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Endogenous Sulphate Acceptors in Rat Liver

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There is now abundant evidence to show that adenosine 3′-phosphate 5′-sulphatophosphate (Robbins & Lipmann, 1957) is an active form of sulphate from which the sulphate group can be transferred to various acceptors under the influence of specific sulphokinases to form sulphate esters or sulphamino compounds (Lipmann, 1958; Gregory & Robbins, 1960). In the presence of added adenosine triphosphate, Mg^{2+} and SO_4^{2-} ions, yeast preparations synthesize adenosine 3′-phosphate 5′-sulphatophosphate by a two-stage reaction. The first stage involves the reversible action of an adenosine triphosphate-sulphurylase to give adenosine 5′-sulphatophosphate and pyrophosphate and this is followed by non-reversible phosphorylation of adenosine 5′-sulphatophosphate by adenosine triphosphate in the presence of a specific adenosine 5′-sulphatophosphate-kinase to give adenosine 3′-phosphate 5′-sulphatophosphate (Bandurski, Wilson & Squires, 1956). The synthesis of adenosine 3′-phosphate 5′-sulphatophosphate by the soluble material of the cytoplasm of mammalian liver is also known to be a two-stage process, and although an unequivocal proof is not yet available it seems reasonably certain that the steps are the same as those in the yeast preparations (Robbins & Lipmann, 1958).

On incubation of particle-free high-speed supernatants of iso-osmotic-potassium chloride homogenates of rat liver with adenosine triphosphate, SO_4^{2-} and Mg^{2+} ions, it was observed that the rate of formation of adenosine 3′-phosphate 5′-sulphatophosphate diminished with increasing length of incubation and, eventually, after reaching a maximum concentration, the overall amount of adenosine 3′-phosphate 5′-sulphatophosphate decreased. Some of the diminution in the rate of formation of the compound might be attributable to the accumulation of pyrophosphate, which would tend to reverse the first stage of synthesis of adenosine 3′-phosphate 5′-sulphatophosphate (Robbins & Lipmann, 1958) and actual loss of the compound would be expected owing to the action of a specific 3′-nucleotidase (Brunngraber, 1958). With $^{35}SO_4^{2-}$ ions, followed by chromatography and radioautography of the incubates after various times, it has been shown that a further contributing factor to disappearance of adenosine 3′-phosphate 5′-sulphatophosphate is the transfer of sulphate from the compound to endogenous acceptors to

give sulphate esters which are revealed as radioactive spots on the radioautographs. The evidence for this is now presented and the nature of the acceptors is discussed. A preliminary communication has appeared (Spencer, 1959*a*).

EXPERIMENTAL

Liver preparations. Livers from M.R.C. hooded-strain rats were removed immediately after the animals were killed and were homogenized in 10 vol. of iso-osmotic KCl (11·4 g./l.) at 2° with a glass homogenizer. The homogenate was centrifuged in a Spinco preparative ultracentrifuge at 54 000 *g* (rotor no. 30) for 90 min. After removal of the superficial fatty layer, the supernatant was distributed amongst a number of tubes of 1 ml. capacity and stored at −20°.

Reaction mixtures. The standard reaction mixture was 40 μl. of liver preparation plus 10 μl. of a solution that had been adjusted to pH 7·4 with NaOH and which contained 1 μmole of adenosine triphosphate (ATP), 1 μmole of KH_2PO_4 and 0·3 μmole of $MgSO_4$. In the radioactive-tracer experiments 0·3 μmole of $MgCl_2$ was substituted for the $MgSO_4$ and 5 μc of $Na_2{}^{35}SO_4$ (code SJSI, specific activity greater than 100 mc/mg. of S; The Radiochemical Centre, Amersham, Bucks.) was added. Incubation was at 38° for various time intervals and the enzymic reaction was stopped and the proteins were precipitated by heating the mixture in a boiling-water bath for 2 min. The mixture was then centrifuged.

Chromatography. The supernatant after centrifuging of the deproteinized reaction mixture was applied to Whatman no. 1 paper in amounts varying from 5 to 25 μl. but usually 10 μl. Three solvent systems were used: acetone–water–acetic acid (90:9:1, by vol.) for 3–4 hr., butanol–acetic acid–water (50:12:25, by vol.) for 16 hr. and *iso*butyric acid–aq. 0·3 N-NH_3 soln., (5:3, v/v) for 16 hr. (cf. Suzuki & Strominger, 1959). The R_F values of $SO_4{}^{2-}$ ions, adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate and adenosine 5′-[^{35}S]-sulphatophosphate were all 0·0 in the acetone solvent, 0·08, 0·04 and 0·06 respectively in the butanol solvent and 0·20, 0·26 and 0·34 in the *iso*butyric acid solvent. After air-drying, radioautographs were prepared by placing the papers in contact with Ilford Industrial B X-ray film for 5–10 days. Alternatively, chromatograms were scanned with a C 100 Actigraph automatic chromatogram strip scanner (Nuclear-Chicago Corp., Ill., U.S.A.) at a speed of 6 in./hr. and with a $\frac{1}{8}$ in. slit. Occasionally the radioactivity of the spots was measured (G.M. mica end-window) after elution of the spots with water and drying the eluates on stainless-steel planchets (5 cm.^{2}). Corrections were made for dead time of the instrument and background but no correction for self-absorption was made and the values obtained were considered only relative.

Preparation of adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate. A yeast extract containing an active adenosine 3′-phosphate 5′-sulphatophosphate-synthesizing system was prepared by the method of Nose & Lipmann (1958). The extract (2 ml.), plus 0·5 ml. of a solution containing 50 μmoles of ATP, 50 μmoles of KH_2PO_4 and 250 μc of $Na_2{}^{35}SO_4$, which had been adjusted to pH 7·4 with NaOH, was incubated for 1 hr. at 38° and then heated in a boiling-water bath for 2 min. After cooling and centrifuging the mixture, the total supernatant was placed on Whatman no. 17 paper, 20 cm. wide, and subjected to electrophoresis at 80 v for 16 hr. at 2° with 0·1 M-ammonium acetate–acetic acid, pH 5·6. Under these conditions the $^{35}SO_4{}^{2-}$ ions had run 30 cm. and the strong radioactive band approximately midway between the origin and the $^{35}SO_4{}^{2-}$ ions was eluted with water and the eluate was freeze-dried. The dried material was dissolved in 1 ml. of water and applied as a band on Whatman no. 3 MM paper. The chromatogram was developed for 4 hr. with acetone–water (9:1). Strong radioactivity was shown only at the origin and this was eluted with 1 ml. of water; the eluate was divided into 100 μl. portions and stored at −20°. On chromatography of this material in a number of different solvents, all the radioactivity, apart from a trace of $^{35}SO_4{}^{2-}$ ions, was found in the adenosine 3′-phosphate 5′-sulphatophosphate spot. No ultraviolet-absorbing spots other than adenosine 3′-phosphate 5′-sulphatophosphate were observed. The amount of the compound varied in different preparations from 0·3 to 1·0 μmole/ml.

