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A Procedure for the Quantitative Analysis of the Sulphur Amino Acids of Rat Tissues

By M. K. GAITONDE and G. E. GAULL*
Neuropsychiatric Research Unit, Medical Research Council, Carshalton, Surrey, and the Division of Pediatric Neurology, Columbia–Presbyterian Medical Center, New York, N.Y., U.S.A.

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1. A method is described for the quantitative separation of the sulphur compounds in a single sample of tissue by passing an extract through a serial assembly of ion-exchange resins in the order: Dowex 2 (Cl^- form), Dowex 1 (CO_3^{2-} form), Amberlite CG-50 (H^+ form) and Zeo-Karb 225 (H^+ form). 2. Groups of sulphur amino acids were eluted separately from each column; the recovery of sulphur compounds after their labelling with ^{35}S *in vivo* by injection of L-[^{35}S]-methionine was 91–106%. Individual sulphur compounds were further resolved by one-dimensional or two-dimensional paper chromatography. 3. Evidence is presented on the occurrence of *S*-adenosylmethionine and *S*-adenosylhomocysteine in rat liver and brain. Rat liver and brain contained 83·6 and 31·4 mμ-moles/g. respectively of *S*-adenosylmethionine.

Methionine is metabolized *in vivo* by three main pathways: it may serve as (*a*) a precursor for protein synthesis, (*b*) a methyl donor and source of cyst(e)ine, or (*c*) a source of energy by oxidation to sulphate and carbon dioxide. It is known that for methionine to be available for (*a*) or (*b*), it must first be activated by combination with ATP. For it to be utilized in protein synthesis, the carboxyl group must be activated by the formation of methionyladenylate, which is incorporated into the complex of soluble RNA and the transfer enzyme (Hoagland, Keller & Zamecnik, 1956). When methionine acts as a methyl donor, it is activated at the sulphur atom with the formation of a sulphonium compound, *S*-adenosylmethionine (Cantoni, 1953). The sulphonium compound is labile under most conditions and rapidly donates its methyl group to acceptor compounds such as guanidinoacetic acid or ethanolamine, giving rise to *S*-adenosylhomocysteine (Cantoni & Scarano, 1954). The further metabolism of *S*-adenosylhomocysteine to form cystathionine, cysteine, cysteinesulphinic acid, taurine and sulphate has been demonstrated *in vitro* and *in vivo* (Carroll, Stacy & du Vigneaud, 1949; du Vigneaud, 1952; Bergeret, Chatagner & Fromageot, 1952; Awapara & Wingo, 1953; Fromageot, 1955; Eldjarn, Pihl & Sverdrup, 1956).

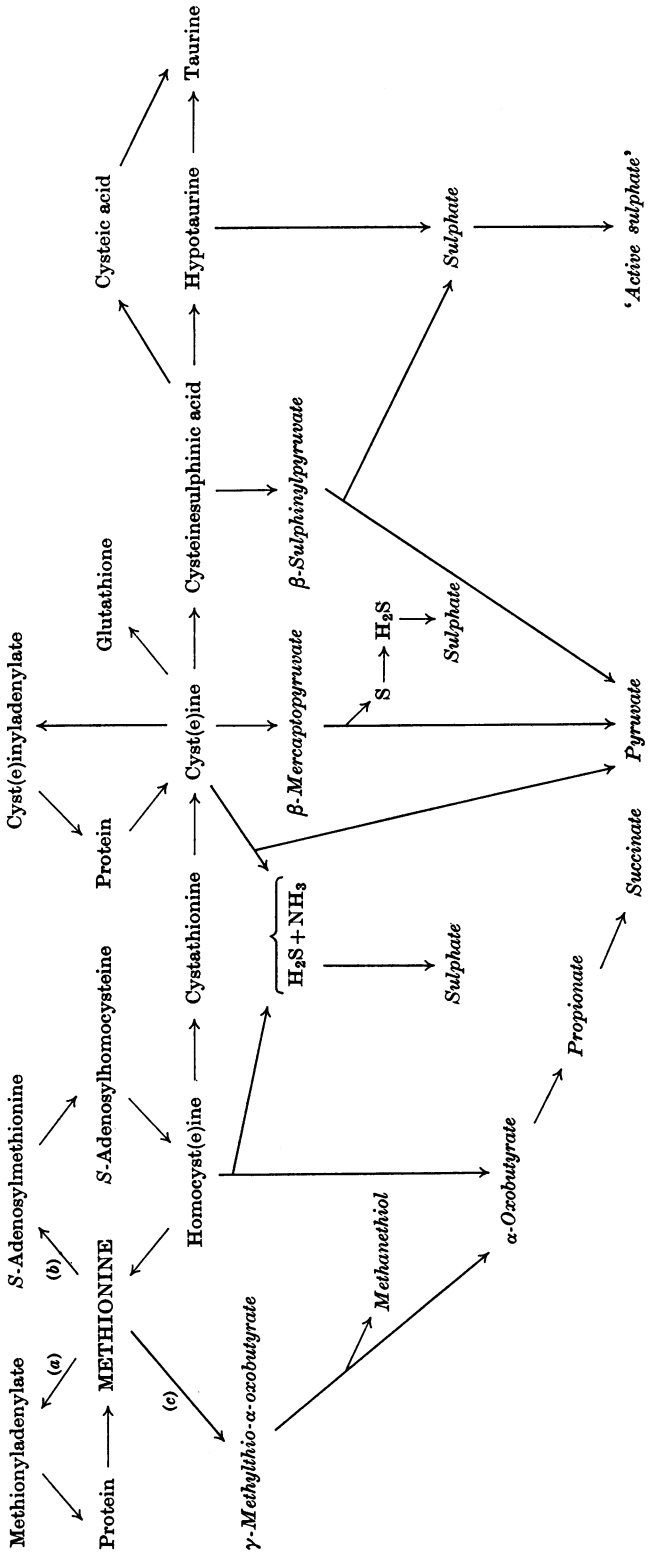
Among the numerous metabolites of methionine a number of sulphur amino acids have been reported to occur in mammalian tissues (Scheme 1). Some of these metabolites have also been shown to be formed in rat tissues after injection of [^{35}S]methionine (Gaitonde & Richter, 1957). Although their content in certain tissues has been reported at various times in the literature, no systematic method for the complete analysis of sulphur compounds in a single sample has hitherto been described. The need for such a method became apparent in a preliminary study of the specific radioactivity of methionine and its metabolites in rat brain and liver after administration of [^{35}S]methionine *in vivo* (Gaull & Gaitonde, 1967).

In the present investigation a procedure was developed for separating the metabolites of [^{35}S]methionine into groups of sulphur compounds many of which were subsequently identified. By using these methods, evidence was obtained of the occurrence of *S*-adenosylmethionine, *S*-adenosylhomocysteine and other sulphur amino acids in rat brain and liver. Paper-chromatographic procedures for the isolation of individual sulphur compounds after their initial group separation were used for the determination of their specific radioactivity.

MATERIALS AND METHODS

Chemicals. The standard HCl and acetic acid were prepared from conc. HCl (10 N) and acetic acid (17·4 N). The standard acids (1 N and 0·1 N) obtained commercially

* Present Address: Division of Pediatric Neurology, Columbia–Presbyterian Medical Center, New York, N.Y., U.S.A.



Scheme 1. Intermediary metabolites of methionine during its utilization as (*a*) a precursor of protein synthesis, (*b*) a methyl donor and (*c*) a substrate for energy generation. Non-nitrogenous metabolites are *italicized*.

contained u.v.-absorbing material and were not used in these studies.

Dowex 1 (AG1; X10; 200–400 mesh) and Dowex 2 (AG2; X8; 200–400 mesh) were preparations of BioRad Laboratories, Richmond, Calif., U.S.A. Amberlite CG-50 (chromatographic grade type 1; 100–200 mesh) was obtained from the British Drug Houses Ltd., Poole, Dorset. Zeo-Karb 225 (14–52 mesh) was a product of The Permutit Co. Ltd., London, W. 4.

Sulphur compounds and other chemicals were obtained from Koch–Light Laboratories Ltd. (Colnbrook, Bucks.), British Drug Houses Ltd. or California Corp. for Biochemical Research (Los Angeles, Calif., U.S.A.). Pure samples of *S*-adenosylhomocysteine were kindly sent 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. Cystathionine sulphoxide was prepared by oxidation with periodic acid (0·2 M). The reaction mixture was passed through a column of Dowex 1 (CO_3^{2-} form): cystathionine sulphoxide was collected in the neutral fraction with 0·1 N-HCl until all the carbonate was exchanged with chloride and free HCl emerged from the column. The solution was concentrated and cystathionine sulphoxide was precipitated with 80–90% (v/v) ethanol. The product was purified further by two more precipitations and finally dried in the desiccator.

The molar extinction of an aqueous solution of *S*-adenosylmethionine chloride supplied by Koch–Light Laboratories Ltd. was ϵ_{260} 11 750. Paper chromatography with ethanol–acetic acid–water (65:1:34, by vol.) gave three u.v.-absorbing spots: *S*-adenosylmethionine (R_F 0·25), *S*-adenosylhomocysteine (R_F 0·33) and methylthioadenosine (R_F 0·67). The first two compounds gave purple spots after a spray with ninhydrin; a third ninhydrin-positive spot (R_F 0·58) was tentatively identified as homoserine. The solution contained approx. 46% of *S*-adenosylmethionine when examined for u.v. absorption at 260 mμ after its passage through a Dowex 1 (CO_3^{2-} form) column. This solution showed no further losses of u.v.-absorbing material after its passage through a second Dowex 1 (CO_3^{2-} form) column.

S-Ribosylmethionine was prepared as described by Parks & Schlenk (1958). A solution (1·5 ml.) of *S*-adenosylmethionine was passed through a column of Dowex 1 (CO_3^{2-} form) to remove impurities and the column was washed with water. The aqueous solution (20 ml.) was cooled in an ice bath and brought to 0·1 N-KOH by addition of 2 N-KOH in the cold room. After 5 hr. the potassium was removed as perchlorate by adding ice-cold 6% perchloric acid to bring the pH to 7·0–7·5. The clear supernatant solution (30 ml.) gave an extinction of 0·41 that was equivalent to 0·77 μmole of *S*-adenosylmethionine (ϵ 16 000). A portion of the solution (25 ml.; 0·64 μmole) was passed through the four-column assembly as described below. Each column was eluted separately in 5 ml. fractions. A portion (1 ml.) of each fraction was tested for ribose and amino N: only fractions 2–6 of eluate from the Amberlite column gave positive tests. The same fractions gave no u.v. absorption at 260 mμ, indicating the presence of *S*-ribosylmethionine and the absence of *S*-adenosylmethionine in these fractions. The recovery was 0·62 μmole of ribose and 0·64 μmole of amino N.

Methylthioadenosine was prepared by hydrolysis of *S*-adenosylmethionine purified as above and was isolated from the reaction mixture by paper chromatography (Schlenk & Ehninger, 1964). Methylthioribose was obtained by acid hydrolysis of methylthioadenosine (Smith & Schlenk, 1952).

