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A Study of the Sulphur Amino Acids of Rat Tissues

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1. In a study of the metabolism of L-[^{35}S]methionine *in vivo*, the labelled sulphur compounds of rat liver and brain were separated first by ion-exchange chromatography into two fractions containing (i) free sulphur amino acids such as methionine, cystathionine, cyst(e)ine and homocyst(e)ine and (ii) glutathione. 2. Two-dimensional paper chromatography with butan-1-ol–acetic acid or propionic acid–water in the first direction and 80% acetone or acetone–ethyl methyl ketone–water in the second direction was found superior to other solvent systems for separating the sulphur amino acids. 3. At 10 min. after injection of [^{35}S]methionine only a small part of the ^{35}S was found combined in free methionine or other free sulphur amino acids. 4. Evidence was obtained of the presence of adenosyl[^{35}S]methionine and adenosyl[^{35}S]homocysteine in perchloric acid extracts of rat liver and brain. 5. The trans-sulphuration pathway was active in brain as well as in liver.

There have been several studies of the incorporation of ^{35}S from L-[^{35}S]methionine into different chemical fractions of the rat brain (Gaitonde & Richter, 1955, 1956, 1957; Palladin, Poliakova & Silich, 1957; Niklas, Quincke, Maurer & Neyen, 1958). The turnover of sulphur in the brain was shown to be relatively rapid in the protein and acid-soluble fractions, whereas its turnover in proteolipids was somewhat slower (see Gaitonde, 1964). Special interest attaches to the sulphur amino acids in view of the demonstration of high concentrations of cystathionine in the human brain (Tallan, Moore & Stein, 1958) and its absence from the brain of patients with homocystinuria (Brenton, Cusworth & Gaull, 1965). This condition, associated generally with mental retardation, is due to an inborn error of methionine metabolism resulting from a deficiency of cystathionine synthase in the brain and certain other organs (Brenton *et al.* 1965; Mudd, Finkelstein, Irreverre & Laster, 1964; Carson, Dent, Field & Gaull, 1965; Gaull & Gaitonde, 1966). The metabolism of the sulphur amino acids has now been re-explored with a view to finding the metabolic basis for the high concentration of cystathionine in the brain.

The study of the sulphur amino acids in tissue extracts involves a number of methodological difficulties. The sulphur amino acids and related compounds readily undergo oxidation: thio ethers are easily oxidized to sulphoxides and sulphones. Thiols are oxidized to disulphides and various mixed disulphides are formed. Of the sulphur amino acids known to occur in extracts of mammalian tissues, only taurine, cystathionine and glutathione are present in easily measurable amounts; the others, including methionine, are present only at relatively low concentrations (see Tallan, 1962). It appeared therefore that, for studies involving the determination of the specific radioactivity of methionine, cyst(e)ine and other sulphur compounds, a paper-chromatographic procedure for their isolation would be the method of choice. The specific radioactivity of free methionine in the rat brain was determined in a previous study by using an ion-exchange procedure (Gaitonde, 1964). In the present investigation an attempt was made to apply this procedure to the measurement of the specific radioactivity of methionine, cystathionine, cyst(e)ine and glutathione. It was found that after administration of [^{35}S]methionine the ^{35}S is incorporated into numerous sulphur compounds, some of which were identified. Some are apparently degradation products of *S*-adenosylmethionine and *S*-adenosylhomocysteine.

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EXPERIMENTAL

Animals and materials. Young adult male rats (approx. 100g., Expts. 1–3) and newborn rats (Expt. 4) from the

Medical Research Council (Carshalton, Surrey) stock were injected with 50μc of L-[^{35}S]methionine containing 78μg. of L-methionine in 0·5ml. of water (specific activity 96mc/m-mole). The labelled compound was obtained from The Radiochemical Centre, Amersham, Bucks. Paper chromatography of 100μl. of this solution revealed no other ninhydrin-positive material (isobutanol–90% formic acid–water, 75:15:10, by vol.). *S*-Adenosylmethionine chloride, L-homocysteine, L-glutathione (GSSG) and L-cystathionine containing trace amounts of L-allocystathionine, were obtained from Koch–Light Laboratories Ltd., Colnbrook, Bucks. All other amino acids were obtained from California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A. Samples of *S*-adenosylhomocysteine were kindly supplied as gifts by Dr S. H. Mudd of the National Institute of Mental Health (Bethesda, Md., U.S.A.) and by Dr W. Sakami of the Western Reserve University (Cleveland, Ohio, U.S.A.).

Methods. The rats were killed by decapitation 10min. after the injection of [^{35}S]methionine. The liver and brain were quickly removed, blotted free of excess of blood and immediately placed in liquid nitrogen, the total time elapsing being 30–40sec.

The frozen tissues were weighed and homogenized in ice-cold 6% perchloric acid (5ml./g. wet wt. of tissue) in an Ultra-Turrax homogenizer (Janke und Kunkel K.G., Stanfen, Breisgau, Germany). The acid-soluble fraction was separated by centrifugation at 800*g* for 1hr. at 0–4°. The precipitate was rehomogenized with ice-cold perchloric acid and centrifuged for 15min. under the same conditions and the supernatants were pooled. Perchloric acid was precipitated by neutralization (pH7·0–7·5) with 2N-KOH and phenolphthalein as indicator. After standing overnight, the precipitate was removed by centrifugation at 800*g* for 45min. The supernatant was filtered through Whatman no. 30 paper into graduated cylinders. The precipitate was washed with ice-cold deionized water and the combined filtrates were made to volume (20–25ml./g. of fresh tissue) and stored at −15° until analysed. All operations before storage of the sample were performed at 0–4°.

