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The Metabolic Fate of the Ester Sulphate Group of Potassium *p*-Nitrophenyl [^{35}S]Sulphate

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The fate of arylsulphates in the animal body has been investigated by a number of workers. Early studies by Rhode (1923), Sperber (1948) and Garton & Williams (1949) suggested that almost the whole of the administered dose of potassium phenyl sulphate was rapidly eliminated unchanged in the urine. Hawkins & Young (1954), using radioactive techniques, observed only slight hydrolysis (4–12%) of orally administered and intraperitoneally injected phenyl [^{35}S]sulphate and naphthyl [^{35}S]-sulphate. In contrast, the results of Hanahan & Everett (1950) indicate that the bulk of the dose of oestrone [^{35}S]sulphate is desulphated by adult female rats, and there is indirect evidence which suggests that appreciable hydrolysis of stilboestrol disulphate (Bishop, Richards & Perry, 1951) and of sulphated insulin (containing sulphated tyrosine residues, see Glendening, Greenberg & Fraenkel-Conrat, 1947) occurs *in vivo*. Michel, Roche, Closon & Michel (1958) noted that ^{131}I-labelled 3:5:3′-tri-iodo-L-thyronine sulphate is partially desulphated *in vivo* by the rat and eliminated as the corresponding glucosiduronic acid derivative. At first sight these collected results might suggest that only the sulphate esters of phenols which can be regarded as 'physiologically active' are extensively hydrolysed *in vivo*.

Mammalian tissues contain at least three distinct enzymes which are capable of hydrolysing arylsulphates. The specificity of these enzymes is not yet clearly defined, but there are indications that two of them, arylsulphatases A and B, show high affinity and activity towards arylsulphates such as dipotassium 2-hydroxy-5-nitrophenyl sulphate and monopotassium 4-chlorocatechol monosulphate (Dodgson & Spencer, 1957; Dodgson & Wynn, 1958), but comparatively low activity towards simpler arylsulphates such as potassium *p*-acetylphenyl sulphate and potassium *p*-nitrophenyl sulphate (cf. Dodgson, Rose & Tudball, 1959). In contrast, the third enzyme, arylsulphatase C, is particularly active towards these simpler arylsulphates. In spite of these differing specificities, all three enzymes show only comparatively feeble activity and affinity towards phenyl sulphate (Dodgson *et al.* 1959), and this can probably be attributed to the absence of an *ortho* or *para* electrophilic substituent group in the substrate molecule (cf. Dodgson, Spencer & Williams, 1956). It is perhaps not surprising therefore that phenyl sulphate is not extensively hydrolysed *in vivo*. Similar considerations probably apply to naphthyl sulphate.

In attempts to elucidate the physiological role of the arylsulphatase enzymes, recent work in these Laboratories has been directed towards a study of the ability of the sulphate esters of 'physiologically active' phenols to act as substrates for the various arylsulphatases both *in vivo* and *in vitro*. In many cases, the chemical synthesis of radioactive (^{35}S) specimens of these particular sulphate esters presents formidable problems. It was therefore considered desirable, in order to gain experience with various techniques, to conduct preliminary investigations with potassium *p*-nitrophenyl [^{35}S]sulphate, an arylsulphate which is readily prepared on a small scale and which, from theoretical considerations, might be expected to undergo appreciable desulphation *in vivo*.

The results of this preliminary study are presented in this paper.

MATERIALS AND METHODS

Potassium p-*nitrophenyl* [^{35}S]*sulphate*. This was prepared by a modification of the method of Burkhardt & Lapworth (1926). Carrier-free chloro[^{35}S]sulphonic acid (0·261 g., specific activity 10·4 mc/m-mole) was mixed with 0·28 g. of

unlabelled chlorosulphonic acid at 0°. To this was added 2 ml. of carbon disulphide followed by 1·72 ml. of diethylaniline, care being taken that the temperature did not rise above 5° during the addition. The temperature was then allowed to rise to that of the room, when 0·59 g. of *p*-nitrophenol suspended in 2 ml. of carbon disulphide was added with stirring. After stirring intermittently over a period of 2 hr. the whole was cooled in an ice–salt mixture, when the supernatant carbon disulphide could be poured off. Sufficient 10% KOH was added to make the residual syrup slightly alkaline, care being taken to keep the temperature below 35°. Water (10 ml.) was added to the alkaline syrup and the whole was extracted with three 20 ml. portions of ether to remove excess of diethylaniline. The cooled aqueous residue was adjusted to approx. pH 3 with N-H_2SO_4 before removal of excess of *p*-nitrophenol by extraction with three 20 ml. portions of ether. The pH of the aqueous residue was raised to about 11 with 10% KOH, and the whole poured into 4 vol. of acetone at 0°. The precipitated $K_2{}^{35}SO_4$ was kept at 0° for 1 hr. and removed by centrifuging; the supernatant was concentrated *in vacuo* at 37°. The residue was recrystallized twice from 90% ethanol and dried *in vacuo* at 0°. The yield of potassium *p*-nitrophenyl [^{35}S]sulphate (NP^{35}S) was 0·75 g. The radioactivity of the preparation was of the order of 15·2 μc/mg. Descending paper chromatography on Whatman no. 1 paper with butan-1-ol–acetic acid–water (50:12:25) showed the material to be homogeneous and free from inorganic [^{35}S]sulphate.