S-Ribosylhomocysteine was prepared by hydrolysis of *S*-adenosylhomocysteine with 0·1 N-HCl at 100° for 90 min. (Duerre, 1962). Under these conditions about 87% of *S*-adenosylhomocysteine was hydrolysed.

Ion-exchange resins and columns. Dowex 2 (supplied in the Cl^- form) was washed by suspending for 2–4 hr. in 2 N-HCl; the suspension was filtered through a Buchner funnel, and the dry material was resuspended a further three times in 2 N-HCl and finally washed free of HCl with distilled water. Dowex 1 (supplied in the Cl^- form) was converted into the CO_3^{2-} form by suspending it in water and adding an equal volume of a saturated solution of Na_2CO_3. The process was repeated four times and finally the resin (in the CO_3^{2-} form) was washed until all the free carbonate was removed. Amberlite CG-50 (supplied in the H^+ form) was washed by suspending four times in N-HCl and finally washed with distilled water. Zeo-Karb 225 (supplied in the H^+ or Na^+ form) was converted into the H^+ form by four alternate treatments (2–3 hr. each) with 4 N-NaOH and 6 N-HCl. The final treatments with NaOH and HCl were each for 16–20 hr. The supernatant liquid was decanted and the resin was poured into a long glass tube (1 in. × 25 in.) and washed with 1–2 l. of 6 N-HCl at a flow rate of 200–300 ml./hr. The resin was then washed with distilled water and stored as a suspension in 0·1 N-HCl. A portion (10 cm. column) of this resin was checked by titration to ensure that all exchangeable ions were in the H^+ form.

The glass columns used for Dowex 1 and 2 resins were approx. 9 mm. × 300 mm. and those for Amberlite and Zeo-Karb resins were approx. 9 mm. × 130 mm. The column with B10 socket at the lower end and B14 socket at the upper end was prepared by inserting an adaptor cone fitted with a sinter (B10, 0 porosity; supplied by Quickfit and Quartz Ltd., Stone, Staffs.); the adaptor was also provided with a cone (B14) at the other end to connect, when required, to a second column. Each type of resin was repeatedly washed with distilled water after packing in the appropriate column. They were then used separately or as a vertical serial assembly.

Administration of L-[^{35}S]*methionine.* Male rats weighing 100 g. were injected intraperitoneally with 0·2 ml. of aqueous solution containing 30 μc in 30 μg. of L-[^{35}S]-methionine (supplied by The Radiochemical Centre, Amersham, Bucks.). The animals were killed at times from 5 min. to 60 min. after the injection. Brain and liver were rapidly taken out, blotted to remove blood and immediately frozen in liquid nitrogen.

Extraction of sulphur compounds with perchloric acid. The frozen tissues were homogenized in ice-cold 6% (w/v) perchloric acid (5 ml./g.) and suspension was centrifuged for 1 hr. at 2000 *g* at 0–4°. The clear supernatant solution was collected by decantation, the residue was washed twice with perchloric acid and the washings were combined with the main extract. The combined extract was neutralized in an ice bath by adding 2 N-KOH until the pH was 7·0–7·5. Care was taken to avoid making the extract alkaline during this procedure. The neutralized extract was left overnight in the cold room. The supernatant solution was decanted and filtered into a measuring cylinder. The precipitate of

potassium perchlorate was washed once with ice-cold water, the suspension centrifuged and the supernatant combined with the main neutral extract. The extract was made up to volume (25 ml./g.) and stored in polythene bottles at $-10°$ to $-15°$ until analysed.

Group separation of sulphur compounds. The neutral perchloric acid extract was passed first through a 20 cm. column of Dowex 2 (Cl^- form), and the resin was washed with 40 ml. of water. The ^{35}S-labelled compounds adsorbed on the resin were eluted first with 0·05 N-HCl or 0·1 N-acetic acid (fraction Dowex 2–A). The residual ^{35}S-labelled compounds were eluted with 0·5 N-HCl (fraction Dowex 2–B).

The solution containing ^{35}S-labelled compounds not adsorbed on Dowex 2 was transferred to a 20 cm. column of Dowex 1 (CO_3^{2-} form) and the resin washed with 40 ml. of water. The labelled compounds adsorbed on Dowex 1 were eluted first with 150 ml. of 0·1 N-acetic acid to give (i) a neutral amino acid fraction (fraction Dowex 1–A), usually 4–4·5 times the resin volume, until the eluate gave an acid reaction to a wide-range indicator paper (British Drug Houses Ltd. or Johnsons), and (ii) an acid fraction collected from the emergence of free acetic acid (fraction Dowex 1–B). The elution was then continued with 150 ml. of 0·1 N-HCl and (iii) fraction Dowex 1–C was collected until all the acetate from the column was replaced by chloride; this point was ascertained by observing the change of colour of resin from brown to light yellow and more precisely with the aid of a dimethyl yellow indicator paper (yellow → dark red); beginning at this stage (iv) fraction Dowex 1–D was collected by elution with 40–50 ml. of 0·1 N-HCl.

In previous studies the elution of compounds from Dowex 1 (CO_3^{2-} form) was performed directly with 0·1 N-HCl to give a neutral fraction and an acid fraction. The acid fraction so obtained contained aspartate, glutamate and glutathione (GSH + GSSG). By using 0·1 N-acetic acid and 0·1 N-HCl in that order as described above it was possible to obtain a further separation of the constituents of the acid fraction, since it was found that aspartate and glutamate were eluted in fraction Dowex 1–B and glutathione in fraction Dowex 1–D.

The compounds adsorbed on Dowex 1 and Dowex 2 were eluted under pressure of nitrogen gas. The application of positive pressure from above prevented extensive breaking of the resin column on Dowex 1 (CO_3^{2-} form) due to CO_2 evolved during elution with acid. Most of the CO_2 liberated was forced downwards in narrow streams, through the resin, but the disruption of the resin so caused did not affect the separation of groups of compounds.

The solution containing the neutral and the cationic compounds of the original perchloric acid extract from the Dowex 2 and Dowex 1 columns was then transferred to a 10 cm. column of Amberlite CG-50 (H^+ form) and the resin washed with 30 ml. of water. The compounds retained by this resin were eluted into ten 10 ml. fractions with 0·05 N-HCl.

The solution of the neutral and the cationic compounds not retained by the above resin was finally passed through a 10 cm. column of Zeo-Karb 225 (H^+ form) and the resin washed with water. The final solution contained neutral compounds such as glucose, glycerol, glycogen and inositol. The compounds adsorbed by the Zeo-Karb resin were eluted into ten 10 ml. fractions with N-NH_3.

Rapid separation of fractions. The above procedure for resolving the sulphur compounds into six major groups could be carried out by using the four types of resins in a single assembly of four columns arranged vertically in the order (from top to bottom); Dowex 2 (Cl^- form), Dowex 1 (CO_3^{2-} form), Amberlite CG-50 (H^+ form) and Zeo-Karb 225 (H^+ form). A known volume of neutralized perchloric acid extract (20–30 ml., 1 g. equivalent) was allowed to pass under pressure of nitrogen gas through this assembly beginning with Dowex 2 (Cl^- form). After the passage of the sample, the assembly was washed with distilled water until the pH of the wash emerging from Zeo-Karb 225 (H^+ form) was neutral: usually a total volume of 75 ml. was collected to obtain compounds not adsorbed by any of the above resins. The procedure finally adopted is given in Table 2.

Paper chromatography. The eluates of different fractions obtained after ion-exchange chromatography were dried under reduced pressure (50–55°) in a rotary evaporator. The residue was suspended in water, mixed thoroughly, filtered through Whatman no. 30 paper and collected in a cone made by sealing the tapered end of a micro-funnel (35 mm. × 35 mm.). The solution was dried in a desiccator *in vacuo* over NaOH. The residue in the cone was dissolved in water (100 μl.) and portions were taken for paper chromatography. Chromatography in the descending direction was performed on Whatman no. 1 paper (chromatographic grade; 46 cm. × 57 cm.). The following solvents were used: 1, butan-1-ol–propionic acid–water (a, 10:5:7, by vol.; b, 10:5:4, by vol.) (Gaitonde, Dahl & Elliott, 1965); 2, butan-1-ol–acetic acid–water (a, 4:1:5, by vol.; upper phase used) (Partridge, 1948) (b, 12:3:5, by vol.) (see Smith, 1962); 3, butan-1-ol–formic acid–water (15:3:2, by vol.) (Gaitonde *et al.* 1965); 4, acetone–water (4:1, v/v) (Gaull & Gaitonde, 1967); 5, ethanol–acetic acid–water (65:1:34, by vol.) (Schlenk & DePalma, 1957); 6, propan-1-ol–aq. NH_3 (sp.gr. 0·88)–water (16:1:3, by vol.) (Gaitonde, 1965); 7, methanol–ethanol–0·1 N-HCl (2:1:1, by vol.) (see Kirby, 1955); 8, methanol–ethanol–0·1 N-NH_3 (2:1:1, by vol.)

After one-dimensional development the chromatograms were heated at 40–50° for 6–16 hr. to remove most of the solvent and the chromatography was continued in the second direction (Gaitonde, 1961*a*; Gaull & Gaitonde, 1967). Finally they were dried, heated for 2 hr. at 40° and inspected in u.v. light. They were sprayed with or dipped in one of the following reagents: (*a*) Ninhydrin. A solution containing a mixture of 0·5 g. of ninhydrin in 10 ml. of methanol and 20 mg. of $SnCl_2,2H_2O$ in 6·25 ml. of 0·1 M-citrate buffer, pH 5·0, in a final volume made to 100 ml. with methanol (Gaitonde, 1961*a*). (*b*) Iodoplatinate. This reagent was prepared as described by Toennies & Kolb (1951) with the exception that 6 N-HCl was used instead of 2 N-HCl as recommended by these authors. It was applied to chromatograms previously sprayed with ninhydrin. (*c*) Benzidine reagent. A 2% (w/v) solution in 16 ml. of N-HCl and 84 ml. of 50% (v/v) ethanol was prepared as described by Gaitonde & Richter (1955). The chromatogram was dipped in this solvent and the position of sulphate was located from the bright yellow fluorescence observed in the u.v. light.

Measurement of radioactivity. Paper chromatograms were scanned with a Beta Probe (type Bp4) fitted to a portable monitor (type 1; E.M.I. Electronics Ltd., Hayes, Middx.) or with a Radioactive Chromatogram Counter (Baird and Tatlock Ltd., Chadwell Heath, Essex).

For quantitative assay the solution (1·0–1·5 ml.) containing ^{35}S was pipetted into a plastic planchet and dried in

a desiccator over NaOH. The radioactivity (counts/min.) was determined by using a Nuclear-Chicago gas-flow counter fitted with a Micromil window. The overall efficiency of the counting was 12%. All samples were counted at infinite thinness and corrected for background count and decay.

Analytical methods. (i) Amino nitrogen. All samples were brought to pH 8·0–8·5 and desiccated over H_2SO_4 and NaOH. The dry residue was dissolved in water and a portion was taken for amino N determination by the method of Yemm & Cocking (1955). For further details see Gaitonde *et al.* (1965).