Ion-exchange chromatography. A sample equivalent to 1g. wet wt. of tissue was transferred to a Zeo-Karb 225 column (H^+ form, 14–52 mesh, 10mm.×100mm.). The column was then washed with 30ml. of deionized water and the effluent collected. This fraction contained taurine as well as free acids and neutral compounds. The amino acids adsorbed on Zeo-Karb were eluted with 100ml. of N-NH_3 solution. Free NH_3 was removed by repeated evaporation under reduced pressure (50–60°) and the residue dissolved in water (10ml.). A portion of this solution was transferred on a Dowex 1 column (AG 1, X10, CO_3^{2-} form, 200–400 mesh, 9mm.×120mm.) and washed with water. The amino acids adsorbed on the column were eluted with 0·1N-HCl in two fractions: (i) a neutral amino acid fraction, collected until all the carbonate was replaced by Cl^-, and (ii) a dicarboxylic amino acid fraction, collected from the beginning of the emergence of free HCl from the column (Gaitonde, 1961*a*). The neutral amino acid fraction contained methionine, cystathionine, homocyst(e)ine and cyst(e)ine as well as other non-sulphur amino acids. The dicarboxylic amino acid fraction was found to contain glutathione, together with aspartate and glutamate.

Oxidation of sulphur amino acids with hydrogen peroxide. The residue of the sulphur amino acids in the neutral amino acid fraction was oxidized with 0·5ml. of H_2O_2 (30%, w/v) or 0·5ml. of performic acid (1 part of H_2O_2+9 parts of formic acid) for 2–16hr. at 4°. The excess of reagent was removed by repeated evaporation under reduced pressure at 50–60°.

Oxidation of sulphur amino acids with periodic acid. In some experiments the amino acid residue obtained by elution of the Zeo-Karb column was treated with 1ml. of 0·2M-periodic acid; the reaction mixture was neutralized to pH8·0 with NH_3 solution and phenolphthalein as indicator. After oxidation at room temperature for 1–2hr., the reaction mixture was transferred to a column of Dowex 1 (CO_3^{2-} form) and the amino acids were fractionated into a neutral amino acid fraction and a dicarboxylic amino acid fraction as described above. The neutral amino acid fraction then contained the sulphoxides of methionine and cystathionine, and the dicarboxylic acid fraction contained cysteic acid and homocysteic acid and the sulphonic acid of glutathione (GSO_3H) together with aspartate and glutamate.

Hydrolysis. The neutral amino acid fraction was evaporated to dryness and the residue was dissolved in 10ml. of 6N-HCl. Glutamine was hydrolysed to glutamate by boiling under reflux for 1hr. The excess of HCl was removed by repeated evaporation under reduced pressure. The residue was dissolved in 10ml. of water: 1ml. was taken for the assay of ^{35}S and the rest of the solution was used for paper chromatography.

Paper chromatography. The amino acid fractions were evaporated to dryness and the residue was dissolved in water and transferred quantitatively into small evaporating cones (prepared by sealing the tapered end of 35mm.× 35mm. microfunnels). The solution in the cones was evaporated to dryness in a vacuum desiccator over NaOH pellets. The residue in the cone was dissolved in water (100μl.) and a portion (80μl.) was transferred to Whatman no. 1 paper (46cm.×57cm.) for two-dimensional paper chromatography in glass tanks.

A number of solvent systems were tried to separate the sulphur amino acids in the neutral amino acid fraction. Depending on the conditions used, the neutral amino acid fraction contained, among other neutral amino acids, the following sulphur amino acids: (i) methionine, cystathionine, cyst(e)ine, homocyst(e)ine; (ii) the sulphoxides or sulphones (or both) of methionine and cystathionine, cysteic acid and homocysteic acid; (iii) the sulphoxides or sulphones (or both) of methionine and cystathionine (cysteic acid and homocysteic acid being obtained in the dicarboxylic amino acid fraction). One of the following solvent systems was found suitable for use in the first direction: (*a*) butan-1-ol–formic acid–water (75:15:10, by vol.); (*b*) butan-1-ol–acetic acid–water (4:1:5, by vol.; upper phase used); (*c*) butan-1-ol–propionic acid–water (10:5:7, by vol.). In all these systems cystathionine sulphoxide and sulphone were the slowest moving compounds. The mobilities of the various sulphur amino acids were: cystathionine sulphoxide and sulphone < cysteic acid and homocysteic acid < methionine sulphoxide and sulphone. The relative merits of these solvent systems for resolving other amino acids have been discussed by Gaitonde, Dahl & Elliott (1965).