Adenosine 5′[^{35}S]-sulphatophosphate of low specific radioactivity was a gift from Dr N. Ringertz. Adenosine 5′[^{35}S]-sulphatophosphate with a specific activity of 7000 counts/sec./mg. was prepared by the method of Reichard & Ringertz (1959).

Estimation of adenosine 3′-phosphate 5′-sulphatophosphate. Use was made of the method of Robbins & Lipmann (1957) in which the formation of *p*-nitrophenyl sulphate by transfer of the sulphate group of adenosine 3′-phosphate 5′-sulphatophosphate to *p*-nitrophenol under the influence of phenolsulphokinase is estimated from the decrease in absorption by the *p*-nitrophenol anion at 400 mμ. Fresh, crude rat-liver KCl-homogenate supernatant was used as a source of phenolsulphokinase and ethylenediaminetetra-acetate (EDTA) was added to suppress formation of adenosine 3′-phosphate 5′-sulphatophosphate (Brunngraber, 1958). The EDTA also partially inhibited the specific 3′-nucleotidase of rat liver (Brunngraber, 1958) which degrades adenosine 3′-phosphate 5′-sulphatophosphate (Fig. 2). As will be shown, crude liver preparations contain endogenous sulphate acceptors but when an added acceptor is present in high concentration only a very small proportion of the sulphate group of adenosine 3′-phosphate 5′-sulphatophosphate is transferred to the endogenous acceptors. Under the conditions employed at least 98% of the sulphate group of adenosine 3′-phosphate 5′-sulphatophosphate was transferred to *p*-nitrophenol.

To 10 μl. of the adenosine 3′-phosphate 5′-sulphatophosphate-containing solution was added 10 μl. of liver preparation, 5 μl. of *p*-nitrophenol solution at pH 7·4 (15 μm-moles of *p*-nitrophenol) and 10 μl. of 0·04 M-EDTA containing 0·025 M-cysteine at pH 7·4. After incubation for 90 min. at 38° the mixture was deproteinized with 200 μl. of ethanol and centrifuged. A 100 μl. portion of the supernatant was diluted with 50 μl. of 0·1 N-NaOH and the extinction at 400 mμ measured with the Hilger Uvispek spectrophotometer and micro-cell attachment. A blank determination, in which 5 μl. of water was substituted for the *p*-nitrophenol solution, and a control, in which the *p*-nitrophenol solution was added immediately before deproteinization, were also carried out. The difference between the control and blank readings at 400 mμ represents 15 μm-moles of *p*-nitrophenol and the difference between this extinction and that between the test and

blank readings represents, proportionally, the amount of *p*-nitrophenol which has been sulphated, and thus the amount of adenosine 3′-phosphate 5′-sulphatophosphate.

Extraction of phenols from rat liver. An extract of liver containing phenols and from which neutral and basic compounds were eliminated was prepared in the following manner. The supernatant (10 ml.) of the KCl homogenate of rat-liver was made 0·1 N with respect to HCl, extracted with ether (3 × 10 ml.) and the combined ether extracts were washed with 0·1 N-NaOH (2 × 5 ml.). The aqueous alkaline solution was washed with ether (2 × 10 ml.), then made 0·1 N with respect to HCl and extracted with ether (3 × 10 ml.). The combined ether extracts were washed once with 5 ml. of water, dried over anhydrous Na_2SO_4 and evaporated to dryness. The extract, which was dissolved in 0·5 ml. of water, gave strong positive $FeCl_3$, 2:6-dichloroquinonechloroimide and Millon's tests for phenols.

Enzymic hydrolysis of the sulphated endogenous acceptors. The arylsulphatase of *Aspergillus nidulans* (C.M.I. 16643) was a preparation used by Spencer (1959*b*). Crude digestive juice of *Helix pomatia* was collected from the crops of freshly dissected snails. A purified preparation of the arylsulphatase of the digestive gland of *H. pomatia* was obtained from Dr G. M. Powell.

The enzyme preparation (10 µl.) plus 10 µl. of M-sodium acetate–acetic acid buffer, pH 6·0, was incubated at 38° for 4 hr. with 20 µl. of the incubated liver preparation. The mixture was then heated at 100° for 2 min., cooled and 20 µl. was applied to paper for chromatography.

Since PO_4^{3-} ion is a potent inhibitor of many sulphatases the incubated liver preparation was made with the standard reaction mixture incorporating $Na_2{}^{35}SO_4$ and adjusted to pH 7·4 but without the KH_2PO_4. After incubation for 2 hr. at 38° the mixture was heated at 100° for 2 min., cooled and centrifuged. The radioautograph pattern of the ester sulphates in the supernatant, as shown by chromatography with all three of the standard chromatographic solvents, was the normal pattern and in every way th ame as when KH_2PO_4 was present in the incubated reaction mixture.

Sulphation of added acceptors. To a mixture of 30 µl. of the liver preparation and 10 µl. of a solution at pH 7·4 containing 1 µmole of ATP, 1 µmole of KH_2PO_4, 0·3 µmole of $MgCl_2$ and 5 µc of $Na_2{}^{35}SO_4$, was added 10 µl. of a solution of the acceptor. After incubation for 1 hr. the mixture was heated in a boiling-water bath for 2 min., cooled and centrifuged and 10 µl. of the supernatant was spotted on Whatman no. 1 paper for chromatography.

The acceptors were dissolved in water or in 0·05 M-acid or -alkali, the pH was adjusted to 7·4 with HCl or NaOH and the resulting solution or suspension was added to the incubation mixture. Steroids were dissolved in ethanol and the requisite amounts of the solutions were added to empty tubes. The solvent was evaporated off and 10 µl. of water was added, followed by the liver preparation and solution containing ATP etc.

Collection of urine and bile. The 24 hr. urine from rats that had each received an intraperitoneal injection of 500 µc of $Na_2{}^{35}SO_4$ in NaCl was collected by placing the rats in large Büchner funnels. Bile was collected from bile cannulae inserted in rats which had been anaesthetized firstly with ether and then with 30 mg. of Nembutal injected subcutaneously. A 500 µc dose of $Na_2{}^{35}SO_4$ in NaCl was injected into the right jugular vein. Bile was collected for 3 hr. after injection of the radioactive compound.