(ii) Ribose was determined according to Mejbaum (1939) as described by Albaum & Umbreit (1947). A 1 ml. sample was mixed with 1 ml. of 12 N-HCl containing $FeCl_3$ (0·1%) and orcinol (1%) to give the final concentration of 6 N-HCl in the reaction mixture. The colour was developed by heating the reaction mixture in a boiling-water bath for 40 min. The extinction of the clear solution was measured at 670 mμ.

Ultraviolet spectroscopy. Aqueous samples and eluates of columns were scanned with a Unicam SP. 700 recording spectrophotometer.

Treatment with hydrogen peroxide. The oxidation of sulphur compounds was effected by treatment of the sample with hydrogen peroxide (30%, w/v) or performic acid prepared by mixing 1 vol. of hydrogen peroxide and 9 vol. of 90% formic acid (Toennies, 1942; Toennies & Homiller, 1942; Sanger, 1949). When treated on paper, hydrogen peroxide (20 μl.) was used to obtain sulphoxides and performic acid (20 μl.) was used to obtain sulphones; otherwise the material to be oxidized to the sulphone was dried in a flask and treated with hydrogen peroxide (0·5 ml.) or performic acid (1 ml.) overnight at 0°. The excess of reagent was removed by repeated volatilization at 50–60° under reduced pressure (see Gaull & Gaitonde, 1967).

Treatment with periodate. A solution of sulphur compounds (20 ml.) was treated with 1 ml. of 0·2 M-periodic acid at pH 8·0 as described previously (Gaull & Gaitonde, 1967).

RESULTS

Ion-exchange separation of sulphur compounds according to their charge

The main object of these studies was the quantitative separation of different sulphur compounds for subsequent analysis and determination of specific radioactivity. To study the recovery and identify the compounds in the fractions containing sulphur after ion-exchange chromatography, a neutralized perchloric acid extract was prepared from the liver of rats killed at different times after injection of L-[^{35}S]methionine. Measurements of the recovery of ^{35}S (Table 1) showed that practically all of the ^{35}S in the perchloric acid extract was recovered from the ion-exchange resins used. The nature of the sulphur compounds was studied in all fractions except B, C and D from Dowex 1 (CO_3^{2-} form), which contained only small amounts of ^{35}S.

Ion-exchange chromatography on Dowex 2 (Cl^- form)

After the passage of sample and washing of the resin with distilled water, the compounds were eluted from the column in 10 ml. fractions with 0·05 N-hydrochloric acid, 0·1 N-hydrochloric acid or 0·1 N-acetic acid and finally with 0·5 N-hydrochloric acid. The elution pattern with respect to ^{35}S is shown in Fig. 1. The specific radioactivity of each fraction, expressed as counts/min./μg. of amino N, is also shown in Fig. 1. The specific radioactivity values for tubes 2–10 were very similar to that of tube 11 when

Table 1. *Recovery of ^{35}S in the perchloric acid extract of rat liver after ion-exchange chromatography on a four-column serial assembly*

Rats were injected intraperitoneally with L-[^{35}S]methionine. For description of the fractions see the text and Table 2.

Fraction	Time after injection (min.)	Percentage recovery of ^{35}S in $HClO_4$ extract					
		5	10	20	30	40	60
Dowex 2–A		10·4	11·7	14·1	40·2	29·3	64·8
Dowex 2–B		9·8	17·2	25·9	36·3	27·9	23·0
Dowex 1–A		38·6	43·8	28·6	11·8	17·5	5·2
Dowex 1–B		*	2·1	2·1	*	*	*
Dowex 1–C		*	0·4	0·3	*	*	*
Dowex 1–D		*	0·4	0·4	*	*	*
Amberlite CG-50		30·4	13·7	16·7	10·8	16·7	3·8
Zeo-Karb 225		16·7	12·3	11·6	4·7		1·9
Unadsorbed		0·4	0·3	0·3	0	*	0·2
Total recovery		106·3	101·9	100·0	103·8	91·4	98·9

* Fractions discarded.

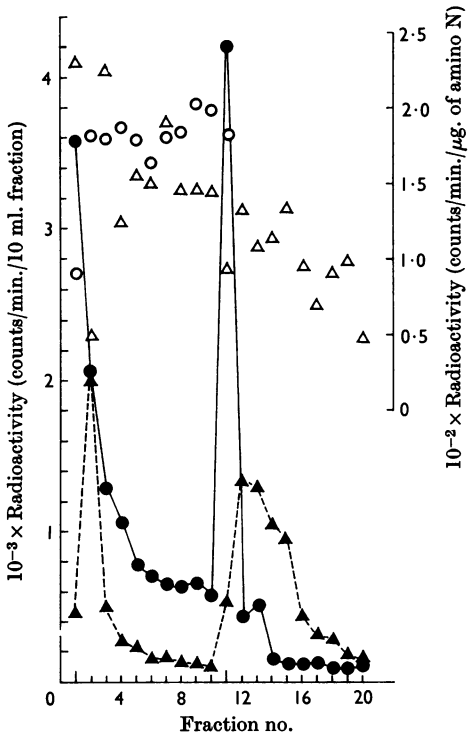


Fig. 1. Elution of ^{35}S-labelled compounds adsorbed on Dowex 2 (Cl^- form) was with 0·05 N-HCl or 0·1 N-acetic acid and 0·5 N-HCl. Fractions 1–10 were collected by elution with 0·1 N-acetic acid (▲) or 0·05 N-HCl (●) and then in each case fractions 11–20 with 0·5 N-HCl. The specific radioactivities (per μg. of amino N) of the fractions after the start of elution with 0·1 N-acetic acid (△) or 0·05 N-HCl (○) are also shown.

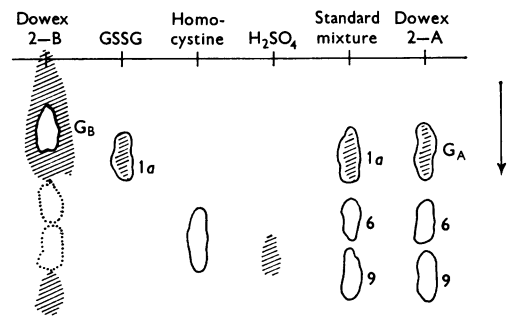


Fig. 2. Separation of ^{35}S-labelled compounds of fractions Dowex 2–A and Dowex 2–B. Chromatograms were developed with the propionic acid system (solvent 1b). The standard mixture contained: 1*a*, GSSG; 6, aspartate; 9, glutamate. The solid line shows the main ninhydrin-positive spot and the broken line shows trace amounts of ninhydrin-positive compound. Hatched areas represent u.v.-absorbing material detected before ninhydrin spray.

the elution of the column was begun with 0·05 N-hydrochloric acid; tube 1 gave a specific radioactivity that was much lower than that of any other tube. The values showed considerable variation when the elution was started with 0·1 N-acetic acid. These results indicated the heterogeneity of sulphur compounds adsorbed on Dowex 2.

Fraction Dowex 2–A [glutathione (GSH or GSSG), mixed disulphides, cysteinesulphinic acid]. In view of the above findings it was decided to begin elution of Dowex 2 with 0·1 N-acetic acid. Known volumes from tubes 1–10 were pooled, evaporated to dryness and chromatographed on paper with the propionic acid system (solvent 1b). About 68% of the ^{35}S in this fraction was recovered in the ninhydrin-positive spot G_A, which had the same R_F as GSSG (Fig. 2). The spot from a parallel chromatographic strip that was not sprayed with ninhydrin also gave the same recovery of isotope. Fraction Dowex 2–A appeared to contain GSSG as the main radioactive sulphur compound. The following observations gave further evidence for its identification:

(i) The mean specific radioactivities (counts/min./μg. of amino N) of tubes 1–6 after elution with 0·1 N-acetic acid were respectively 133, 204, 175, 159, 111 and 169. These values may be compared with 209 for compound G_A isolated after paper chromatography (Fig. 2).

(ii) One-dimensional paper chromatography of fraction Dowex 2–A with different solvent systems showed that the R_F of the major radioactive spot G_A and that of GSSG were similar. In the two-dimensional paper chromatography the major radioactive spot G_A was located very nearly in the position of GSSG.

(iii) Performic acid oxidation of fraction Dowex 2–A decreased the mobility of spot G_A, which behaved similarly to GSSG after oxidation under the same conditions (Fig. 3). Other weakly ninhydrin-positive spots 17 and 18 were also radioactive (Fig. 3).

(iv) The spot G_A, like GSH or GSSG (100–200 μg.), showed absorption of u.v. light before the chromatogram was sprayed with ninhydrin.

(v) A pure sample of glutathione at neutral or alkaline pH was adsorbed on Dowex 2 (Cl^- form). Elution of the resin with 0·1 N-acetic acid, 0·05 N-hydrochloric acid or 0·1 N-hydrochloric acid gave a complete recovery of glutathione in the first 30–40 ml. fraction.

(vi) When the fraction Dowex 2–A was rechromatographed on a Dowex 1 (CO_3^{2-} form) column, and the compounds were eluted with 0·1 N-acetic acid (fractions Dowex 1–A and 1–D) and finally with 50 ml. of 0·5 N-hydrochloric acid (fraction

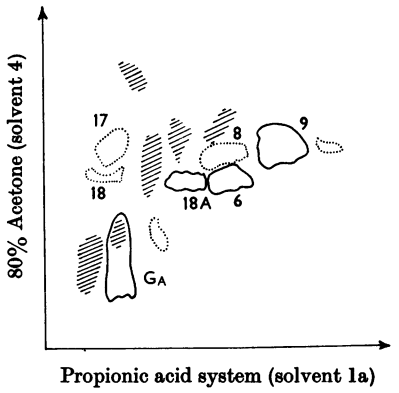


Fig. 3. Two-dimensional separation of ^{35}S-labelled compound G_A after performic acid oxidation of fraction Dowex 2–A. Spot G_A overlapped with glutathionesulphonic acid on treatment with performic acid; 6, aspartate; 8, glycine; 9, glutamate; 17, homocysteic acid; 18, cysteic acid; 18A, cysteinesulphinic acid (marker).

Dowex 1–E), the distribution of ^{35}S in the fractions was as follows: unadsorbed (wash), 4%; Dowex 1–A, 9%; Dowex 1–B, 12%; Dowex 1–C, 4%; Dowex 1–D, 41%; Dowex 1–E, 6%; the total recovery was 76%. Normally this kind of chromatography of a mixture of aspartate, glutamate and glutathione results in the elution of glutamate and aspartate in fraction Dowex 1–B and glutathione in fraction Dowex 1–D. These results provide evidence that glutathione was one of the main ^{35}S-labelled compounds present in the original fraction Dowex 2–A.