Of the various solvent systems tried for development in the second direction, only the following were found successful for the complete resolution of all the sulphur amino

acids: (*a*) methanol–ethanol–chloroacetic acid (1N)–water (50:25:6:19, by vol.); (*b*) 80% (w/w) phenol (in the presence of NH_3); (*c*) 80% (v/v) acetone or acetone–ethyl methyl ketone–water (40:40:20, by vol.). The second-direction solvent system (*a*) was suggested from observations of Fischer (1957), Kirby (1955), Wyatt (1951) and Schram, Moore & Bigwood (1954). It was, however, abandoned for routine use in view of its toxic nature. Further, the complete removal of chloroacetic acid, which was not always satisfactory, was essential for the reproducibility of colour reaction after spraying with ninhydrin. The intensity of the purple spots was never sufficiently reproducible for them to be eluted for quantitative purposes.

The other two solvents (*b*) and (*c*) were both satisfactory for most purposes. As a solvent, phenol gave an excellent separation of most amino acids but acetone was found superior in that it gave a separation of (i) cysteic acid from homocysteic acid and (ii) methionine sulphone from methionine sulphoxide and other adjacent amino acids on a paper chromatogram. Besides these advantages, the acetone system was pleasant to handle, it required no purification of solvents, it did not mask the ultraviolet-absorbing compounds, it did not require the time-consuming removal of solvent before spraying with ninhydrin and it gave a perfectly white background well suited for the quantitative determination of amino acids after spraying with ninhydrin.

The chromatograms were always developed with the formic acid, acetic acid or propionic acid system for 24–30hr. and dried in a fume cupboard (40–50°) for 6–16hr. to remove most of the acid. The dried chromatograms were then developed in the second direction with 80% acetone for 10–14hr.

The use of 80% acetone as the first solvent for the resolution of amino acid mixtures was not found to be satisfactory, since its resolving power was dependent on the presence of trace amounts of formic acid, acetic acid or propionic acid used in the first solvent system. The presence of these acids in excessive amounts caused all the amino acids to travel faster in the acetone system. The amino acid spots, especially those of cysteic acid and homocysteic acid, were found to be compact if the vapour phase of aqueous acetone was maintained in the glass tanks before development of the chromatogram with 80% acetone. In subsequent studies a mixture of acetone, ethyl methyl ketone and water was preferred to 80% acetone for overnight development of the chromatograms since the rate of movement of amino acids in this solvent was slower than in 80% acetone.

The papers were sprayed with ninhydrin reagent immediately after drying and the amino acids estimated as described previously (Gaitonde, 1961*a*). Higher colour yields were, however, obtained if the ninhydrin spots were cut out after heating at 60° for 20min. and allowed to stand overnight in the dark in tightly stoppered tubes.

Measurement of ^{35}S. A portion of the solution was transferred to a planchet for counting at infinite thinness with a Nuclear–Chicago gas-flow counter fitted with a Micromil window. Further details on the preparation of samples is given elsewhere (Vrba, Gaitonde & Richter, 1962). Under the conditions used the counter gave an efficiency of 12%. In some experiments the samples were counted directly in a solution prepared according to Bray (1960), but without ethylene glycol. An unrefrigerated dual-channel automatic liquid-scintillation counter (Isotope Developments Ltd., Reading, Berks.) giving 60% efficiency was used. Correction for quenching was applied by the channels-ratio method (Bush, 1963).

Measurement of the ^{35}S *content of sulphur amino acids after treatment with ninhydrin.* The production of a purple compound when an α-amino acid is treated with ninhydrin involves the deamination and decarboxylation of the α-amino acid to form the corresponding aldehyde. The aldehyde resulting from aspartate is somewhat volatile, but that from glutamate is non-volatile (see Gaitonde, 1965). The product obtained from L-[^{35}S]methionine by eluting the purple spot and evaporating for counting in the solid state showed a considerable loss of ^{35}S (50–75%). However, under the same conditions eluates of spots from [^{35}S]methionine sulphoxide or the sulphone had lost only 8–15% of the ^{35}S and those of cysteic acid, taurine and glutathione (GSSG) gave 95–100% recovery of ^{35}S. These results suggest that the presence of the acidic groups $-CO_2H$, $-SO-$, $-SO_2-$ or $-SO_3H$ in the aldehyde molecules decreases the volatility of the latter at room temperature (22°). The observed losses were due mainly to volatilization of the aldehyde during the drying of the sample prepared for counting in the solid state. In view of these findings it was considered essential to oxidize thio ethers to their sulphoxides or sulphones, and thiols or disulphides to the corresponding sulphonic acids before paper chromatography. It was preferable to assay the ^{35}S in eluates of the purple spot, developed with ninhydrin, by a liquid-scintillation counting technique.

RESULTS

Oxidation of sulphur amino acids with hydrogen peroxide and periodic acid. Hydrogen peroxide, performic acid and peracetic acid have been commonly used for oxidation of sulphur amino acids (Toennies, 1942; Toennies & Homiller, 1942; Sanger, 1949; Alexander, Hudson & Fox, 1950). Methionine is oxidized to the sulphoxide in a few minutes at room temperature (20–25°) and, under the conditions used in this work, to the sulphone at 50–100°. However, the samples of methionine applied at the origin of the paper chromatograms were oxidized to the sulphoxide on treatment with hydrogen peroxide even at 50–60°. Under the same conditions performic acid allowed to react for 15–20min. oxidized methionine to its sulphone. Other peracids such as permolybdate (hydrogen peroxide + ammonium molybdate) have also been used for this purpose (Dent, 1948). Although the exact nature of the oxidation product of cystathionine is not known, it is presumed that cystathionine is also converted into its sulphoxide and into the sulphone under parallel conditions. Cyst(e)ine is oxidized to cysteic acid by a solution of performic acid (Schram *et al.* 1954), by peracetic acid (Alexander *et al.* 1950) or by hydrogen peroxide (Gaitonde, 1961*b*). Other amino acids such as threonine, serine, leucine, phenylalanine and tyrosine are also partially decomposed as a result

of performic acid oxidation (Toennies & Homiller, 1942; Dunstone & Morgan, 1965).

