cystine.

4. The distribution of ^{35}S in the faeces and the sulphur fractions of the urine has been studied after the administration of 1-naphthyl[^{35}S]-mercapturic acid to rats. Little of the ^{35}S was excreted in the faeces. Most of it appeared in the neutral sulphur fraction of the urine, and about one-third was accounted for in the urine as 1-naphthylmercapturic acid. Less than 4% of the administered ^{35}S was present in the urine as inorganic sulphate, and less than 1% as ethereal sulphate.

5. The excretion of ^{35}S by rats dosed with phenyl-^{35}[S]mercapturic acid or *p*-bromophenyl[^{35}S]mercapturic acid resembled that observed when 1-naphthyl[^{35}S]mercapturic acid was administered.

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A Cation Carrier in the Yeast Cell Wall

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The active transport of K^+ ions in exchange for H^+ ions during fermentation has been described in various publications (e.g. Conway & O'Malley, 1946; Rothstein & Enns, 1946; Conway, Brady & Carton, 1950). Without any added buffering, the pH of the external fluid when the volume of this is restricted as far as possible can fall as low as 1·5.

When 1 ml. of external fluid, containing 5% glucose (w/v) but no potassium chloride or buffer, is present/g. of yeast the pH external to the cells is in the region of 3, owing to the excretion of succinic acid. When under such conditions the external fluid contains sodium chloride, lithium chloride or other inorganic chloride, even in high concentration, no appreciable uptake of the inorganic cation species occurs, with the exception of rubidium. When, however, the external fluid is buffered to pH 6–7, not only is the uptake of potassium markedly increased, but also that of sodium, lithium, magnesium and other metal cations. There is then marked competition between cation species.

In this paper such uptake and competition are studied at pH 6–7 and the evidence leads to the conclusion that only one carrier system is involved (Conway & Duggan, 1956).

Since K^+ ions are taken up much more readily than is any other metal cation, the carrier may be regarded as the physiological K^+ ion carrier, but is not the only mechanism involved in the uptake of cations. Magnesium, for example, can be actively transported also, by a different system operating at pH 3–5, and the transport is not then inhibited by small concentrations of K^+ ions, the magnesium being taken up in association with phosphate (Rothstein, 1955).

This physiological K^+ ion carrier is to be distinguished from that which transports sodium from within outwards, when, for example, a considerable quantity of Na^+ ions has been introduced (Conway, Ryan & Carton, 1954). It also appears to be operative when external cations exchange for Na^+ ions within the cells of sodium-rich yeast.

An account is given of the determination of the concentration of the carrier in terms of milli-equivalents of combining power/kg. of washed and centrifuged yeast.

EXPERIMENTAL

Chemical analysis and materials

Sodium, potassium, caesium, rubidium and lithium. The concentration of these in yeast was determined by means of the Beckman flame photometer, with wavelengths of 589, 769, 852, 795 and 670·8 mμ for Na^+, K^+, Cs^+, Rb^+ and Li^+ ions respectively. The yeast for analysis was first centrifuged, washed twice and then 1 g. (moist wt.) was suspended in 20 ml. of water. This suspension was then brought to 100° for 1–2 min., cooled, centrifuged and suitably diluted. The readings were compared with those obtained with standard solutions.

pH *determinations.* These were carried out by means of the Beckman model G meter.

Magnesium determinations. (i) Titan-yellow method: Determinations of Mg were carried out mainly by the method of Young & Gill (1951). (ii) Flame-photometer method: This method was also used in the earlier determinations. Small quantities of yeast (0·8–1·0 g.) were treated with a few drops of 4 N-H_2SO_4 and ashed in platinum crucibles. The ash was dissolved in 2 ml. of warm N-HCl, the solution then neutralized with dilute aq. NH_3 soln. and Mg was precipitated by the addition of 1 ml. of 8-hydroxyquinoline reagent (Cruess-Callaghan, 1935). The separation of the Mg in this way from Na and K was found necessary because of interference by Na^+ and K^+ ions with the photometer readings for Mg. The precipitate was then dissolved in very dilute HCl and water was added so that the final concentration of Mg in the solution was between 25 and