It appeared that spot G_A was most likely a mixture of GSSG and a disulphide of GSH and cysteine. The presence of the disulphide in liver extracts was shown previously by Tallan, Moore & Stein (1954). To test this possibility a solution of GSH (5 μmoles) and cysteine (7 μmoles) was mixed with 4ml. of ice-cold 6% perchloric acid. The neutralized solution prepared as described for tissue extracts was then transferred to a Dowex 2 (Cl^- form) column, which was eluted with 0·1N-acetic acid and 0·5N-hydrochloric acid as in Fig. 1. Paper chromatography in 80% phenol (Livermore & Muecke, 1954) of the eluate obtained with 0·1N-acetic acid (fraction Dowex 2–A) suggested that all the GSH was present as a disulphide with cysteine. The R_F values were as follows: cystine, 0·11; GSSG, 0·11; disulphide of cysteine and GSH, 0·18; GSH, 0·41. Performic acid treatment provided evidence for the presence of glutathionesulphonic acid (R_F 0·34) and cysteic acid (R_F 0·42) in the ethanol system (solvent 5).

The presence of spots 17 and 18 (Fig. 3) containing ^{35}S with R_F values corresponding to homocysteic acid and cysteic acid respectively was consistent with the presence in liver extracts of mixed disulphides of GSH + cysteine and GSH + homocysteine.

Observation (vi) indicated that there were other ^{35}S-labelled compounds in fraction Dowex 2–A. A pure sample of cysteinesulphinic acid behaved like glutathione on Dowex 2 (Cl^- form) and on Dowex 1 (CO_3^{2-} form), but unlike glutathione it was not adsorbed on a cation-exchange resin (Zeo-Karb 225, H^+ form).

Fraction Dowex 2–B (cysteic acid, homocysteic acid, sulphate, adenosine phosphate sulphatophosphate). The elution curve in Fig. 1 showed that the ^{35}S-labelled compounds in this fraction were much more firmly bound to Dowex 2 than those in fraction Dowex 2–A. The ^{35}S-labelled compounds in fraction Dowex 2–B were not eluted with 0·1N-acetic acid, but were partially eluted with 0·05N- or 0·1N-hydrochloric acid and were almost quantitatively eluted with 20–30ml. of 0·5N-hydrochloric acid. At first it was thought that the ^{35}S-labelled compound might be *S*-adenosylhomocysteine since this has been isolated previously from an anion-exchange column (Dowex 1; formate form) (Duerre, 1962). But the paper and ion-exchange chromatographic behaviour of a pure sample of *S*-adenosylhomocysteine ruled out its presence in this fraction. The following observations were made during a study of the nature of the ^{35}S-labelled compounds in this fraction:

(i) The specific radioactivity (counts/min./μg. of amino N) of this fraction (tube 11) was similar to that of fraction Dowex 2–A (tubes 2–10; Fig. 1).

(ii) Eluates of tubes 11–12 were pooled, evaporated to dryness and portions were taken for paper chromatography: (*a*) The use of the propionic acid system (solvent 1b) showed a number of spots that absorbed u.v. light. When sprayed with ninhydrin it showed the presence of a single prominent purple spot G_B overlapping the ultraviolet-light-absorbing area (Fig. 2). The compound G_B had a mobility somewhat less than that of GSSG and contained most of the radioactivity of the original sample (Fig. 4). (*b*) The chromatograms developed in an ammonia system (solvent 8) when dipped in iodoplatinate reagent gave after some hours a positive reaction showing a white spot against a brown background. Subsequent spray with ninhydrin showed a ninhydrin spot in the same place after 24hr. and it contained most of the radioactivity. (*c*) The R_F of the ninhydrin-positive ^{35}S-labelled compound in 80% acetone was 0·51; it decreased to 0·33 when this solvent was used in the second direction after prior development with acetic acid system (solvent 2a) in the first direction. Elution of the radioactive spot and repeated

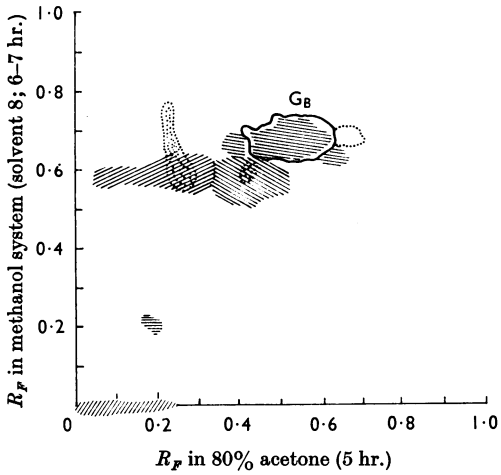


Fig. 4. Two-dimensional separation of ^{35}S-labelled compound G_B from fraction Dowex 2–B. Key to spots is as given in Fig. 2. Stippled areas also contained ^{35}S.

chromatography with 80% acetone further reduced its mobility.

(iii) After removal of free hydrochloric acid, or directly in the presence of 0·5N-hydrochloric acid, most of the ^{35}S may be adsorbed on a cation-exchange resin (Zeo-Karb 225, H^+ form). About 18% of the ^{35}S passed unhindered through the column; of the remainder only 40% was eluted with 10vol. of N-ammonia and 32% with 3vol. of 6N-ammonia solution. In this respect the ^{35}S-labelled compound in fraction Dowex 2–B, unlike that in fraction Dowex 2–A, was more firmly bound to the cation-exchange resin. The ammonia eluates did not show the presence of compounds with an absorption maximum at 260mμ when scanned with a Unicam SP. 700 spectrophotometer.

(iv) When the fraction Dowex 2–B was rechromatographed on Dowex 1 (CO_3^{2-} form) as described above in (vi) for fraction Dowex 2–A, the distribution of ^{35}S in the fractions was as follows: unadsorbed (wash), 1%; Dowex 1–A, 6%; Dowex 1–B, 7%; Dowex 1–C, 2%; Dowex 1–D, 40%; Dowex 1–E, 11%; the total recovery was 67%. The specific radioactivity of compounds in fraction Dowex 1–D was 119counts/min./μg. of amino N, which was identical with that obtained from pooled eluates of tubes 3–10 (part of fraction Dowex 2–A; see Fig. 1) when chromatographed under the same conditions. Two-dimensional paper chromatography of fraction Dowex 1–D (described above) with the propionic acid system (solvent 1a) in the first direction and 80% acetone in the second direction gave a ^{35}S-containing ninhydrin-positive spot in the position very nearly that occupied by GSSG. It had R_F 0·24 in the ammonia system (solvent 8) and R_F 0·48 in the ethanol system (solvent 5); it gave a positive reaction with iodoplatinate reagent.

The nature of the compound G_B is now under investigation.

The observations reported above also showed that there were other ^{35}S-labelled compounds in fraction Dowex 2–B. The most likely sulphur compounds to occur in this fraction are cysteic acid, homocysteic acid, inorganic sulphate (and probably adenosine phosphate sulphatophosphate). Under the conditions used the two amino acids and sulphate may be adsorbed on Dowex 2 (Cl^- form) and subsequently eluted with 0·5N-hydrochloric acid in fraction Dowex 2–B. Further, all these compounds, unlike GSH, and the disulphide of GSH and cysteine, passed unhindered through a column of cation-exchange resin, as described in observation (iii) above. Hence about 17% of the amino N of the original fraction Dowex 2–B might be considered attributable to cysteic acid or homocysteic acid or both.

Cysteic acid and homocysteic acid adsorbed on Dowex 2 (Cl^- form) were also eluted directly with 0·05N-hydrochloric acid but not with 0·1N-acetic acid. It is therefore likely that a considerable amount of other strongly adsorbed ^{35}S-labelled compounds, such as compound G_B, was also eluted with 0·05N-hydrochloric acid (see Fig. 1).

Ion-exchange chromatography on Dowex 1 (CO_3^{2-} *form*)

The sample containing compounds not adsorbed on Dowex 2 (Cl^- form) was passed through Dowex 1 (CO_3^{2-} form) and the compounds adsorbed were eluted first with 0·1N-acetic acid (fractions Dowex 1–A and 1–B) and subsequently with 0·1N-hydrochloric acid (fractions Dowex 1–C and 1–D).

Fraction Dowex 1–*A* (*all neutral amino acids including those containing sulphur,* S-*adenosylhomocysteine,* S-*ribosylhomocysteine, methylthioadenosine*). The neutral sulphur amino acids such as methionine, cyst(e)ine, homocyst(e)ine, cystathionine, their sulphoxides and sulphones, taurine and hypotaurine were eluted in this fraction if originally present in the perchloric acid extract. In addition to these sulphur amino acids other neutral amino acids such as glycine, alanine, glutamine, γ-aminobutyrate etc. were also present in this fraction. Oxidation of the fraction obtained from perchloric acid liver extracts with hydrogen peroxide or performic acid showed the presence of only taurine among sulphur-containing amino acids on two-dimensional paper chromatograms: cysteic acid, homocysteic acid, sulphoxides and/or sulphones of methionine and cystathionine were not detectable, suggesting the absence of detectable

amounts of cyst(e)ine, homocyst(e)ine, methionine and cystathionine in liver. The paper chromatograms prepared under similar conditions from brain extracts gave evidence for the presence of cystathionine sulphoxide (sulphone) and taurine: other above-mentioned sulphur amino acids were not detectable. These results extend and confirm previous observations (Gaull & Gaitonde, 1967).

The solution (60–70ml.) of the neutral amino acids (fraction Dowex 1–A) was treated with 0·5ml. of hydrogen peroxide overnight in the cold room and transferred directly to another Dowex 1 (CO_3^{2-} form) column. After the passage of the sample and washing of the column with water, fractions Dowex 1–A′, Dowex 1–B′, Dowex 1–C′ and Dowex 1–D′ were collected. Fractions Dowex 1–B′ and Dowex 1–C′ contained negligible amounts of ^{35}S and therefore they were discarded. The recovery of ^{35}S in fractions Dowex 1–A′ and Dowex 1–D′ was more than 90%; fraction Dowex 1–D′ contained 5–7% of the total ^{35}S. The solution of fraction Dowex 1–A′ was dried and paper-chromatographed in two directions with propionic acid system (solvent 1a) and 80% acetone (solvent 4) and sprayed with ninhydrin. The main ^{35}S-labelled spots obtained with extracts from liver and brain were (Fig. 5) cystathionine sulphoxide (spot 1), taurine (spot 16), methionine sulphoxide (spot 10) and unidentified ^{35}S-labelled compounds in the area of spots 3, 7 and 8 and 11*a*. Subsequent experiments with pure samples of cystine and homocystine indicated that under these conditions no significant amounts of cysteic acid and homocysteic acid were detected.