Periodate is known to oxidize all α-amino acids, but the rate of oxidation is particularly rapid with serine, threonine, tryptophan, methionine, cyst(e)ine and homocyst(e)ine (Nicolet & Shinn, 1939; Artom, 1945; Gaitonde & Gordon, 1958; Stevens & Sakami, 1959; Stephan & Erdman, 1964; Clamp & Hough, 1965). The treatment of sulphur amino acids with periodic acid between pH 5 and 8 at room temperature was found to oxidize the sulphur atom to the sulphoxide, sulphone or sulphonic acid: thus methionine was oxidized to its sulphoxide and traces of sulphone (see also Stevens & Sakami, 1959; Stephan & Erdman, 1964), cyst(e)ine to cysteic acid, homocyst(e)ine to homocysteic acid and glutathione to its sulphonic acid. Under the conditions used, cystathionine was also found to give an oxidation product; in paper chromatography its presence was revealed on spraying with ninhydrin. The intensity of the purple colour was much less than that produced by an equivalent amount of untreated cystathionine. The product was provisionally identified as cystathionine sulphone: it is presumed that the first oxidation product was probably the sulphoxide which in the course of chromatography was converted into the sulphone.

Although a number of oxidation products of sulphur amino acids were identified, the quantitative aspects of the oxidation reaction have not yet been investigated. Clamp & Hough (1965) found that very little carbon dioxide was formed during the oxidation of methionine and cyst(e)ine with periodate. From these observations it appears that the oxidation of sulphur amino acids with periodate affects mainly the sulphur atom and not significantly the α-carbon atom according to the reaction:

$$R \cdot CH(NH_2) \cdot CO_2H + O \rightarrow R \cdot CHO + NH_3 + CO_2$$

Ion-exchange chromatography of ^{35}S-labelled amino acids in tissue extracts. In two experiments (Table 1) the ^{35}S-labelled compounds in perchloric acid extracts of rat brain and liver were separated initially into two fractions by using a strongly acidic resin, Zeo-Karb 225. The ^{35}S-labelled compounds which were not adsorbed on the resin were washed out from the column with water; on paper chromatography it was found that taurine was the only strongly labelled amino acid in this fraction. The other ^{35}S-labelled amino acids adsorbed on the resin were eluted with ammonia. The overall recovery of ^{35}S after ion exchange on Zeo-Karb 225 was 87–106% for liver and 95–116% for brain perchloric acid extracts. Small differences in the recovery are probably due to errors involved in applying the correction for quenching of liquid scintillation by samples containing yellow pigment. The main ^{35}S-labelled compounds in the eluate from Zeo-Karb 225 were fractionated further on a Dowex 1 (CO_3^{2-} form) column. Small but measurable amounts of ^{35}S (0·8–1·7%) passed unhindered through this column. The radioactivity adsorbed on Dowex 1 was eluted separately in the neutral amino acid and in the dicarboxylic amino acid fractions. The neutral amino acid fraction contained, among other neutral amino acids, the following free sulphur amino acids: methionine, cystathionine and their sulphoxides or sulphones, cyst(e)ine, homocyst(e)ine; the dicarboxylic amino acid fraction contained glutathione (GSH and GSSG) together with aspartate and glutamate.

Table 1. *Percentage distribution and recovery of ^{35}S by ion-exchange chromatography of perchloric acid extracts of rat liver and brain at 10 min. after injection of [^{35}S]methionine*

Rats were killed by decapitation 10 min. after the intraperitoneal injection of L-[^{35}S]methionine. Figures give the percentages of the total ^{35}S in the perchloric acid extract.

		^{35}S in fractions (% of total in perchloric acid extract)					
		Zeo-Karb 225		Zeo-Karb eluate on Dowex 1 (CO_3^{2-})			
Expt. no.	Perchloric acid extract (10^{-5} × counts/min./g. of fresh tissue)	Wash (taurine)	Eluate (sulphur amino acids and glutathione)	Wash	Neutral (sulphur amino acids)	Acid (glutathione)	Recovery from Dowex 1 (CO_3^{2-})
				Liver			
1	43·31	16·0	71·1	1·0	52·3	12·1	65·4
2	5·89	7·7	98·7	1·7	70·7	16·6	89·0
				Brain			
1	2·02	2·4	92·7	0·8	82·5	3·8	87·1
2	0·73	5·4	110·3	1·1	88·4	6·6	96·1

Most of the ^{35}S adsorbed on Dowex 1 was recovered in the free sulphur amino acids and glutathione. There were small losses (5–14%) of ^{35}S after ion exchange on Dowex 1. The proportion of ^{35}S incorporated into the glutathione fraction was greater in liver (12–17%) than in brain (4–7%); the ^{35}S content of the free sulphur amino acids was greater in brain (82–88%) than in liver (52–71%) at 10min. after injection of L-[^{35}S]methionine (Table 1).