RESULTS

Synthesis of adenosine 3′-phosphate 5′-sulphatophosphate. At 38° in the presence of added ATP, Mg^{2+}, PO_4^{3-} and SO_4^{2-} ions the particle-free rat-liver preparation synthesized adenosine 3′-phosphate 5′-sulphatophosphate at a rate which diminished with time. The concentration of adenosine 3′-phosphate 5′-sulphatophosphate in the system was maximum at 90 min. and thereafter decreased until, after 6 hr. incubation, the amount remaining was negligible (Fig. 1). The highest concentration attained was 20 µM and since the added ATP concentration was 0·02 M and that of the SO_4^{2-} ions was 6 mM, neither of these reactants was rate-limiting, at least in the initial stages.

Breakdown of adenosine 3′-phosphate 5′-sulphatophosphate. As well as the slowing down of the rate of synthesis of adenosine 3′-phosphate 5′-sulphatophosphate in the liver system it was evident that breakdown of the compound had taken place. This was investigated separately by incubating the ^{35}S-labelled compound with a rat-liver supernatant (which had been stored at −20° for 3 months) in the presence of Mg^{2+} and PO_4^{3-} ions. Portions of the incubation mixture were removed after various times, boiled for 2 min., centrifuged and the supernatant was examined chromatographically with the *iso*butyric acid–aq. NH_3 soln. solvent. The chromatograms were scanned with the Actigraph automatic strip scanner and the amounts of adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate, adenosine 5′[^{35}S]-sulphatophosphate and $^{35}SO_4^{2-}$ ions were assessed by measuring the areas under the curve. A more accurate estimation of the $^{35}SO_4^{2-}$

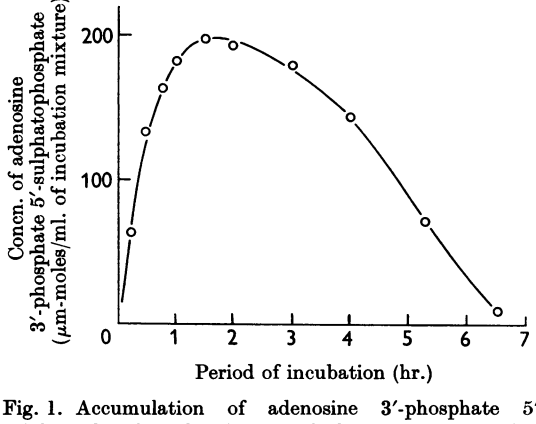

Fig. 1. Accumulation of adenosine 3′-phosphate 5′-sulphatophosphate by the particle-free supernatant of an iso-osmotic-KCl rat-liver homogenate at 38° and pH 7·3 in the presence of 0·02 M-ATP, 0·02 M-KH_2PO_4 and 6 mM-$MgSO_4$.

ions produced was obtained by elution of the $^{35}SO_4^{2-}$ ions from the chromatograms and measurement of the radioactivity by a plating technique. After 1·5 hr. all the adenosine 3′-phosphate 5′-[^{35}S]-sulphatophosphate had disappeared (Fig. 2), but only approx. 95% of the initial amount of ^{35}S could be accounted for as $^{35}SO_4^{2-}$ ions. A further amount of SO_4^{2-} ions, equal to approx. 4% of the adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate introduced, was recovered after hydrolysis of the 1·5 hr. incubation mixture with N-HCl for 1 hr. No breakdown of adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate occurred in the control which contained boiled liver preparation.

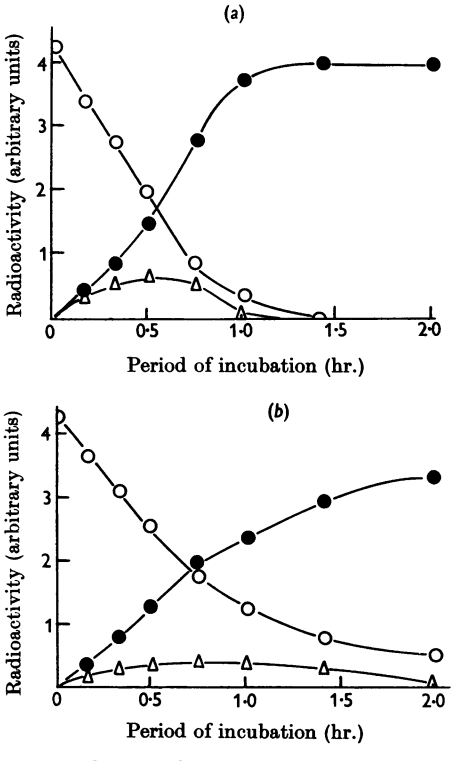

Fig. 2. Degradation of adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate by the rat-liver supernatant which had been stored at −20° for 3 months. (*a*) Adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate (0·1 mM) was incubated at 38° in the presence of 0·02 M-KH_2PO_4 and 6 mM-KH_2PO_4 at pH 7·3. After the required period a portion of the incubation mixture was heated at 100° for 2 min., cooled, centrifuged and 10 μl. of the supernatant was chromatographed on Whatman no. 1 paper for 16 hr. with *iso*butyric acid–aq. 0·3 N-NH_3 soln. (5:3). Radioactivity of the spots corresponding to $^{35}SO_4^{2-}$ ions, adenosine 5′[^{35}S]-sulphatophosphate and adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate was assessed by measuring the area under the curve obtained by scanning the chromatograms with a C 100 Actigraph automatic strip scanner. The amount of radioactivity is expressed in arbitrary units. (*b*) Conditions were the same as in (*a*) but EDTA was incorporated at a concentration in the incubation mixture of 0·08 M. ○, Adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate; ●, $^{35}SO_4^{2-}$ ion; △, adenosine 5′[^{35}S]-sulphatophosphate.