Experimental animals. Mature M.R.C. hooded rats were used throughout. These animals were injected intraperitoneally under light ether anaesthesia and were subsequently placed in metabolism cages designed to permit separate collection of urine and faeces. The animals were allowed water without restriction and were removed from the cages for 30 min. in each 24 hr. period, when they were permitted to feed. Any urine and faeces voided during this period were rejected. Urine and faeces were collected every 24 hr., the cages being washed down with 150 ml. of water. The urine and washings were pooled, filtered through a plug of cotton wool and, whenever possible, were used immediately; otherwise they were stored at 2° until required.

Measurement of radioactivity of fractions of urine. The inorganic sulphate fraction of the urine was precipitated as $Ba^{35}SO_4$ by a modification of the method of Folin (1905). A portion of the urine (15 ml.) was transferred to a tared, specially designed (see Walkenstein & Knebel, 1957) 50 ml. centrifuge tube and a known amount of carrier M-K_2SO_4 solution was added to give a final precipitate weight of approx. 100 mg. of $Ba^{35}SO_4$. A portion (4 ml.) of 2·5 N-HCl was then added, followed by 4 ml. of aqueous 10% $BaCl_2$, added dropwise. The tube was capped with Parafilm (A. Gallenkamp and Co. Ltd., London, E.C. 2) and allowed to stand without disturbance at room temperature for 16–20 hr. The precipitated $Ba^{35}SO_4$ was separated by centrifuging and washed three times by suspending it in 40 ml. portions of water, the suspension being kept for 1 hr., before each re-centrifuging. The precipitate was finally washed with 40 ml. of acetone and dried at 110° before weighing. Preliminary experiments showed that NP^{35}S was not hydrolysed during this procedure nor did it interfere in any way.

The precipitation of the combined inorganic and ester sulphate fraction of urine was carried out as described above, after hydrolysis of the urine (15 ml.) with 5 ml. of 2·5 N-HCl for 90 min. at the temperature of the steam bath. Preliminary experiments showed that no significant hydrolysis of NP^{35}S occurred during the period between the voiding of the urine and the analysis thereof.

The total sulphur fraction was also precipitated as $Ba^{35}SO_4$, after oxidation of the urine with fuming HNO_3 (sp.gr. 1·5) according to the method of Young, Edson & McCarter (1949).

The precipitated $Ba^{35}SO_4$ was plated for counting by a technique similar to that described by Eldjarn & Nygaard (1954). The amount of $Ba^{35}SO_4$ present in each planchet was determined by weighing the latter before and after plating. Radioactivity measurements were made with a thin end-window Geiger–Müller tube. Corrections for background, self-absorption, decay and coincidence were made and sufficient counts were recorded to give a standard error of less than 2%.

Measurement of ^{35}S content of faeces and tissues. The total sulphur content of faeces, individual tissues or of the whole rat was precipitated and counted as $Ba^{35}SO_4$ after oxidation with fuming HNO_3 according to the method of Young *et al.* (1949).

Detection of radioactivity on paper chromatograms. Radioactive spots were located on chromatograms with the aid of the C. 100 Actigraph automatic chromatogram-strip scanner (Nuclear-Chicago Corp., Ill., U.S.A.). Alternatively, radioautograms were obtained with Ilford Industrial B X-ray film. The exposure time was usually 21 days.

EXPERIMENTAL AND RESULTS

Rats were injected intraperitoneally with 5 or 40 μmoles of NP^{35}S in 0·5 ml. of water. In initial experiments, urine and faeces were collected every 24 hr. for a total of 96 hr. However, the amount of ^{35}S appearing in urine and faeces during the second half of this period was insignificant (less than 0·7% of the injected dose) and in subsequent experiments urine and faeces were collected during 48 hr. only. After 48 hr. the animals were killed by a blow on the back of the head and the whole carcass was taken for the determination of residual ^{35}S. Table 1 records the results. The faecal ^{35}S values are not included since, for the whole 48 hr. period, these were always less than 0·6% of the injected dose.

With male rats given the lower dose approx. 90% of the radioactivity of the dose was recovered from urine, faeces and carcass. A substantial amount (25%) of the initial dose of ^{35}S was present as inorganic sulphate. With the higher dose the recoveries of ^{35}S were rather low (80%) and the degree of hydrolysis of NP^{35}S was somewhat reduced. With the female rats the recovery of ^{35}S was complete, with the inorganic [^{35}S]sulphate accounting for 29·5% of the initial dose of NP^{35}S. No great significance can be attached to the variations between the three groups of animals in view of the small number of animals tested. It is, however, clear that appreciable hydrolysis of NP^{35}S occurs *in vivo*.