Paper chromatography of the ^{35}S-labelled amino acids of rat tissues. In view of the small amounts of methionine, cyst(e)ine and homocyst(e)ine in rat tissues, and also in view of the partial oxidation of methionine to its sulphoxide or sulphone in the course of preparation of samples and during paper chromatography, it was decided to oxidize the samples (of neutral amino acid fractions) before paper chromatography. Two-dimensional chromatograms prepared from the extracts of 1g. of rat liver or of brain showed either the absence or the presence of only traces of methionine, cyst(e)ine and homocysteine; cystathionine was not detectable in liver samples; but chromatograms prepared from brain samples gave a distinct spot of cystathionine sulphone. The distribution and recovery of ^{35}S from different sulphur amino acids in the rat tissues was studied by adding carrier amounts (25μg. each) of methionine sulphone, cystathionine sulphone, cysteic acid and homocysteic acid to the sample before paper chromatography (Fig. 1). The recovery of ^{35}S in the neutral amino acid fraction was obtained after elution of the ninhydrin-positive spots from the chromatogram. The known sulphur amino acids such as methionine, cyst(e)ine, homocyst(e)ine and cystathionine accounted for 23% and 29% of the ^{35}S in the two neutral amino acid fractions of rat liver (Table 2, columns 2 and 4). The recovery of ^{35}S from rat brain was 13% of the ^{35}S in the neutral amino acid fraction (Table 2, column 3).

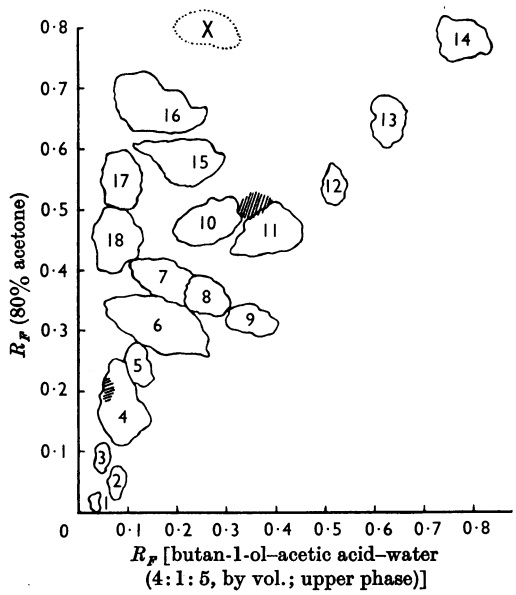


Fig. 1. Two-dimensional separation of amino acids present in the neutral amino acid fraction of rat liver. After application of the sample previously oxidized with H_2O_2 a mixture of carrier amounts (25μg. each) of methionine sulphone, cystathionine sulphone, cysteic acid, homocysteic acid and taurine was added on top of the sample. 1, Unknown; 2, cystathionine sulphone; 3, unknown; 4, histidine; *S*-adenosylhomocysteine sulphoxide; 5, asparagine(?); 6, glutamine; 7, serine; 8, glycine; 9, glutamate; 10, methionine sulphoxide and threonine; 11, α-alanine; 12, tyrosine; 13, valine; 14, leucine; 15, methionine sulphone; 16, taurine; 17, homocysteic acid; 18, cysteic acid. Spots shown with hatched lines were areas showing absorption of ultraviolet light (254mμ). Reference to spot X is made in the Results section. The R_F scale given above indicates approximate R_F values of the compounds.

In addition to 29% of the ^{35}S that could be recovered from the above-mentioned known sulphur amino acids in rat liver, about 24% of the ^{35}S was elutable from other unidentified ninhydrin-positive spots on the chromatogram of rat liver (Fig. 1 and Table 2, column 4).

These findings gave evidence that the neutral amino acid fraction contained other unknown ^{35}S-labelled compounds. To obtain more information on the nature of these compounds, the whole amino acid fraction of liver eluted from Zeo-Karb 225 was subjected to one of the following two treatments (Scheme 1): (i) Expt. 3: the ammonia eluate (about 35ml.) was treated directly with 0·5ml. of 30% hydrogen peroxide at 0° for 1hr. and then brought to 100° by immersion in the boiling-water bath: 0·5ml. of 1N-sodium hydroxide was added to keep the pH alkaline (pH 13) and the heating was continued for 20min. The reaction mixture was evaporated to dryness; the residue was dissolved in water and transferred to a Dowex 1 (CO_3^{2-} form) column to obtain the neutral and dicarboxylic amino acid fractions. (ii) Expt. 4: the ammonia eluate of the Zeo-Karb 225 was evaporated to dryness. The residue was dissolved in water: one-half of the solution was fractionated directly on Dowex 1 (CO_3^{2-} form) and the other half was oxidized with periodic acid. The reaction mixture was transferred directly to a Dowex 1 (CO_3^{2-} form) column to obtain neutral and dicarboxylic amino acid fractions. The ^{35}S content of the amino acid fractions obtained from the experiments (Expts. 3 and 4) are given in Table 3.