The presence of ^{35}S reported previously in the position of histidine with the acetic acid system (solvent 2) and 80% acetone (solvent 4) (Gaull & Gaitonde, 1967) and again observed in this study in the position of spot 3 was attributed to the sulphoxide of *S*-adenosylhomocysteine present in fraction Dowex 1–A. This was supported from the following observations:

(i) A solution of *S*-adenosylhomocysteine was treated under the conditions used for extracting the tissue. The neutralized extract was passed through Dowex 2 (Cl^- form), which, after repeated washing with water, gave about 80% of the *S*-adenosylhomocysteine. *S*-Adenosylhomocysteine in the water washings was adsorbed by Dowex 1 (CO_3^{2-} form) from which it was eluted with 0·1 N-acetic acid in fraction Dowex 1–A (65%) and fraction Dowex 1–B (25%). The eluate (82ml.) was divided into two equal parts: (*a*) One half was evaporated to dryness and subjected to paper chromatography in the usual way (Fig. 5). On inspection in u.v. light two spots that absorbed u.v. light were detected; the main spot 4 and the minor spot 3 were both found to react with ninhydrin. The two spots were identified as (Fig. 5): spot 4, *S*-adenosylhomocysteine; spot 3, *S*-adenosylhomocysteine sulphoxide (see below). (*b*) The second half of the eluate was treated with 0·5ml. of hydrogen peroxide and the solution was left overnight in the cold room. It was then transferred directly on Dowex 1 (CO_3^{2-} form) and fractions Dowex 1–A′, Dowex 1–B′, Dowex 1–C′ and Dowex 1–D′ were collected. Fraction Dowex 1–A′ contained 76% and fraction Dowex 1–B′ contained 24% of the u.v.-absorbing material added to the column. Fractions Dowex 1–A′ and Dowex 1–B′ were evaporated to dryness (45–50°) separately and chromatographed on paper as in (*a*) above (Fig. 5). Both fractions Dowex 1–A′ and Dowex 1–B′ gave a single u.v.-absorbing spot in the position of spot 3 that also gave a purple spot with ninhydrin. The spot 3 was identified as *S*-adenosylhomocysteine sulphoxide. The presence of the sulphoxide in (*a*) may be attributed to the partial oxidation of *S*-adenosylhomocysteine either during the extraction procedure used (Duerre, 1962) or during paper chromatography with solvents containing butanol that was not redistilled to remove traces of peroxides. *S*-Ribosylhomocysteine sulphoxide (spot 17) was most likely derived from the labile *S*-adenosylhomocysteine sulphoxide (Duerre, 1962) during evaporation of the sample.

(ii) When *S*-adenosylhomocysteine was oxidized with periodate and rechromatographed on Dowex 1 (CO_3^{2-} form), the u.v.-absorbing material was elutable in fractions Dowex 1–A′ (42%) and Dowex 1–B′ (18%) but the amino N-positive material was

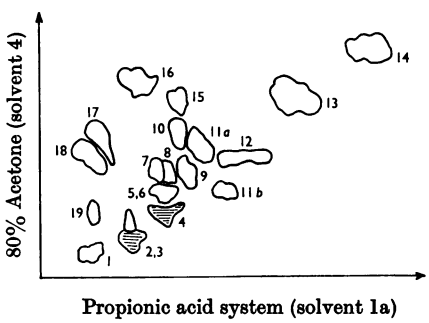


Fig. 5. Separation of amino acids of fraction Dowex 1–A′ by two-dimensional paper chromatography. Amino acids present in liver: 2, 7, 8, 10, 11*a*, 11*b*, 13, 14 and 16; amino acids present in brain: 1, 2, 3, 5, 7, 8, 11*a*, 11*b*, 12, 13, 14 and 16. Key to spots: 1, L-cystathionine sulphoxide and sulphone; 2, histidine; 3, *S*-adenosylhomocysteine sulphoxide; 4, *S*-adenosylhomocysteine; 5, glutamine; 6, aspartate; 7, serine, *S*-ribosylhomocysteine, 8, glycine; 9, glutamate; 10, methionine sulphoxide, hypotaurine, threonine; 11*a*, α-alanine; 11*b*, β-alanine; 12, γ-aminobutyrate; 13, methionine, valine; 14, leucine, isoleucine; 15, methionine sulphone; 16, taurine; 17, homocysteic acid; 18, cysteic acid; 19, *S*-ribosylhomocysteine sulphoxide.

eluted in fractions Dowex 1–C′ and Dowex 1–D′. One-dimensional paper chromatography in solvent systems 1a, 4 and 5 was used to identify the compounds in these fractions. Fractions Dowex 1–A′ and Dowex 1–B′ showed streaks of absorption of u.v. light; the R_F of these streaks was different from that of adenine or adenosine, which moved as discrete spots in these solvents. Fractions Dowex 1–C′ and Dowex 1–D′ showed the presence of homocysteic acid well separated from the u.v.-absorbing spots of iodate and periodate also present in these fractions. These findings suggested that the u.v.-positive streak in fractions Dowex 1–A′ and Dowex 1–B′ is probably the 2′,3′-dialdehyde of adenosine (Whitfeld & Markham, 1953) formed in the reaction. The oxidation of the neutral amino acids of fraction Dowex 1–A of liver with periodic acid and rechromatography on Dowex 1 (CO_3^{2-} form) gave about 81% of the ^{35}S in fraction Dowex 1–A′, 2% in Dowex 1–B′, 6% in Dowex 1–C′ and 9% in Dowex 1–D′, suggesting that about 15% of ^{35}S was in sulphonic acids derived from cyst(e)ine, homocyst(e)ine and *S*-adenosylhomocysteine.

(iii) Hydrolysis of *S*-adenosylhomocysteine (1 μmole) by refluxing with 50ml. of 2N-hydrochloric acid for 1hr. showed the presence of *S*-ribosylhomocysteine (spots 7 and 8; Fig. 5), homocystine, adenine and traces of a u.v.-absorbing compound that also reacted with ninhydrin. Oxidation of a portion of this sample with hydrogen peroxide on the paper chromatogram and subsequent development in the two directions showed the presence of *S*-ribosylhomocysteine sulphoxide (spot 19) and adenine.

With the ion-exchange procedure used, pure hypotaurine, another metabolite of methionine, was adsorbed on Dowex 1 (CO_3^{2-} form) and eluted in fraction Dowex 1–A. On two-dimensional paper chromatograms it migrated to the position of spots 7 and 8 (Fig. 5). The presence of ^{35}S in the position of spots 7 and 8 on paper chromatograms of rat liver and brain extracts may therefore be attributed mainly to hypotaurine and to small amounts of ^{35}S of *S*-ribosylhomocysteine.

Ion-exchange chromatography on Amberlite CG-50 and Zeo-Karb 225

The solution of compounds that passed unhindered through Dowex 2 (Cl^- form) and Dowex 1 (CO_3^{2-} form) contained a considerable amount of ^{35}S; this fraction contained all cations including amines, sulphur compounds such as *S*-adenosylmethionine, *S*-ribosylmethionine, ergothioneine and neutral compounds such as glucose and inositol. The following observations were made with regard to the nature of ^{35}S-labelled compounds in this solution:

Expt. 1. The solution was evaporated to dryness (60–70°) under reduced pressure. The residue was dissolved in water and transferred to a 10cm. column of Zeo-Karb 225 (H^+ form). After the column had been washed with water to remove unadsorbed ^{35}S, the ^{35}S-labelled compounds were eluted with 1N- and 2N-ammonia solution (Fig. 6). The overall recovery of ^{35}S was as follows: unadsorbed, 10%; N-ammonia eluate, 78%; 2N-ammonia eluate, 6%. The unadsorbed ^{35}S was probably due to methylthioribose since this fraction contained neutral compounds such as ribose, glucose and glycerol. More than 95% of the amino N adsorbed on the Zeo-Karb was eluted with N-ammonia solution.

The material eluted in tubes 4–7 (Fig. 6) giving an absorption maximum at 260mμ, ^{35}S and amino N may be attributed to the presence of *S*-adenosylmethionine. Two-dimensional paper chromatography (Fig. 7) showed the presence of three spots with radioactive areas, which after elution gave ^{35}S in spot 1 (methionine sulphoxide?), 25%, spot 2, 5%, and spot 3, 6%; the total recovery was 36%.

Expt. 2. The sample was hydrolysed with barium hydroxide at pH 12–13 for 5min. in a boiling-water bath. Under these conditions *S*-adenosylmethio-

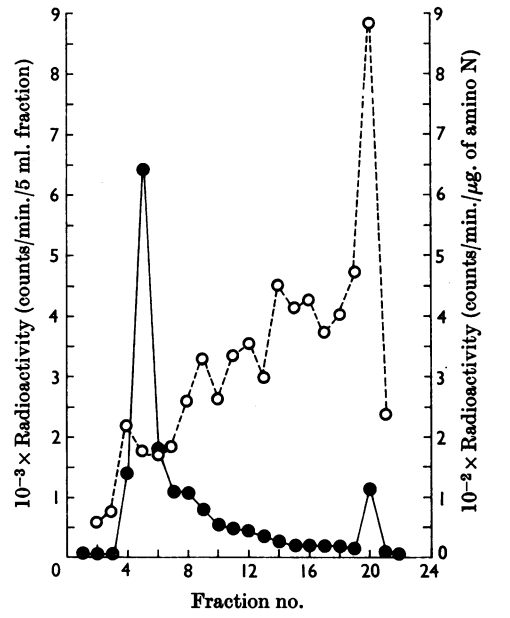


Fig. 6. Elution of ^{35}S-labelled compounds adsorbed on Zeo-Karb 225 (H^+ form) with aq. NH_3. Fractions 1–19 were collected by elution with N-NH_3 and fractions 20–22 with 2N-NH_3, alkaline reaction beginning at tube 4. The radioactivity in each fraction (●) and the specific radioactivities (○) (per μg. of amino N) are shown.

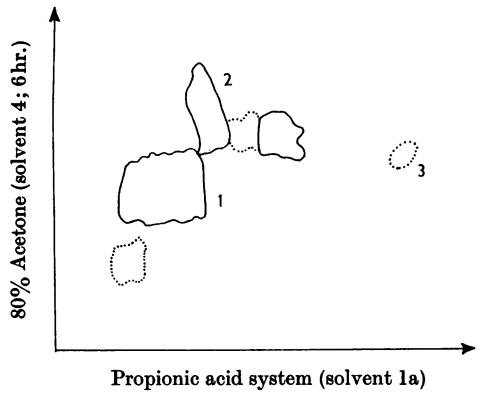


Fig. 7. Separation of ^{35}S-labelled compounds from the cationic fraction eluted from Zeo-Karb 225 with aq. NH_3. Spots shown were observed after spraying the chromatograms with ninhydrin. Spots 1, 2 and 3 contained ^{35}S.

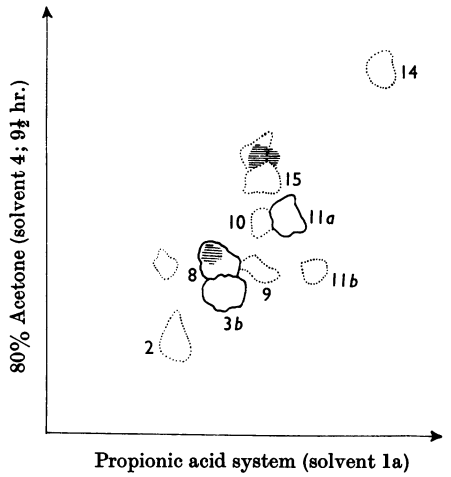


Fig. 8. Separation of ^{35}S-labelled compounds after $Ba(OH)_2$ hydrolysis of compounds in the Dowex 1 (CO_3^{2-} form) wash. The amino acids in the hydrolysate were separated after further ion exchange on Dowex 1 (CO_3^{2-} form) and oxidized with performic acid before paper chromatography. ^{35}S was found in the area of spot 8 (14%) and spot 9 (23%); amounts in the range 1–2% were measured in spots 3*b*, 14 and 15. Key to the spots is as given in Fig. 5.

nine and *S*-ribosylmethionine are hydrolysed mainly into free methionine (Schlenk & Ehninger, 1964; Parks & Schlenk, 1958). After removal of Ba^{2+} with sulphuric acid, the clear aqueous extract was rechromatographed on Dowex 1 (CO_3^{2-} form) column as described above. The distribution of ^{35}S in the fractions was as follows: unadsorbed > 32%; Dowex 1-A, 39; Dowex 1-B, 1%; Dowex 1-C, < 1%; and Dowex 1-D, 4%. This suggested that the hydrolysis of the ^{35}S-labelled compound was only partial, giving 39% as [^{35}S]methionine and other degradation products such as methylthioadenosine.