These results suggested that at least two mechanisms were responsible for breakdown of adenosine 3′-phosphate 5′-sulphatophosphate, the major one leading to the formation of inorganic sulphate and the other to a new form or forms of 'bound' sulphate. The formation of inorganic sulphate is readily explained as being the result of the action of the specific 3′-nucleotidase (Brunngraber, 1958) or phosphatase (Robbins & Lipmann, 1958) which is known to be present in rat liver, followed by a splitting of the phosphosulphate link of the adenosine 5′-sulphatophosphate so formed (cf. Reichard & Ringertz, 1959). In the chromatograms of the liver–adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate experiments a radioactive spot with the same R_F as synthetic adenosine 5′-sulphatophosphate appeared after incubation for 10 min. The concentration of this spot rose during the incubation but eventually it all disappeared (Fig. 2). Its identity with synthetic adenosine 5′-sulphatophosphate was confirmed by radioautography after paper electrophoresis under the conditions used for the preparation of adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate (see Experimental section). The breakdown of adenosine 5′-sulphatophosphate to give inorganic sulphate was shown by incubation of 0·1 μmole of the synthetic ^{35}S-labelled compound in 10 μl. of water with 30 μl. of the liver preparation and 10 μl. of water containing 0·3 μmole of Mg^{2+} ions and 1 μmole of PO_4^{3-} ions. After chromatography with the *iso*butyric acid–aq. NH_3 soln. solvent the presence of $^{35}SO_4^{2-}$ ions was demonstrated by radioautography. Measurements of the radioactivity of the $^{35}SO_4^{2-}$ ions and adenosine 5′[^{35}S]-sulphatophosphate spots by elution and plating showed that the major fate of the sulphate of adenosine 5′-sulphatophosphate in the system used (i.e. in the absence of added ATP) was to be split off as SO_4^{2-} ions, all of the ^{35}S-labelled compound being hydrolysed in 2·5 hr. On examination under u.v. light (253 mμ) all the ^{35}S-labelled compound had disappeared after 2·5 hr. and new u.v.-absorbing spots with R_F values corresponding to adenylic acid (R_F 0·45) and adenosine (R_F 0·50) were seen. Approx. 1% of the ^{35}S of the adenosine 5′[^{35}S]-sulphatophosphate was incorporated into a new spot at R_F 0·45 (*iso*butyric acid–aq. NH_3 soln.).

When 0·08 M-EDTA was incorporated into the incubation mixtures containing rat-liver supernatant which had been stored at −20° for 3 months, the rate of breakdown of adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate and adenosine 5′[^{35}S]-

sulphatophosphate was inhibited (Fig. 2). With the latter compound the non-specific 5′-nucleotidase activity at pH 7·3 was completely inhibited (cf. Spencer, 1958), and apart from residual adenosine 5′[^{35}S]-sulphatophosphate the only u.v.-absorbing spot observed was adenylic acid. With fresh liver preparations the breakdown of adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate was so rapid that adenosine 5′[^{35}S]-sulphatophosphate was not seen on chromatograms of the liver–adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate incubation mixtures. In the presence of 0·08 M-EDTA the fresh liver preparations did produce adenosine 5′[^{35}S]-sulphatophosphate.

'Bound' sulphate. Radiochromatographs of the standard radioactive reaction mixture after different incubation times revealed the presence of a number of radioactive spots with R_F greater than that of adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate (Fig. 3). The concentration of these unknown spots increased with the length of time of incubation of the reaction mixture while the adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate concentration declined (cf. Fig. 1).

With the *iso*butyric acid–aq. NH_3 soln. solvent, $^{35}SO_4^{2-}$ ions, adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate, adenosine 5′[^{35}S]-sulphatophosphate and up to seven other distinct radioactive spots were observed (Table 1 and Fig. 3). With butanol–acetic acid–water solvent, adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate and adenosine 5′[^{35}S]-sulphatophosphate were sometimes obscured by the strong $^{35}SO_4^{2-}$ ion spot at R_F 0·08, but eight other radioactive spots were observed. With a band of the incubation mixture and the acetone–acetic acid–water solvent, 15 radioactive bands were seen, apart from $^{35}SO_4^{2-}$ ions, adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate and adenosine 5′[^{35}S]-sulphatophosphate, all of which remained at the origin. Many of the bands on the one-way chromatograms were not single entities. Elution of the single band showing the highest R_F in acetone–acetic acid–water followed by rechromatography with *iso*butyric acid–aq. NH_3 soln. revealed the presence of five different radioactive spots. A two-way chromatogram with the acetone and *iso*butyric acid solvents showed 15 radioactive spots apart from $^{35}SO_4^{2-}$ ions, adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate and adenosine 5′[^{35}S]-sulphatophosphate. In the two-dimensional chromatogram many of the weak radioactive components previously observed when the acetone–acetic acid–water solvent was used alone were not seen. Thus the total number of radioactive compounds in the incubated material must be greater than 15. However, most of the work to be reported was of an exploratory nature and was carried out by one-way chromatography. The *iso*butyric acid–aq. NH_3 soln. solvent was preferred since it distinguished $^{35}SO_4^{2-}$ ions, adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate and adenosine 5′[^{35}S]-sulphatophosphate and this solvent has been used throughout unless otherwise stated.

The most feasible suggestion about the nature of the unknown spots was that they were sulphate esters formed by transfer of sulphate from adenosine 3′-phosphate 5′-sulphatophosphate to endogenous acceptors in the rat-liver supernatant. The evidence to support this suggestion was as follows.

After prolonged dialysis (24 hr. against six changes of distilled water) the rat-liver supernatant

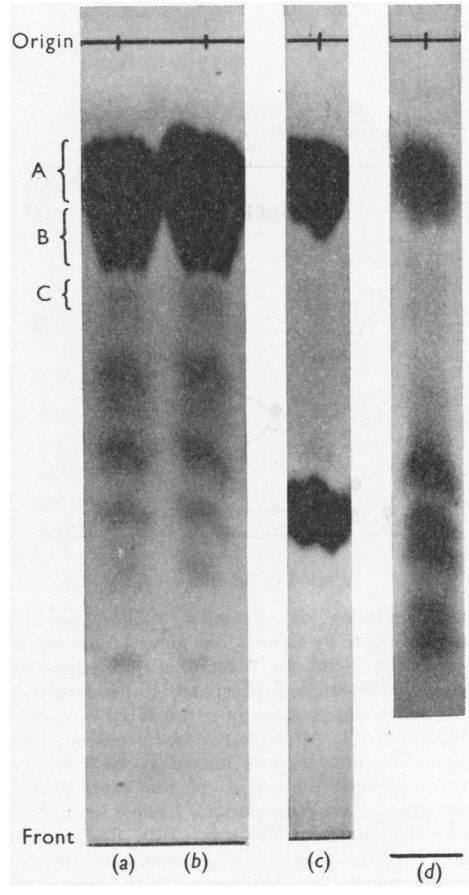

Fig. 3. Radioautographs of *iso*butyric acid–aq. NH_3 soln. chromatograms. (*a*) Standard incubation mixture incorporating $Na_2{}^{35}SO_4$ after 1 hr. incubation at 38°. (*b*) As (*a*), with the incorporation of 0·01 M-hydroxyproline. (*c*) As (*a*), with the incorporation of 0·01 M-*p*-cresol. (*d*) Bile collected from a rat which had been injected with 500 μc of $Na_2{}^{35}SO_4$. A, $^{35}SO_4^{2-}$ ions; B, adenosine 3′-phosphate 5′-[^{35}S]-sulphatophosphate; C, adenosine 5′[^{35}S]-sulphatophosphate.

could still produce adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate under the standard-incubation conditions, but the unknown radioactive spots were either missing on the chromatograms or very weak in comparison with those given by the undialysed preparation. Addition of the supernatant of boiled fresh rat-liver KCl supernatant to the dialysed preparation followed by incubation under the standard conditions resulted in the appearance of all the unknown spots.