On oxidation with hydrogen peroxide (Expt. 3), there was a decrease in the ^{35}S content of the

Table 2. *Distribution and recovery of ^{35}S from paper chromatograms of the neutral amino acid fraction eluted from Dowex 1 (CO_3^{2-} form) column*

Young adult rats (Expts. 1, 2 and 3) and newborn rats (Expt. 4) were injected with [^{35}S]methionine. The amino acid spots referred to are indicated in Fig. 1. For conditions of Expts. 3 and 4 see Scheme 1.

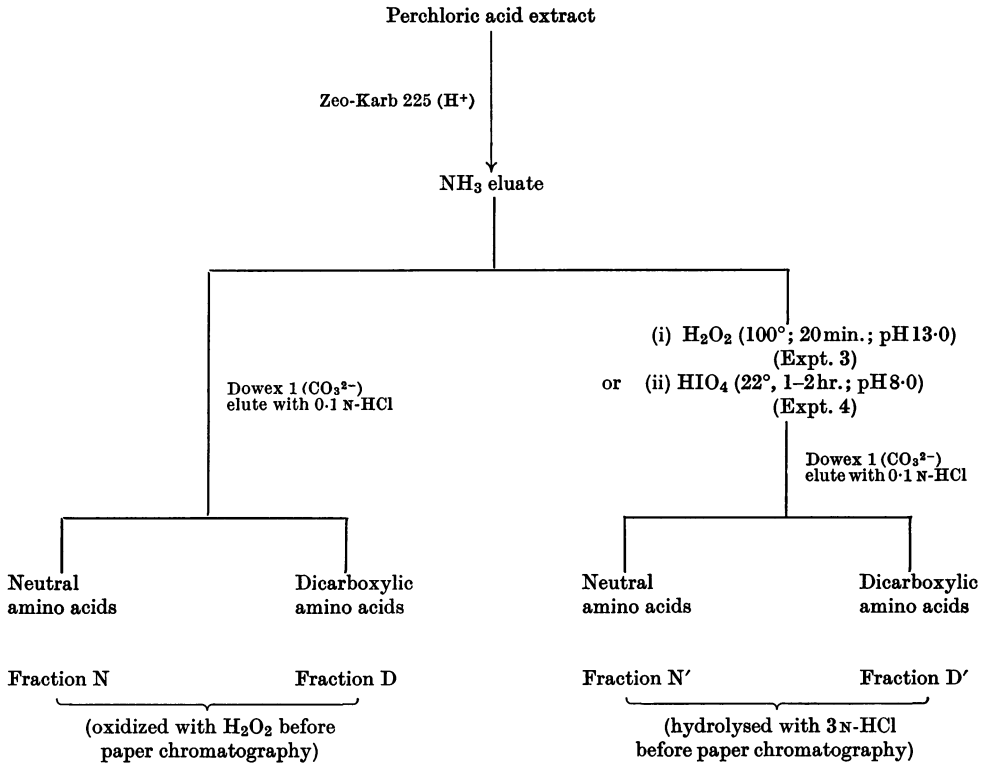
	(10^{-2} × Counts/min.)					
			Expt. 3 (liver)		Expt. 4 (liver)	
Compound	Expt. 1 (liver)	Expt. 2 (brain)	No treatment	H_2O_2	No treatment	HIO_4
(1)	(2)	(3)	(4)	(5)	(6)	(7)
Methionine as sulphone	2185·8	4·8	41·3	88·9		
Cystathionine					12·8	
Cystathionine as sulphone	528·0	3·0	3·8	5·1		7·6
Cyst(e)ine as cysteic acid	672·0	4·8	2·4			
Homocyst(e)ine as homocysteic acid	1780·2	207·0	3·1			
Other ^{35}S compounds at						
(i) origin	67·80	1·8				
(ii) spot 3			6·7	0·8		
(iii) histidine (spot 4)			10·5			
(iv) glutamine (spot 6)			2·6		15·1	10·1
(v) glycine (spot 8)			9·2	0·5	2·2	2·3
(vi) threonine and alanine (spots 10 and 11)			4·5			
(vii) taurine (spot 16)			7·4	2·3	1·5	5·1
(viii) valine (spot 13)					13·4	13·5
Recovered: counts/min.	5233·8	221·4	91·5	97·6	45·0	38·6
Present: counts/min.	22665·0	1663·2	170·9	143·4	166·8	126·7
Percentage recovery	23	13	53	68	27	30

neutral amino acid fraction and an increase in the dicarboxylic amino acid fraction with a total 95% recovery of ^{35}S. Similar results (84% recovery) were obtained after oxidation with periodic acid (Expt. 4). The overall recovery of ^{35}S after paper chromatography of samples treated as in (i) above was the same as of samples which had been oxidized with hydrogen peroxide immediately before transfer to the paper chromatogram (Table 3, Expt. 3; columns N and N'). However, the loss of ^{35}S on paper chromatography was less in samples treated with hydrogen peroxide than in (i), i.e. it was 15% instead of 28%. Under the same conditions the dicarboxylic amino acid fraction showed changes in the opposite direction: the loss of ^{35}S was greater, i.e. 33% instead of 19% after paper chromatography of the respective samples (Table 3, Expt. 3; columns D' and D).