^{35}S-labelled compounds referred to as unadsorbed included *S*-ribosylmethionine or intact *S*-adenosylmethionine together with 5–6% of methylthioribose. Two-dimensional paper chromatography of the neutral fraction Dowex 1-A (Fig. 8) after performic acid oxidation gave ^{35}S in the position of spot 8 (glycine position), 14%; spot 9, 23%; spot 15 (methionine sulphone), 1·3%; spot 14 (leucine position), 2%; the total recovery after paper chromatography was 40%. The u.v.-absorbing compound in the position of spot 8 was probably the sulphoxide of methylthioadenosine, indicating that the hydrolysis with barium hydroxide had resulted also in the breakdown of *S*-adenosylmethionine into methylthioadenosine. The measurement of ^{35}S in the eluate of spots after the ninhydrin spray would result only in 15% loss of ^{35}S from methionine sulphone or sulphoxide (Gaull & Gaitonde, 1967). The losses (if any) of ^{35}S from the sulphoxide of methylthioadenosine under similar conditions are likely to be of the same order. It is therefore likely that losses of ^{35}S approaching 50% after paper chromatography are due to other unknown sulphur compounds formed during performic acid oxidation, during drying of the eluates of their spots after ninhydrin spray, or both.

In view of the heterogeneity of the ^{35}S-labelled compounds in the solution after Dowex 2 and Dowex 1 chromatography it was decided to obtain further fractionation with two types of cation-exchangers such as Amberlite CG-50 (H^+ form, weakly acidic) and Zeo-Karb 225 (H^+ form, strongly acidic). Therefore the solution was passed through the Amberlite and the solution of compounds unadsorbed was then allowed to pass through the Zeo-Karb. The compounds adsorbed on Amberlite were eluted with 0·05 N-hydrochloric acid and those adsorbed by Zeo-Karb were eluted with N-ammonia.

Amberlite CG-50 (S-adenosylmethionine, S-ribosylmethionine). The hydrochloric acid eluates (10ml. fractions) were collected in separate tubes and the eluate in each tube was assayed for ^{35}S, amino N and u.v. absorption. The first tube showed a large amount of amino N, a negligible amount of ^{35}S and u.v. absorption ($E_{max.}$ 278mμ). Amines such as histamine, 5-hydroxytryptamine ($E_{max.}$ 278mμ), ethanolamine, amino acids such as arginine and lysine, and cytidine ($E_{max.}$ 278mμ) when present in the original perchloric acid extract were also found in this fraction. The eluates in tubes 2 and 3 gave positive tests for amino N and ^{35}S, and had u.v. absorption ($E_{max.}$ 260mμ), which suggested the presence of *S*-adenosylmethionine in these two

tubes. These fractions (tubes 2 and 3) also gave positive tests for ribose.

To confirm these findings a solution of pure *S*-adenosylmethionine was treated with 6% perchloric acid. After neutralization in the cold the solution was passed through Dowex 2 (Cl^- form), Dowex 1 (CO_3^{2-} form) and Amberlite CG-50 (H^+ form). *S*-adenosylmethionine was not adsorbed by the first two resins but was quantitatively adsorbed by Amberlite, from which it was quantitatively eluted with 0·05N-hydrochloric acid. *S*-Ribosylmethionine behaved similarly under the same conditions. It was therefore convenient to assay for ribose for the determination of specific radioactivity of *S*-adenosylmethionine in the pooled acid eluate of Amberlite.

Zeo-Karb 225 (*amino acids?*, *ergothioneine*). A part of the radioactivity that was not retained by Amberlite was adsorbed on Zeo-Karb 225 (H^+ form), from which it was quantitatively eluted with 5vol. of N-ammonia solution. The eluates after removal of ammonia showed the presence of amino N-positive compounds in fractions also containing ^{35}S. The compounds could not be eluted with 0·1N-hydrochloric acid but were eluted easily with N-ammonia solution, beginning with the emergence of free ammonia from the Zeo-Karb. Paper chromatography of the residue obtained after removal of ammonia showed the presence of a number of amino acids, as indicated in Fig. 8. The ^{35}S-labelled compounds present in this fraction included traces of methionine, which was detected as methionine sulphone after performic acid oxidation. A simulated 'perchloric acid extract' of pure amino acids that occur in tissues was passed through the four-column assembly. The amino acids in this extract were completely adsorbed by the first three resins and the ammonia eluate of the fourth resin column (Zeo-Karb 225) showed no traces of free amino acids. In the light of these findings the reasons for the presence of amino acids in this fraction from perchloric acid extracts of rat tissues are not known.

Under the conditions of ion-exchange chromatography pure ergothioneine passed through Dowex 2 (Cl^- form), Dowex 1 (CO_3^{2-} form) and Amberlite CG-50 (H^+ form). In a serial assembly of these three resins a weak affinity of ergothioneine towards the resins was observed. Pure ergothioneine or ergothioneine added to perchloric acid extract of the tissue emerged after collection of 75–80ml. of water effluent, by which time the sample volume was completely displaced from the column assembly. The recovery of ergothioneine was about 80% of that transferred to the column assembly. The procedure eliminates a number of compounds that apparently interfere in the determination of ergothioneine in extracts of tissues contaminated with blood (Hunter, 1949; Melville & Lubschez, 1953; Melville, Horner & Lubschez, 1954). Ergothioneine in the water effluent may be quantitatively adsorbed on Zeo-Karb 225 (H^+ form), from which it may be eluted with 5vol. of N-ammonia solution. The recovery of ergothioneine from Zeo-Karb was not always reproducible, and further the compound eluted with ammonia solution gave a much lower recovery on reaction with diazotized sulphanilic acid than when analysed by u.v. spectroscopy.

In view of the small volume (75ml.) of water used to wash out unadsorbed compounds, it is unlikely that ergothioneine if present in the original perchloric acid extract had passed through Dowex 2 (Cl^- form), Dowex 1 (CO_3^{2-} form) and Amberlite CG-50 (H^+ form) to be adsorbed on Zeo-Karb 225.

DISCUSSION

The stability of sulphur amino acids in solution depends on the pH: (i) *S*-Adenosylmethionine, although very labile at any pH, is most stable to storage at about pH 2·0 (see Schlenk, 1965). This is also true of methionyladenylate (Moldave, Castelfranco & Meister, 1959; Berg, 1958). (ii) *S*-Adenosylhomocysteine is stable over a wide pH range, but it is hydrolysed slowly at acid pH; hence it is preferable to handle it in alkaline solution (Duerre, 1962; Schlenk, 1965). (iii) In general, thio ethers such as methionine and cystathionine are more stable at alkaline pH than at acid pH. (iv) Cysteine and homocysteine are more stable at acid pH; they are unstable at alkaline pH. Therefore one of the main considerations in the isolation and subsequent determination of sulphur amino acids in tissue extracts should be the conditions used for extraction of the tissue. It is common practice to extract tissues with ice-cold trichloroacetic acid, ice-cold perchloric acid or picric acid. After the preliminary centrifugation and filtration of the suspension, trichloroacetic acid is removed by its repeated extraction with ether or by ion exchange on Dowex 2, picric acid by ion exchange and perchloric acid by neutralization in the cold with potassium hydroxide.

In the present studies four types of ion-exchange columns were used to separate sulphur compounds according to their charge. The perchloric acid extract (pH 7·0–7·5) was passed through a column assembly in the order: Dowex 2 (Cl^- form), Dowex 1 (CO_3^{2-} form), Amberlite CG-50 (H^+ form) and Zeo-Karb 225 (H^+ form). The types of sulphur compounds and other compounds that may be separated and subsequently eluted are recorded in Table 2. The following modifications of the procedure may also be used: (i) Tissue extracts maintained at an acid pH after careful removal (below

Table 2. *Ion-exchange procedure for the group separation of sulphur compounds and other amino acids*

Sulphur compounds tested are designated *a*; sulphur compounds not tested but expected to behave as in *a* are designated *b*; other non-sulphur compounds tested are designated *c*. The individual members of the groups are as follows: 1*a*, GSH, GSSG, cysteinesulphinic acid, mixed disulphides (e.g. of cysteine and GSH); 1*b*, homoglutathione, *S*-alkylglutathione, (ophthalmic acid); 1*c*, aspartate, glutamate; 2*a*, cysteic acid, homocysteic acid, sulphate, unknown sulphur compound(s); 2*b*, adenosine phosphate sulphatophosphate, β-mercaptopyruvate, β-sulphinylpyruvate, γ-methylthio-α-oxobutyrate, AMP-2′(3′)-amino acid esters; 3*a*, methionine, cystathionine, cyst(e)ine, homocyst(e)ine, *S*-adenosylhomocysteine, *S*-ribosylhomocysteine, their sulphoxides and sulphones, hypotaurine, taurine; 3*b*, *S*-alkylcysteine and its sulphoxide; 3*c*, neutral amino acids, e.g. alanine, serine, histidine, glutamine, γ-aminobutyrate and others; 4*a*, *S*-adenosylmethionine, *S*-ribosylmethionine; 4*c*, basic amino acids lysine, arginine, and amines such as histamine, glucosamine, ethanolamine and 5-hydroxytryptamine; 5*a*, unknown cationic sulphur compounds, ergothioneine.