Hydrolysis of the incubated standard radioactive reaction mixture with 0·1 N-HCl for 15 min. at 100° before chromatography resulted in the disappearance of the majority of radioactive spots, including the adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate and adenosine 5′[^{35}S]-sulphatophosphate. The spots that remained were very much weaker than the corresponding ones on the chromatograms of the unhydrolysed liver incubates. After hydrolysis with N-HCl for 30 min. at 100° only $^{35}SO_4^{2-}$ ions were detected on the chromatograms. Most sulphate esters are known to be hydrolysed under these conditions.

In the experiment in which adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate was incubated with the liver preparation approximately 4% of the added compound was converted into the new forms of 'bound' sulphate. Radioautographs of the incubation mixture showed the 'bound' sulphate as barely discernible radioactive spots, which were, however, distinct from $^{35}SO_4^{2-}$ ions, adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate and adenosine 5′[^{35}S]-sulphatophosphate. The experiment was repeated with mM-adenosine 5′-[^{35}S]-sulphatophosphate in the incubation mixture, i.e. about ten times the amount used previously, and the mixture after incubation for 1 hr. was examined chromatographically in the three solvent systems. The radioautographs prepared with an exposure time of 3 weeks showed all the radioactive spots previously observed in the radioautographs of the incubation mixture containing liver preparation, ATP, PO_4^{3-}, Mg^{2+} and $^{35}SO_4^{2-}$ ions, i.e. when adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate was being continuously formed.

With the standard reaction mixture the particle-free supernatants prepared from 10% (w/v) homogenates in iso-osmotic KCl (11·4 g./l.) of rat kidney, spleen, lung, heart and testis were all able to produce adenosine 3′-phosphate 5′-sulphatophosphate. The number of sulphate acceptors in each of the tissue preparations, as revealed by the radio-

Table 1. *R_F values of radioactive spots on chromatograms of the incubated standard reaction mixture*

Standard reaction mixture, incorporating $Na_2{}^{35}SO_4$, was incubated for 4 hr. at 38°, then heated at 100° for 2 min., centrifuged and 10 µl. samples of the supernatant were chromatographed in three different solvent systems. The radioactive spots were localized by radioautography. The experiment was repeated with the iso-osmotic KCl (11·4 g./l.) supernatants of other rat tissues instead of liver. Male rats were used throughout.

*iso*Butyric acid–aq. NH_3 soln.				Acetone–water–acetic acid		Butanol–acetic acid–water	
Liver	Kidney	Spleen	Assignment	Liver	Assignment	Liver	Assignment
0·18	0·18	0·18	$^{35}SO_4^{2-}$	0·00	$^{35}SO_4^{2-}$, adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate, adenosine 5′[^{35}S]-sulphatophosphate	(0·04)	Adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate
						(0·06)	Adenosine 5′[^{35}S]-sulphatophosphate
0·24	0·24	0·24	Adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate	0·04	—	0·08	$^{35}SO_4^{2-}$
0·31	0·31	—	Adenosine 5′[^{35}S]-sulphatophosphate	0·07	—	0·27	—
0·39	0·39	—	—	0·09	—	0·37	—
0·45	—	—	—	0·11	—	0·42	Steroid sulphates?
0·53	0·53	0·53	—	0·14	—	0·46	Steroid sulphates?
0·61	0·61	0·61	Arylsulphates?	0·15	—	0·53	—
0·69	0·69	0·69	—	0·21	—	0·60	Arylsulphates?
0·74	0·74	—	—	0·29	—	0·73	—
0·79	0·79	0·79	Steroid sulphates and arylsulphates?	0·39	—	0·80–0·88	—
				0·46	—		
				0·56	—		
				0·64	—		
				0·74	Arylsulphates?		
				0·84	Arylsulphates?		
				0·93	Steroid sulphates?		

active spots on the chromatogram, was in all cases very much less than in the liver preparations. The spots which were observed were all common with those of the liver preparation but less intense. Formation of adenosine 3′-phosphate 5′-sulphatophosphate and sulphate transfer to endogenous acceptors has also been shown for sheep-trachea epithelium (D. K. Watkins, unpublished observation).

Nature of the endogenous sulphate acceptors. Some of the endogenous acceptors of normal liver might have been derived directly from the food supply or from the blood present in the liver. Supernatants were therefore prepared from the iso-osmotic-KCl (11·4 g./l.) homogenates of livers from rats that had been starved for 48 hr., but given water without restriction, and from livers that had been perfused with cold iso-osmotic KCl. These preparations were incubated for 4 hr. in the standard reaction mixture. In each case the patterns of radioactive spots on the chromatograms prepared with all three standard solvents were the same as those derived from normal liver.

Evidence accumulated which suggested that some of the endogenous acceptors were phenols. The ether extract of rat liver (10 μl.) was incubated for 15 min. at 38° with 50 μl. of the standard reaction mixture containing $Na_2{}^{35}SO_4$, then it was heated at 100° for 2 min., centrifuged and 10 μl. of the supernatant was applied for chromatography. The radioautographs showed that some radioactive spots, in particular those with R_F 0·61 in *iso*butyric acid–aq. NH_3 soln., R_F 0·84 in acetone–water–acetic acid and R_F 0·60 in butanol–acetic acid–water, were greatly increased in intensity compared with those given under the same conditions in the absence of the extract. Some of these intensified spots were presumably arylsulphates formed from the phenols present in the ether extract.

A portion (2 ml.) of the standard reaction mixture that had been incubated for 4 hr. was chromatographed as a band on Whatman no. 3MM paper with the acetone–water–acetic acid solvent. The five radioactive bands with the highest R_F values, i.e. 0·93, 0·84, 0·74, 0·64 and 0·55, were eluted with 0·5 ml. of water and their ultraviolet-absorption spectra were examined. The spectra in each case showed a peak or inflexion in the 280 mμ region and high absorption at lower wavelengths, although this was somewhat masked by non-specific end-absorption. Spectra of this type are characteristic of aromatic compounds.