The neutral amino acid fractions obtained in Expt. 4 (Table 3) were hydrolysed with 3N-hydrochloric acid and subjected to paper chromatography for the detection of the type of ^{35}S-labelled amino acids. The ^{35}S recovered after chromatography of the hydrolysed sample was 27%, showing a loss of $97-27=70\%$ of the ^{35}S. The neutral amino acid fraction obtained after treatment with periodic acid as in (ii) and subsequent paper chromatography of the hydrolysed sample gave a loss of $74-25=49\%$ of the ^{35}S.

When the acid hydrolysis of the neutral amino acid fraction was omitted there was a striking difference in the nature of the oxidation products obtained with hydrogen peroxide and with periodic acid. Two-dimensional paper chromatograms of the neutral amino acid fraction after its oxidation on paper with hydrogen peroxide showed a considerable amount of ^{35}S near the origin (cf. Table 2 and Fig. 1). In contrast, the paper chromatograms of the neutral amino acid fraction obtained from the whole amino acid fraction (i.e. Zeo-Karb eluate) after oxidation with periodic acid showed an absence of intense radioactivity (^{35}S) near the origin.

These results established two points. (*a*) Treatment with hydrogen peroxide results in the oxidation of sulphur compounds to new products which are then elutable in the dicarboxylic amino acid fraction. This was confirmed by the disappearance of ^{35}S from various other ^{35}S-labelled compounds (indicated in Table 2, columns 4 and 5) after paper chromatography of the neutral amino acid fraction, and the appearance of a ninhydrin-negative compound X, which moved rapidly in the 80% acetone system used in the second direction (this compound



Scheme 1. Group separation of sulphur compounds formed after oxidation with hydrogen peroxide or periodic acid.

is shown at position X in Fig. 1). It was usually lost when acetone was allowed to drip off the paper in the course of prolonged irrigation lasting 8–12hr. (*b*) Hydrolysis and paper chromatography of the neutral amino acid fraction results in a loss of ^{35}S. The observed loss was attributed mainly to the appearance of a ninhydrin-negative compound, which, in view of its fast mobility, was normally lost during paper chromatography during development in propionic acid or acetic acid solvent systems used in the first direction.

DISCUSSION

The main object of these studies was to isolate free ^{35}S-labelled methionine, cystathionine, cyst(e)ine, homocyst(e)ine and glutathione from rat tissue after administration *in vivo* of L-[^{35}S]-methionine. In the first stage of analysis of these compounds, glutathione was separated from other above-mentioned sulphur amino acids by ion-exchange chromatography. These experiments confirmed the previous finding that the trans-sulphuration pathway is active in brain as well as in liver.

Two-dimensional paper chromatography confirmed the presence of cystathionine in brain. Its presence in liver was not observed on chromatograms sprayed with ninhydrin, but it was measurable by ^{35}S analysis. This is probably a reflection of the extreme rapidity with which trans-sulphuration occurs in liver. Methionine was either detectable in trace amounts (1–2μg.) or not detectable at all in perchloric acid extracts of brain and liver. The only previous figures for the methionine content of these tissues are those of Schurr, Thompson, Henderson, Williams & Elvehjem (1950), who reported values obtained by a microbiological method of 12·2μg. of methionine/g. of brain and 18·4μg. of methionine/g. of liver. Under the conditions used in the present work, these amounts of methionine would have been sufficient for satisfactory detection and isolation by paper chromatography.

The present studies have shown the presence in liver and brain of ^{35}S-labelled compounds other than those referred to above. These compounds were rapidly labelled after injection of L-[^{35}S]methionine. Their nature was studied by oxidation with hydrogen peroxide and periodic acid and by acid

Table 3. *Analysis of products of oxidation by hydrogen peroxide or periodic acid of* ^{35}S*-labelled amino acids eluted after ion-exchange chromatography on Zeo-Karb 225*

In each experiment ^{35}S-labelled amino acids eluted from Zeo-Karb 225 were separated into two fractions on a Dowex 1 column (CO_3^{2-} form) as shown in Scheme 1.

	Percentage distribution											
	In fractions of Expt. 3						In fractions of Expt. 4					
	Untreated			H_2O_2			Untreated			HIO_4		
	N	D	Recovery (N+D)	N′	D′	Recovery* (N′+D′)	N	D	Recovery (N+D)	N′	D′	Recovery* (N′+D′)
Ion-exchange chromatography (*a*)	60·9	39·1	100·0	45·7	49·3	95·0	97	3	100	73·6	10·2	84·0
Paper chromatography (*b*)	33·0†	20·0†	53·0	31·0	16·0	47·0	27‡	—	—	25·0‡	—	—
Loss of ^{35}S (*a*−*b*)	27·9	19·1	47·0	14·7	33·3	48·0	70	—	—	48·6	—	—

* Recoveries are given as percentage of the sum of ^{35}S in fraction N and fraction D (see Scheme 1).
† Treated with H_2O_2 before paper chromatography.
‡ Hydrolysed with 3N-HCl before paper chromatography.