Type of resin	Solvents for eluting columns separately	Fraction	Group of compounds	
			Four-column assembly	One column only*
Dowex 2 (Cl^- form) (20 cm.)	0·1 N-Acetic acid (50 ml.)	Dowex 2–A	1*a*, 1*b*, 1*c*	1*a*, 1*b*, 1*c*
	0·5 N-HCl (50 ml.)	Dowex 2–B	2*a*, 2*b*	2*a*, 2*b*
Dowex 1 (CO_3^{2-} form) (20 cm.)	0·1 N-Acetic acid (150 ml.)	Dowex 1–A (neutral)	3*a*, 3*b*, 3*c*	3*a*, 3*b*, 3*c*
		Dowex 1–B (acetic acid)	—	1*c*
	0·1 N-HCl (150 ml.)	Dowex 1–C (acetic acid)	—	—
		Dowex 1–D (HCl)	—	1*a*, 1*b*, 2*a*, 2*b*
Amberlite CG-50 (H^+ form) (10 cm.)	0·05 N-HCl (50 ml.)	Amberlite	4*a*, 4*c*	
Zeo-Karb 225 (H^+ form) (10 cm.)	N-NH_3 (100 ml.)	Zeo-Karb	5*a*	1*a*†, 1*b*, 1*c*, 3*a*‡, 3*b*, 3*c*, 4*a*, 4*c*, 5*a*

* Group of compounds not listed would pass unhindered through the column and be collected in the wash.
† Except cysteinesulphinic acid.
‡ Except taurine.

pH 7·0) of the excess of acid precipitant may be put directly on the assembly of four columns as described in this work. The compounds normally present in fraction Dowex 2–A would be washed out and retained by Dowex 1 (CO_3^{2-} form). The elution of Dowex 1 in the usual way gave aspartate and glutamate in fraction Dowex 1–B and glutathione and cysteinesulphinic acid in fraction Dowex 1–D. The compounds obtained as fraction Dowex 2–B would still be retained by Dowex 2 and their elution should follow as before, with 0·5 N-hydrochloric acid. (ii) Under some conditions the introduction of the Dowex 2 (Cl^- form) column is not necessary since the ^{35}S-labelled compounds adsorbed on this column can also be adsorbed by Dowex 1 (CO_3^{2-} form). Under these conditions compounds previously obtained as Dowex 2–B would be eluted in fraction Dowex 1–D and thus increase the complexity of this fraction. In either of the modifications due allowance must be made for ion exchange by increasing the length of Dowex resins (25–30 cm./g. of fresh tissue extract).

The Dowex 2 column was used primarily in the early stages for rapid elution of sulphur compounds from the resin. The retention of aspartate, glutamate and glutathione on Dowex 2 (Cl^- form) observed in the present work was possible since the tissue extracts used had pH above 7. However, it is known that, when tissue extracts used are acid, these compounds together with other amino acids pass unhindered through the column (Stein & Moore, 1954). If Dowex 2 is used, the compounds adsorbed on Dowex 1 may preferably be eluted directly with 0·1 N-hydrochloric acid rather than with 0·1 N-acetic acid, since fractions Dowex 1–B, Dowex 1–C and Dowex 1–D eluted as described in this work did not contain significant amounts of ^{35}S (Table 1). Also, some overlapping of the neutral fraction Dowex 1–A with the next acidic fraction may be allowed for convenience of its rapid isolation: the elution of Dowex 1 may be then terminated.

One of the main features of the procedure is that the method gives a preliminary group separation (Table 2) of a variety of sulphur compounds that may be isolated after paper chromatography. This procedure gave quantitative recovery of ^{35}S, showing that labelled sulphur compounds present in neutralized perchloric acid extracts of rat brain and liver were not volatile at 20–60°. The results also showed that sulphur compounds in extracts (pH 7·0–7·5) stored at −10° to −15° were not

extensively decomposed. Several of the sulphur compounds mentioned below were separated and isolated from the same sample of rat brain and liver extracts. The R_F values of sulphur compounds in a few solvent systems used are given in Table 3.

S-*Adenosylmethionine.* The presence of this

Table 3. *R_F values of sulphur amino acids and other relevant compounds*

All R_F values were obtained in solvent systems without pre-equilibration of the paper in the chromatographic tank. Owing to the rapid change of solvent composition the R_F values in solvent system 4 (acetone) were considerably less than those reported if chromatograms were irrigated for 4–5 hr. Compounds containing amino N were detected by spraying with ninhydrin; sulphate was located by dipping the paper in benzidine reagent; compounds containing adenine were located in u.v. light; compounds containing ribose were tested for the presence of pentose by the method of Albaum & Umbreit (1947). Non-sulphur compounds are *italicized.*

Compound / Solvent system ...	$100 \times R_F$ 1*a* (propionic acid)	2*b* (acetic acid)	4 (acetone)	5 (ethanol)
Glutathione (reduced)	35	30	39	56
Glutathione (oxidized)	13	12	28	38
Glutathionesulphonic acid*	13	11	33	56
Cysteinesulphinic acid	16	18	41	44
Cysteic acid	12	12	46	42
Homocysteic acid	12	14	52	41
Sulphate (free acid)	43	46	73	79
Aspartate	25	27	34	50
Glutamate	36	34	40	60
Methionine	58	56	53	66
Methionine sulphoxide	34	22	38	55
Methionine sulphone	34	24	52	56
L-Cystathionine	18	12	20	29
L-Cystathionine sulphoxide*	13	7	17	17
L-Cystathionine sulphone*	13	6	18	17
Cysteine†	17	41	67 (12, 36)	58 (23)
Cystine	12	10	18	33
Cystine disulphoxide	21	17	41	17
S-Methylcysteine	23	44	58	39
Homocysteine†	50	48	72 (39)	62 (34)
Homocystine	28	24	35	40
S-Adenosylhomocysteine	36	21	29	33
S-Adenosylhomocysteine sulphoxide*	18	9	27	31
S-Ribosylhomocysteine‡	25	24	38	53
S-Ribosylhomocysteine sulphoxide*‡	16	9	35	44
Taurine	21	16	59	60
Hypotaurine	28	22	49	54
Methylthioadenosine	80	70	72	67
Methylthioadenosine sulphoxide*	57	40	56	57
Homoserine	31	30	48	58
Histidine	19	12	29	35
Adenine	70	56	47	58
S-Adenosylmethionine‡	17	8	7	25
S-Ribosylmethionine		10§		35§
Methylthioribose‡	71	68	82	79
Methylthioribose sulphoxide*‡	42	38	60	73
Ribose‡	38	37	62	76

* The sulphoxide was prepared by treatment with H_2O_2 or periodic acid and the sulphone or sulphonic acid was prepared by treatment with performic acid (see Gaull & Gaitonde, 1967).

† Freshly prepared solutions gave more than one spot on paper chromatograms. Their R_F values are given in parentheses. Also, homocysteine in solvent system 1a gave additional spots of R_F 0·29, 0·35 and 0·43 and a streak of R_F 0·61.

‡ Located with $AgNO_3$ reagent (Trevelyan, Procter & Harrison, 1950).

§ Parks & Schlenk (1958).

compound in rat brain was indicated in previous studies (Gaitonde, 1961*b*). Recently its concentration in rat tissues was estimated indirectly by an isotope-dilution technique involving the combined use of *S*-adenosyl[*Me*-^{14}C]methionine, *N*[^{3}H]-acetylserotonin and *O*-methyltransferase (Baldessarini & Kopin, 1963). In the present studies this compound was isolated from rat brain and liver. *S*-Ribosylmethionine, if present as a breakdown product of *S*-adenosylmethionine in the perchloric acid extract, was also retained on Amberlite CG-50 and eluted together with *S*-adenosylmethionine. The procedure outlined gave a quick method for estimating the specific radioactivity of *S*-adenosylmethionine. The quantitative assay of the compound was based on the ribose determination since: (i) it takes into account both *S*-adenosyl- and *S*-ribosyl-methionine; (ii) free ribose may be used as a standard for the determination of *S*-adenosyl- and *S*-ribosyl-methionine (Baddiley, Cantoni & Jamieson, 1953; Parks & Schlenk, 1958); (iii) except for cytidine other ribose-containing tissue constituents such as nucleosides and nucleotides are removed by adsorption on Dowex 2 (Cl^- form) and Dowex 1 (CO_3^{2-} form); cytidine is adsorbed on Amberlite and may be eluted with 0·05N-hydrochloric acid together with *S*-adenosylmethionine, but it does not react in the orcinol reaction for ribose; (iv) it is more sensitive than u.v. absorption at 260mμ; (v) cytidine and other u.v.-absorbing compounds such as 5-hydroxytryptamine ($E_{max.}$ 278mμ) present in this fraction make measurements at 260mμ unreliable. From the ribose determination, the *S*-adenosylmethionine concentration (mμmoles/g. $\pm$ S.D.) of rat brain was 31·4 $\pm$ 3 and that of liver was 83·6 $\pm$ 6. These values are comparable with 30·0 and 78·0mμmoles of *S*-adenosylmethionine/g. reported for rat brain and liver respectively by Baldessarini & Kopin (1963, 1964).

S-*Adenosylhomocysteine*. The presence of this compound in tissue extracts was indicated in a previous paper (Gaull & Gaitonde, 1967). Further observations made in this study gave evidence of its occurrence in tissue extracts. It is now possible to isolate it in fraction Dowex 1–A and separate it from other sulphur amino acids by paper chromatography (Fig. 5). Even in the absence of treatment with hydrogen peroxide, a considerable proportion of *S*-adenosylhomocysteine, under the conditions used, was oxidized to the corresponding sulphoxide, which partially decomposed to the sulphoxide of ribosylhomocysteine (Fig. 5). By employing conditions such as the use of thiodiglycol (Duerre, 1962) during the preparation of the sample for paper chromatography and by the use of peroxide-free solvents during development in the first direction, to prevent oxidation of *S*-adenosylhomocysteine to its sulphoxide, it is possible to obtain *S*-adenosylhomocysteine. This may also be isolated as its sulphoxide by using a solvent system containing hydrogen peroxide in the second direction. The sulphoxide moves slower than the parent compound and was well separated from the neighbouring compounds (see Fig. 5). Alternatively, *S*-adenosylhomocysteine may be isolated directly from fraction Dowex 1–A on its adsorption on a formate column at pH 10·0 as described by Duerre (1962).

Sulphur amino acids. The presence of various other sulphur compounds such as methionine, cystathionine, cyst(e)ine, homocyst(e)ine and taurine has been reported in the literature at various times. In the present work all these sulphur amino acids were obtained in the neutral amino acid fraction Dowex 1–A. The results obtained in this work and those reported previously (Gaull & Gaitonde, 1967) showed that the amounts of methionine, cyst(e)ine and homocyst(e)ine were too small for detection as ninhydrin-positive spots on paper chromatograms prepared from either rat brain or rat liver extracts.

The results therefore suggest that most of the methionine in rat tissues is present as *S*-adenosylmethionine and also probably as methionyladenylate, which, depending on the methods used for their isolation, are hydrolysed to methionine or decomposed to methylthioadenosine (Cantoni, 1953; Baddiley *et al.* 1953; Schlenk & DePalma, 1957; Berg, 1958; Moldave *et al.* 1959; Schlenk & Ehninger, 1964).

Cyst(e)ine and homocyst(e)ine were not found in measurable amounts. The presence of ^{35}S in a fraction containing cysteic acid and homocysteic acid obtained after peroxide or periodate oxidation indicated that they were present in fraction Dowex 1–A. A considerable amount of free cysteine and homocysteine in the neutralized perchloric acid extract was in the form of a mixed disulphide with GSH. Paper-chromatographic evidence obtained in this work for the presence of such a disulphide in the glutathione fraction (fraction Dowex 2–A; Fig. 3) of rat liver and brain, and its formation when a mixture of cysteine and GSH was subjected to the procedure for the preparation of tissue extracts, is in agreement with the findings of previous workers (Wikberg, 1953; Livermore & Muecke, 1954; Tallan *et al.* 1954; Frimpter, 1961). The presence of such mixed disulphides in tissue extracts raises the question whether they are present *in vivo* or formed *in vitro* as artifacts.