After hydrolysis of the standard reaction mixture which had been incubated for 2 hr. with the aryl-sulphatase preparations from *A. nidulans* and *H. pomatia*, chromatography followed by radioautography showed that certain of the radioactive spots were considerably diminished in intensity. With the acetone–water–acetic acid solvent this applied particularly to the fast-moving spots and in the *iso*butyric acid–aq. NH_3 soln. solvent the spot R_F 0·61 was greatly affected. Adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate and adenosine 5′-[^{35}S]-sulphatophosphate were unaffected by the enzymes and none of the other spots completely disappeared. After hydrolysis with the crude digestive juice of *H. pomatia*, known to contain aryl-, glyco-sulphatase and steroid sulphatase, the majority of the radioactive spots completely disappeared. Those remaining (R_F 0·79, 0·74 and 0·31 in *iso*butyric acid–aq. NH_3 soln.) were greatly diminished in intensity. The adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate spot had also disappeared and a new radioactive spot (R_F 0·0 in butanol–acetic acid–water; R_F 0·12 in *iso*butyric acid–aq. NH_3 soln; R_F 0·0 in acetone–water–acetic acid) of similar intensity appeared. Further work, which will be reported elsewhere, has shown that a transfer of $^{35}SO_4{}^{2-}$ ions from the adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate of the liver incubation mixture to an endogenous acceptor present in the snail juice had taken place under the influence of a specific sulphokinase in the juice.

Sulphation of known compounds by the rat-liver preparation. Another method of approach towards the identification of the unknown spots was made by attempting to recognize the classes of compounds that could be sulphated by the liver preparation. The procedure was to incubate possible acceptors with the standard reaction mixture for 1 hr., followed by chromatography and radioautography of the protein-free supernatant of the boiled reaction mixture that had been incubated. The radioautographic pattern was then compared with that obtained in the absence of added acceptor. When transfer of $^{35}SO_4{}^{2-}$ ions from adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate to an added acceptor took place, little or no adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate remained; the sulphated endogenous acceptors were either completely absent or very weak and the presence of the sulphated acceptor was shown as a strong new radioactive spot or spots (Fig. 3*c*). When no such transfer took place, substantial amounts of adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate were present and the endogenous ester sulphate pattern (Fig. 3*b*) was the same as that of the standard reaction mixture (Fig. 3*a*). In initial experiments the steroids were dissolved in propane-1:2-diol (Nose & Lipmann, 1958) and diluted with an equal volume of water. After incubation of each of the steroids with the liver preparation four radioactive spots were observed on the chromatograms developed with butanol–acetic acid–water. Three of the spots were found to be formed from

Table 2. *R_F values of new radioactive spots on chromatograms of the standard reaction mixture incubated with various acceptors for 1 hr.*

R_F values in parentheses indicate that the spot was faint relative to the other spots.

Acceptor	Concn. of acceptor in incubation mixture (M)	R_F *iso*Butyric acid–aq. NH_3 soln.	R_F Acetone–water–acetic acid	R_F Butanol–acetic acid–water
Phenols				
Phenol	0·01	0·53	0·86	—
o-Cresol	0·01	0·60	0·85	0·60
m-Cresol	0·01	0·60	0·85	0·60
p-Cresol	0·01	0·60	0·85	0·60
m-Hydroxybenzoic acid	0·01	0·36	0·39	0·51
p-Hydroxybenzoic acid	0·01	0·48	0·46	—
Thymol	0·01	—	0·79	—
L-Adrenaline	0·01	0·38, 0·41, 0·50	—	—
Tyramine	0·01	0·38, 0·47, 0·50	0·07, 0·58, 0·72	0·37, (0·45), 0·49
Steroids				
Androsterone	0·01	0·79	0·93	0·45
Dehydro*iso*androsterone	0·01	0·79	0·93	0·45
Pregnenolone	0·01	0·79	—	0·45
Oestrone	0·01	0·79	—	0·45
Oestradiol	0·01	0·79	—	0·45
Alcohols				
Ethanol	0·68	0·35	—	0·45
Butanol	0·40	—	—	0·73
Propane-1:3-diol	0·31	—	—	0·37, (0·46), 0·73
Propane-1:2-diol	0·31	0·30, 0·51, 0·62	—	0·54, 0·64, 0·85
Aromatic mines				
α-Naphthylamine	0·01	0·59	—	(0·45), 0·71
β-Naphthylamine	0·01	—	—	(0·45), 0·88

propane-1:2-diol in the absence of the steroid (Table 2). In subsequent experiments the steroids were treated as described in the Experimental section.

Table 2 lists the acceptors added and the R_F values of the new radioactive spots detected on the chromatograms. That the sulphation of the added acceptors occurred through the transfer of $^{35}SO_4^{2-}$ ions from adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate was shown by repetition of the experiments but with substitution of 10 μl. of the adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate solution for the ATP–KH_2PO_4–$MgCl_2$–$Na_2{}^{35}SO_4$ mixture. The radioautographs in all cases showed the same new spots as those found when the liver preparation was allowed to produce the adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate *in situ*. The following compounds did not appear to be sulphated when incorporated into the standard reaction mixture at a final concentration of 0·01 M: bilirubin, cholic acid, L-threonine, L-serine, L-hydroxyproline, L-hydroxyglutamic acid and choline chloride. Rat bile diluted with 4 vol. in the incubation mixture also failed to give new radioactive spots.

Rat urine and bile after injection of $Na_2{}^{35}SO_4$. More than 95 % of the radioactivity of the 24 hr.-urine sample collected from rats that had been injected with 500 μc of $Na_2{}^{35}SO_4$ was present as $^{35}SO_4^{2-}$ ions. On chromatography with the *iso*butyric acid–aq. NH_3 soln. solvent, the pattern of radioactive spots was very similar to that previously observed with the incubation mixtures with liver (Tables 1 and 3). One spot (R_F 0·48), which did not occur in the liver pattern, was seen in the urines from both male and female rats and an additional spot (R_F 0·51) was seen only in the radiochromatograph of urine from males. The spots at R_F 0·79 and 0·74 were very weak in the urine from males compared with that from females. In the acetone–water–acetic acid solvent, distinctions between the chromatograms of both urines were seen, particularly amongst the spots with low R_F values (Table 3). Several spots which were present in the liver pattern were not found in the radiochromatographs of urine from rats of either sex. All the radioactive spots seen on radioautographs of *iso*butyric acid–aq. NH_3 soln. chromatograms of urine disappeared after hydrolysis of the urines with N-HCl for 30 min. at 100° or with crude snail juice at pH 5·5 for 4 hr. and only $^{35}SO_4^{2-}$ ions remained.