hydrolysis. The behaviour of the oxidation products was consistent with the presence of adenosyl[^{35}S]-methionine and adenosyl[^{35}S]homocysteine in the original fraction eluted with ammonia from Zeo-Karb 225 (H^+). The presence of only small amounts of ^{35}S in the water washes of Dowex 1 (Table 1) and an increase in the ^{35}S of methionine sulphone after direct oxidation of the ammonia eluate with hydrogen peroxide (Table 2, columns 4 and 5) showed that almost all of the adenosyl[^{35}S]methionine eluted from Zeo-Karb was decomposed during the process of evaporation of the ammonia eluate. It is known that under these conditions *S*-adenosylmethionine is decomposed into methylthioadenosine and homoserine (Cantoni, 1953; see also Schlenk, 1965), but *S*-adenosylhomocysteine is stable (Duerre, 1962). These findings were confirmed with samples of pure *S*-adenosylmethionine and *S*-adenosylhomocysteine under the conditions used in these studies.

After removal of the ammonia, methylthioadenosine and *S*-adenosylhomocysteine can be adsorbed on Dowex 1 (CO_3^{2-} form) and then eluted in the neutral amino acid fraction. The latter fraction represented about 83–88% of the ^{35}S in brain and 52–71% in liver of rats injected with L-[^{35}S]methionine (Table 1). This suggested that the greater part of the ^{35}S in this fraction might be attributable to ^{35}S-labelled thiomethyladenosine and *S*-adenosylhomocysteine. Paper chromatography of the neutral amino acid fraction showed the presence of ^{35}S-labelled compound with a fast mobility (R_F 0·60–0·75) in the butan-1-ol–acetic acid or butan-1-ol–propionic acid systems; the R_F of this compound remained relatively unchanged on periodic acid oxidation or acid hydrolysis. These observations are in agreement with the known properties of methylthioadenosine (Table 4). Further, the observed increase in the ^{35}S content of methionine sulphone on oxidation with hydrogen peroxide at pH 13·0 (Expt. 3; see Table 2) suggested the presence of *S*-adenosylmethionine in the ^{35}S-labelled amino acid fraction eluted from Zeo-Karb 225, since under similar conditions *S*-adenosylmethionine was shown to decompose into methionine (Parks & Schlenk, 1958; Schlenk & Ehninger, 1964).

Paper chromatography of the neutral amino acid fraction also showed the presence of a ^{35}S-labelled compound superimposed on the histidine spot, which also coincided with the position of a pure sample of *S*-adenosylhomocysteine sulphoxide (Fig. 1). The persistence of ^{35}S in this spot on paper chromatograms of samples on oxidation with hydrogen peroxide and not on oxidation with periodate is consistent with the findings that *S*-adenosylhomocysteine is oxidized to its sulphoxide by hydrogen peroxide and to homocysteic

Table 4. *Some properties of methylthioadenosine and S-adenosylhomocysteine*

The sulphoxides and dialdehydes referred to above are also elutable in the neutral amino acid fraction and homocysteic acid in the dicarboxylic amino acid fraction from Dowex 1.

Treatment	Methylthioadenosine (*a*)	*S*-Adenosylhomocysteine (*b*)	Reference
None	R_F 0·75†; 0·64*	R_F 0·16*	(*a*) Schlenk & DePalma (1957)
			(*a*) Schlenk & Ehninger (1964)
			(*b*) Duerre (1962)
		0·22*, 0·34‡, 0·35†	(*b*) Present work
H_2O_2	Sulphoxide formed R_F 0·34*	(i) Sulphoxide formed R_F 0·10*	(*a*) Schlenk & Ehninger (1964)
			(*b*) Duerre (1962)
		(ii) R_F overlaps histidine (as shown in Fig. 1)	(*b*) Present work
		(iii) Partially decomposed to ribosylhomocysteine sulphoxide of R_F 0·09*; 0·36†	(*b*) Duerre (1962)
HIO_4	2′,3′-Dialdehyde formed	(i) 2′,3′-Dialdehyde and (ii) homocysteic acid formed	(*a*) Baddiley, Cantoni & Jamieson (1953)
			(*b*) Gaitonde & Gaull (1967)
Hydrolysis (HCl)	(i) Methylthioribose formed; R_F 0·73*	(i) Ribosylhomocysteine formed; R_F 0·14*; 0·44†	(*a*) Smith & Schlenk (1952)
	(ii) Other products(?)	(ii) Homocysteine formed	(*b*) Duerre (1962)

* Butan-1-ol–acetic acid–water (12:3:5, by vol.).
† Ethanol–acetic acid–water (65:1:34, by vol.).
‡ Butan-1-ol–propionic acid–water (10:5:7, by vol.).

acid by periodate (Table 4). The ^{35}S of *S*-adenosylhomocysteine is elutable as the sulphoxide in the neutral amino acid fraction and as homocysteic acid in the dicarboxylic amino acid fraction.

These findings suggest strongly that *S*-adenosylmethionine and *S*-adenosylhomocysteine were rapidly labelled *in vivo* and extracted in the perchloric acid extracts of rat tissues. If a part of the methionine in tissue extracts is present as the *S*-adenosyl derivative, this could explain the higher figures for free methionine reported by Schurr *et al.* (1950), who used a microbiological method. Further evidence on the identification of these two compounds is given by Gaitonde & Gaull (1967).

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