Cystathionine was found in measurable amounts on paper chromatograms obtained from rat brain; it was not detectable on paper chromatograms prepared from 1g. equivalent extracts of rat liver after ninhydrin spray, but ^{35}S assay after addition of carrier cystathionine before paper chromatography showed that it was formed *in vivo* in liver. In view of

the possible presence of *S*-adenosylhomocysteine sulphoxide in the same region of the paper chromatogram (Fig. 5), the ^{35}S content of cystathionine sulphoxide in rat liver should be assayed after chromatography with carrier cystathionine sulphoxide.

Taurine was identified as a discrete spot in a unique position on paper chromatograms from both rat liver and brain (Fig. 5). Hypotaurine has been shown to be an intermediate in the conversion of cysteine into taurine in rat liver and brain (Chatagner & Bergeret, 1951; Awapara, 1953). If present in tissue extracts prepared in the present studies, hypotaurine would be obtained in fraction Dowex 1-A, and on paper chromatograms its position was in the area occupied by serine and glycine (spots 7 and 8; Fig. 5). A part of the ^{35}S of this region of the chromatogram might be attributed to hypotaurine of rat liver and brain.

Glutathione. This was easily separated as fraction Dowex 2-A in the first step of ion-exchange chromatography on Dowex 2 (Cl^- form). Mixed disulphides of GSH with cysteine or homocysteine were also found in this fraction (Fig. 3). Cysteinesulphinic acid, which is considered to be an intermediate in the oxidation of cysteine to taurine (Awapara, 1953; Bergeret & Chatagner, 1954*a*,*b*) and sulphate, is likely to be another sulphur amino acid eluted together with glutathione, but paper chromatography in solvent 1a or 1b would resolve it from glutathione (Fig. 3) and also from aspartate and glutamate present in the fraction (Fig. 2). Although the presence of cysteinesulphinic acid was indicated separate studies were not undertaken in this work to obtain unequivocal evidence for its presence.

Cysteic acid and homocysteic acid. These amino acids may be separated as fraction Dowex 2-B in the first stage of ion-exchange chromatography: they may be eluted with 0·5N-hydrochloric acid from the Dowex 2 column after elution of glutathione (fraction Dowex 2-A) with 0·1N-acetic acid. Alternatively, they may also be eluted directly with 0·05N-hydrochloric acid together with glutathione, cysteinesulphinic acid, aspartate and glutamate. The presence of cysteic acid and homocysteic acid in tissue extracts was not studied here although the methods may be successfully applied for this purpose. Sulphate and probably adenosine phosphate sulphatophosphate ('active sulphate') may also be obtained in this fraction. The highly radioactive unidentified sulphur compound G_B present in fraction Dowex 2-B was removed by its adsorption on a cation-exchange resin (Zeo-Karb 225): cysteic acid, homocysteic acid, sulphate (and adenosine phosphate sulphatophosphate) passed unhindered through this resin. One-dimensional paper chromatography in 80% acetone was used to resolve cysteic acid, homocysteic acid and sulphate. The presence of sulphate on paper chromatograms was revealed in u.v. light as a bright fluorescent spot after its precipitation with benzidine hydrochloride.

Ergothioneine. Ergothioneine concentrations of 130μg./g. in liver have been reported by Melville *et al.* (1954). Crossland, Woodruff & Mitchell (1964) have shown its presence in specific regions of rat brain. Under the conditions used in the present work, ergothioneine was not detectable in rat liver or brain either by u.v. spectroscopy or by coupling with diazotized sulphanilic acid (Hunter, 1951).

The group separation of sulphur compounds by ion-exchange chromatography described in the present studies showed that ergothioneine was not retained by Dowex 2 (Cl^- form), Dowex 1 (CO_3^{2-} form) and Amberlite CG-50 (H^+ form). It showed, however, some weak affinity towards these columns, but on continued washing with water 80% of the ergothioneine added to the tissue extract was recovered. Ergothioneine was adsorbed on a Zeo-Karb 225 (H^+ form) column: the results showed that this step should be avoided since its elution from the column gave low recoveries that were never reproducible.

Other sulphur compounds. No definite evidence for the presence of adenylates of methionine and cyst(e)ine was obtained in this work. In the present studies, the perchloric acid extracts (pH 7·0–7·5) were stored at $-10°$ to $-15°$ and it is not unlikely that a considerable proportion of the amino acid adenylates was decomposed (Berg, 1958; Moldave *et al.* 1959). Further, a part of these adenylates would also be converted into the amino acid esters, AMP-2′(3′)-methionine and AMP-2′(3′)-cyst(e)ine (Moldave *et al.* 1959). At alkaline pH these esters would behave as anions and be retained by Dowex 2 (Cl^- form). To what extent the adenylates of methionine and cyst(e)ine remained intact in the tissue extracts is not known.

Methylthioadenosine formed by decomposition of *S*-adenosylmethionine was probably present in small amounts. It may be isolated from fraction Dowex 1-A as the sulphoxide (Fig. 5). The presence of methylthioribose was indicated from negligible amounts of ^{35}S in the solution of compounds that emerged unadsorbed by the four resins used.

^{35}S-containing compounds that remained unidentified were obtained in fraction Dowex 2-B (compound G_B; Figs. 2 and 3) and in the fraction eluted from Zeo-Karb 225 with ammonia solution.

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REFERENCES

Albaum, H. G. & Umbreit, W. W. (1947). *J. biol. Chem.* **167**, 369.

Awapara, J. (1953). *J. biol. Chem.* **203**, 183.

Awapara, J. & Wingo, W. J. (1953). *J. biol. Chem.* **203**, 189.

Baddiley, J., Cantoni, G. L. & Jamieson, G. A. (1953). *J. chem. Soc.* p. 2662.

Baldessarini, R. J. & Kopin, I. J. (1963). *Analyt. Biochem.* **6**, 289.

Baldessarini, R. J. & Kopin, I. J. (1964). *Abstr. int. Conf. Neurochem.* p. 4. Oxford: Pergamon Press Ltd.

Berg, P. (1958). *J. biol. Chem.* **233**, 608.

Bergeret, B. & Chatagner, F. (1954*a*). *Biochim. biophys. Acta*, **14**, 297.

Bergeret, B. & Chatagner, F. (1954*b*). *Biochim. biophys. Acta*, **14**, 543.

Bergeret, B., Chatagner, F. & Fromageot, C. (1952). *Biochim. biophys. Acta*, **9**, 147.

Cantoni, G. L. (1953). *J. biol. Chem.* **204**, 403.

Cantoni, G. L. & Scarano, E. (1954). *J. Amer. chem. Soc.* **76**, 4744.

Carroll, W. R., Stacy, G. W. & du Vigneaud, V. (1949). *J. biol. Chem.* **180**, 375.

Chatagner, F. & Bergeret, B. (1951). *C. R. Acad. Sci., Paris*, **232**, 448.

Crossland, J., Woodruff, G. N. & Mitchell, J. F. (1964). *Nature, Lond.*, **203**, 1388.

Duerre, J. A. (1962). *Arch. Biochem. Biophys.* **96**, 70.

du Vigneaud, V. (1952). *A Trail of Research in Sulfur Chemistry and Metabolism and Related Fields*, p. 27. Ithaca: Cornell University Press.

Eldjarn, L., Pihl, A. & Sverdrup, A. (1956). *J. biol. Chem.* **223**, 353.

Frimpter, G. W. (1961). *J. biol. Chem.* **236**, PC51.

Fromageot, C. (1955). *Harvey Lect.* **49**, 1.

Gaitonde, M. K. (1961*a*). *J. Neurochem.* **8**, 234.

Gaitonde, M. K. (1961*b*). *Biochem. J.* **80**, 277.

Gaitonde, M. K. (1965). *Biochem. J.* **95**, 803.

Gaitonde, M. K., Dahl, D. R. & Elliott, K. A. C. (1965). *Biochem. J.* **94**, 345.

Gaitonde, M. K. & Richter, D. (1955). *Biochem. J.* **59**, 690.

Gaitonde, M. K. & Richter, D. (1957). In *Metabolism of the Nervous System*, p. 449. Ed. by Richter, D. Oxford: Pergamon Press Ltd.

Gaull, G. E. & Gaitonde, M. K. (1967). *Biochem. J.* **102**, 294.

Hoagland, M. B., Keller, E. B. & Zamecnik, P. C. (1956). *J. biol. Chem.* **218**, 345.

Hunter, G. (1949). *Canad. J. Res.* E, **27**, 230.

Hunter, G. (1951). *Biochem. J.* **48**, 265.

Kirby, K. S. (1955). *Biochim. biophys. Acta*, **18**, 575.

Livermore, A. H. & Muecke, E. C. (1954). *Nature, Lond.*, **173**, 265.

Mejbaum, W. (1939). *Hoppe-Seyl. Z.* **258**, 117.

Melville, D. B., Horner, W. H. & Lubschez, R. (1954). *J. biol. Chem.* **206**, 221.

Melville, D. B. & Lubschez, R. (1953). *J. biol. Chem.* **200**, 275.

Moldave, K., Castelfranco, P. & Meister, A. (1959). *J. biol. Chem.* **234**, 841.

Parks, L. W. & Schlenk, F. (1958). *J. biol. Chem.* **230**, 295.

Partridge, S. M. (1948). *Biochem. J.* **42**, 238.

Sanger, F. (1949). *Biochem. J.* **44**, 126.

Schlenk, F. (1965). In *Transmethylation and Methionine Biosynthesis*, p. 48. Ed. by Shapiro, S. K. & Schlenk, F. Chicago and London: University of Chicago Press.

Schlenk, F. & DePalma, R. E. (1957). *J. biol. Chem.* **229**, 1037.

Schlenk, F. & Ehninger, D. J. (1964). *Arch. Biochem. Biophys.* **106**, 95.

Smith, I. (1962). In *Chromatographic and Electrophoretic Techniques*, vol. 1, p. 6. Ed. by Smith, I. London: William Heinemann Medical Books Ltd.

Smith, R. L. & Schlenk, F. (1952). *Arch. Biochem. Biophys.* **38**, 159.

Stein, W. H. & Moore, S. (1954). *J. biol. Chem.* **211**, 915.

Tallan, H. H., Moore, S. & Stein, W. H. (1954). *J. biol. Chem.* **211**, 927.

Toennies, G. (1942). *J. biol. Chem.* **145**, 667.

Toennies, G. & Homiller, R. (1942). *J. Amer. chem. Soc.* **64**, 3054.

Toennies, G. & Kolb, J. (1951). *Analyt. Chem.* **23**, 823.

Trevelyan, W. E., Procter, D. P. & Harrison, J. S. (1950). *Nature, Lond.*, **166**, 444.

Whitfeld, P. R. & Markham, R. (1953). *Nature, Lond.*, **171**, 1151.

Wikberg, E. (1953). *Nature, Lond.*, **172**, 398.

Yemm, E. W. & Cocking, E. C. (1955). *Analyst*, **80**, 209.