About 50 % of the radioactivity of the bile from a male rat which had been injected with $Na_2{}^{35}SO_4$

could be attributed to $^{35}SO_4^{2-}$ ions, although the percentage varied slightly with the time of collection after the injection. Four radioactive spots, R_F 0·27, 0·33, 0·48 and 0·56, which were not previously observed in the liver pattern, were seen on radiochromatographs with *iso*butyric acid–aq. NH_3 soln. of the bile whereas the spots (R_F 0·31 and 0·61) of the liver pattern were not present in the bile (Table 3). Although there were slight changes in the relative intensities of the radioactive spots in the radiochromatographs of bile collected at 15 min. intervals, the overall pattern remained constant up to 3 hr. after the injection of $Na_2{}^{35}SO_4$. Similar, but fewer, radioactive spots have been observed in rabbit bile (Boström & Vestermark, 1959). After hydrolysis of the bile with N-HCl for 30 min. at 100° chromatography showed that at least 95% of radioactivity was present as $^{35}SO_4^{2-}$ ions. The radioactivity remaining was spread throughout the paper and no definite spots were seen. The same result was obtained by hydrolysis with the crude digestive juice of *Helix pomatia*. These results would seem to preclude the possibility that the radioactivity of any of the unknown spots was due to [^{35}S]taurine. Taurine formation from inorganic sulphate in liver after injection of $^{35}SO_4^{2-}$ ions has been noted (Boström & Aqvist, 1952).

DISCUSSION

The accumulation of adenosine 3′-phosphate 5′-sulphatophosphate by particle-free supernatants of iso-osmotic-potassium chloride homogenates of rat liver in the presence of ATP, Mg^{2+} and SO_4^{2-} ions (Fig. 1) was found to be dependent not only on the rate of synthesis of adenosine 3′-phosphate 5′-sulphatophosphate but also on the rate of breakdown. The mechanism of synthesis of adenosine 3′-phosphate 5′-sulphatophosphate has been established (Robbins & Lipmann, 1957) but apart from brief notes to the effect that rat liver contains a phosphatase capable of 3′-dephosphorylating adenosine 3′-phosphate 5′-sulphatophosphate (Robbins & Lipmann, 1958; Brunngraber, 1958) little has been reported about degradation of adenosine 3′-phosphate 5′-sulphatophosphate.

When adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate was incubated with the particle-free liver preparations in the absence of added ATP and Mg^{2+} ions the major fate of the sulphate group of the nucleotide was to be split off as inorganic sulphate. One mechanism of $^{35}SO_4^{2-}$ ion formation in this system was shown to involve 3′-dephosphorylation of adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate to give adenosine 5′-sulphatophosphate followed by cleavage of the phosphate–sulphate linkage to give adenylic acid and $^{35}SO_4^{2-}$ ions. Both the enzymes concerned were inhibited by EDTA. It is possible that the enzyme which desulphates adenosine 5′-sulphatophosphate can also desulphate adenosine 3′-phosphate 5′-sulphatophosphate to yield SO_4^{2-} ions and adenosine 3′:5′-diphosphate, which could then be further degraded by 3′-dephosphorylation. This latter reaction is known to take place in liver extracts (Brunngraber, 1958) but proof of the overall reaction mechanism would depend on the identification of adenosine 3′:5′-diphosphate as an inter-

Table 3. *R_F values of radioactive spots on chromatograms of the urine of male and female rats and the bile of a male rat after injection with* $Na_2{}^{35}SO_4$

Each rat received 500 μc of $Na^{35}SO_4$ in 1 ml. of 0·9% sodium chloride soln.

*iso*Butyric acid–aq. NH_3 soln.				Acetone–water–acetic acid		
Liver	Urine of male	Urine of female	Bile	Liver	Urine of male	Urine of female
0·18	0·18	0·18	0·18	0·00	0·00	0·00
0·24	—	—	—	0·04	—	—
—	—	—	0·27	0·07	—	—
0·31	—	—	—	—	0·08	—
—	—	—	0·33	0·09	0·09	0·09
0·39	0·39	0·39	0·39	—	0·10	—
0·45	0·45	0·45	0·45	0·11	—	0·11
—	0·48	0·48	0·48	0·14	—	0·14
—	0·51	—	—	0·15	—	—
0·53	0·53	0·53	—	—	0·17	—
—	—	—	0·56	0·21	0·21	0·21
0·61	0·61	0·61	—	0·29	0·29	0·29
0·69	—	0·69	0·69	0·39	0·39	0·39
0·74	0·74	0·74	0·74	0·46	0·46	0·46
0·79	0·79	0·79	0·79	0·56	0·56	0·56
				0·64	0·64	0·64
				0·74	0·74	0·74
				0·84	0·84	0·84
				0·93	0·93	0·93

mediate product. No evidence can be offered on this point since the crude liver extract used in the present investigation produced some adenosine 3′:5′-diphosphate when SO_4^{2-} ion was transferred from adenosine 3′-phosphate 5′-sulphatophosphate to endogenous sulphate acceptors. A further reaction that was considered was the formation of SO_4^{2-} ions and ATP from adenosine 5′-sulphatophosphate and pyrophosphate under the influence of the ATP-sulphurylase which is present in the rat-liver supernatant. However, the liver preparation also contained an active pyrophosphatase and it was therefore unlikely that the concentration of endogenous pyrophosphate was sufficient for the above-mentioned mechanism to contribute significantly to the formation of SO_4^{2-} ions from adenosine 5′-sulphatophosphate.

A further fate of adenosine 3′-phosphate 5′-sulphatophosphate when incubated with the liver preparation was the transfer of the sulphate group to endogenous acceptors. This transfer occurred to a slight but significant extent, which was probably limited by the small amounts of acceptors present. When adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate was used in the experiment the sulphated compounds appeared on chromatograms as radioactive spots with R_F values greater than adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate and adenosine 5′[^{35}S]-sulphatophosphate in the three solvent systems used. Identical spots were seen on chromatograms of liver incubates in which adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate was continuously formed from ATP and $^{35}SO_4^{2-}$ ions. Similar spots, but fewer in number, have been previously obtained in comparable circumstances (Vestermark & Boström, 1959*a*) and it is probable that these are also sulphate esters of endogenous acceptors.

It is known (Gregory & Robbins, 1960) that the particle-free rat-liver preparation contains specific enzymes which can transfer the sulphate group of adenosine 3′-phosphate 5′-sulphatophosphate to phenols, phenolic steroids, non-phenolic steroids, arylamines and mono- and di-hydric alcohols. The particle-free liver preparation used in the present investigation transferred sulphate from adenosine 3′-phosphate 5′-sulphatophosphate to all of the established acceptors (Table 2) and it can therefore be expected that many of the unknown endogenous acceptors belong to these classes of compounds. By adding known sulphate acceptors to the liver system followed by chromatography of the liver incubates it has been possible, in certain cases, to apportion areas of the chromatograms to different types of sulphate esters. Thus in *iso*butyric acid–aq. ammonia solution the radioactive spots formed from phenols had R_F 0·48–0·60 and all the steroids had R_F 0·79 (Table 2). The radioactive spots formed from the endogenous acceptors with these R_F values may therefore contain aryl and steroid sulphates respectively. Further evidence for the aryl nature of the spot R_F 0·61 was obtained by u.v. spectroscopy and by hydrolysis with arylsulphatases.

Little further is known about the nature of the endogenous sulphate acceptors. It is significant that many of the acceptors and the enzymes responsible for their sulphation are common to a number of organs (Table 1). This would suggest that either the acceptors are formed in the individual organs by metabolic pathways that are common to all the organs or that they are derived from one particular source and then carried round the body by the blood. The presence, in radiochromatographs of urine and bile, of sulphated compounds with the same R_F values as the sulphated acceptors of liver would further suggest that many acceptors are end products of metabolism which are excreted as sulphate esters rather than as active intermediates. However, the possibility that some of sulphated liver acceptors are low-molecular-weight intermediates in the synthesis of sulphated mucopolysaccharides or that they are inert forms of physiologically active compounds (cf. Spencer, 1956) is deserving of further investigation.

The presence of new radioactive spots in chromatograms of liver incubates to which potential acceptors had been added has been taken as indicating the formation of sulphate esters of the acceptors. A partial confirmation of this supposition was obtained for all the compounds listed in Table 2 by showing that the same new radioactive spots were formed in the absence of ATP when added adenosine 3′-phosphate 5′-sulphatophosphate was used as the sulphate donor. Further, with phenol and *p*-nitrophenol the R_F values of the respective spots were the same as those of authentic specimens of phenyl sulphate and *p*-nitrophenyl sulphate. One pitfall in making this general assumption when crude liver extracts are being used is that the added compound or its metabolites may in some way potentiate the ability of an endogenous compound to accept sulphate, thus giving rise to a new spot. The potentiation of the synthesis of arylamine sulphate esters by steroid sulphates (Roy, 1960) is an example of the kind of reaction that might occur. For this reason particular care should be exercised in interpreting the presence of the two or more new radioactive spots; for example, the naphthylamines (Table 2). In some cases the added compound has sufficient sites which could be sulphated to allow multiple spots to be accounted for solely as sulphate esters of the added acceptor. Thus propane-1:2-diol gives three spots which could be the two isomeric monosulphates

and the disulphate, but such an explanation will not hold for propane-1:3-diol (Table 2). Tyramine and L-adrenaline each give three spots which could be any of the two isomeric ring *O*-sulphates, the *N*-sulphate and, with adrenaline, the side-chain *O*-sulphate, or combinations. Another consideration is that the added acceptor may be metabolized in some way before or after sulphation. For example, in particle-free liver extracts L-adrenaline is known to be *ortho*-methylated (Axelrod, 1957) under certain circumstances.

The failure of the liver extract to sulphate bilirubin is unexpected since bilirubin sulphate has been found in rat bile (Isselbacher & McCarthy, 1959). Choline sulphate, which is formed by fungi and algae (Spencer & Harada, 1959), was not synthesized by the liver extract. None of the hydroxyamino acids was sulphated at the concentration employed (0·01 M) but it should be pointed out that very little sulphation of the primary aliphatic alcohols was observed at this concentration (cf. Table 2 and Vestermark & Bostrom, 1959*b*). One point that should be considered in evaluating these negative results is that sulphation may occur but the sulphate ester is hydrolysed by a sulphatase in the liver extract. However, with choline it is known that mammalian tissues are unable to desulphate choline sulphate (Morimoto, 1937).

SUMMARY

1. In the presence of adenosine triphosphate, Mg^{2+} and SO_4^{2-} ions particle-free supernatants of iso-osmotic-potassium chloride homogenates of rat liver accumulated adenosine 3′-phosphate 5′-sulphatophosphate. The rate of accumulation of adenosine 3′-phosphate 5′-sulphatophosphate depended both on the rate of synthesis and the rate of breakdown.

2. In the absence of adenosine triphosphate the rat-liver preparation degraded adenosine 3′-phosphate 5′-sulphatophosphate to SO_4^{2-} ions and adenosine monophosphate and eventually adenosine. One mechanism of degradation involved 3′-dephosphorylation to give adenosine 5′-sulphatophosphate, which was then desulphated yielding SO_4^{2-} ions and adenosine monophosphate. Both stages of this reaction were inhibited by ethylenediaminetetra-acetic acid.

3. A further fate of the sulphate group of adenosine 3′-phosphate 5′-sulphatophosphate in the presence of the liver extract was to be transferred to endogenous acceptors with the formation of sulphate esters.

4. Extracts of kidney, spleen, heart, lung and testes produced adenosine 3′-phosphate 5′-sulphatophosphate and transferred sulphate from this compound to endogenous acceptors.

5. Evidence has been obtained which suggests that some of the endogenous acceptors are phenols and possibly steroids.

6. The radiochromatograph patterns of urine and bile from rats which had received injections of $Na_2{}^{35}SO_4$ were compared with the radiochromatographs of the sulphated endogenous acceptors of liver. Some common radioactive spots were observed.

7. On incubation of various potential sulphate acceptors with the liver extract in the presence of adenosine triphosphate, Mg^{2+} and $^{35}SO_4^{2-}$ ions new radioactive spots were observed on chromatograms. Evidence suggesting that these spots were the sulphate esters of the acceptors has been presented but pitfalls in the interpretation of the chromatograms have been pointed out.

8. In this system sulphate esters were formed from all the tested phenols, steroids, alcohols and arylamines. Tyramine, adrenaline and propane-1:2-diol each gave three new radioactive spots. Bilirubin, cholic acid, L-threonine, L-serine, L-hydroxyproline; L-hydroxyglutamic acid and choline chloride did not appear to be sulphated.

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