Retention of ^{35}S in tissues

Two male rats were injected with 5 μmoles of NP^{35}S and after 48 hr. they were killed. The individual tissues from each rat were combined, washed with water, dried between

Table 1. *Distribution of* ^{35}S *in the urine and carcass of rats injected intraperitoneally with potassium* p-*nitrophenyl* [^{35}S]*sulphate*

Results are average values with the range in parentheses. ^{35}S was precipitated and counted as $Ba^{35}SO_4$ by the procedures described in the text. The radioactivity of the respective doses was $6{\cdot}2 \times 10^5$ counts/min. (5 μmoles) and 5×10^6 counts/min. (40 μmoles), measured after hydrolysis as an infinitely thick plate of $Ba^{35}SO_4$.

Sex	Dose (μmoles)	No. of animals	Range of body wt. (g.)	Distribution of ^{35}S in urine (% of ^{35}S injected)						Residual ^{35}S in carcass after 48 hr. (% of ^{35}S injected)
				Inorganic sulphate fraction		Combined inorganic and ester sulphate fraction		Total urinary sulphur		
				24 hr.	48 hr.	24 hr.	48 hr.	24 hr.	48 hr.	
Male	5	7	300–400	22·8 (15·4–30·2)	1·5 (1·1–2·0)	81·2 (74·9–89·6)	1·6 (1·3–1·8)	83·2 (76·9–91·6)	3·0 (2·8–3·5)	3·5 (2·8–4·1)*
Male	40	3	220–280	17·5 (13·4–22·2)	1·6 (1·3–2·3)	70·8 (68·4–71·3)	2·4 (1·7–3·2)	72·6 (70·2–73·1)	3·6 (2·5–5·5)	3·0 (1·4–5·5)
Female	5	3	250–310	26·7 (15·9–32·6)	2·8 (1·7–3·1)	99·0 (98·3–99·6)	3·0 (2·1–4·0)	99·5 (99·0–99·5)	3·2 (2·5–4·1)	2·5 (1·8–3·0)

* Average of four rats.

filter paper, weighed and dissolved in a known volume of fuming HNO_3. The ^{35}S content of suitable volumes (usually 1 ml.) was precipitated as $Ba^{35}SO_4$ after oxidation. Blood was collected by heart puncture and treated in a similar fashion. Table 2 shows that all the tissues examined retained some ^{35}S. Almost the whole of the radioactivity of blood was associated with the plasma. Skin and cartilage also retained ^{35}S (see below).

Appearance of ^{35}S *in skin, cartilage and liver*

Since injected inorganic sulphate is incorporated into the sulphated mucopolysaccharides of connective tissue (e.g. Dziewiatkowski, 1951; Boström, 1952) and into the liver taurine (Boström & Åqvist, 1952), it was investigated whether a similar uptake of [^{35}S]sulphate occurred after administration of $NP^{35}S$. Six rats (four male and two female) each received injections of 5 and, 2 hr. later, 10 mg. of $NP^{35}S$. The rats were kept in pairs in metabolism cages for 24 hr. and the urine was collected for chromatographic experiments. The rats were then killed and the skins, livers and sternal, xiphisternal and tracheal cartilage were pooled.

Treatment of skin and cartilage. All hair was removed with a razor before maceration of the skin in acetone at 0°. The defatted material was then dried in air (total wt. from six rats was 53·2 g.). Cartilage was also macerated in acetone at 0° and dried (total wt. 1·53 g.). The dried skin or cartilage was extracted by mechanical shaking for 24 hr. at 27° with 0·5 N-NaOH (15 ml./g. of material). The cloudy supernatant separated by centrifuging for 30 min. at 5000 *g* was stored at 0° while the residue was re-extracted as before with 0·5 N-NaOH (5 ml./g. of original dried material) and centrifuged. The extracts were combined, toluene was added as a preservative and the whole was dialysed against running tap water for 48 hr., followed by distilled water for 48 hr. at 4°. The pH of the extract was adjusted to 7·8 with N-NaOH before incubation with papain (L. Light and Co. Ltd.; 10 mg./g. of original dried material) at 37° for 5 days. The incubation was carried out in dialysis tubing immersed in 0·1 M-NaH_2PO_4 which had been adjusted to pH 7·8 with N-NaOH. After incubation, one-third of the volume of 40% trichloroacetic acid was added and precipitated material separated by centrifuging. The supernatant was dialysed for 48 hr. against several changes of distilled water at 4° before being concentrated

Table 2. *Retention of* ^{35}S *by rat tissues* 48 *hr. after intraperitoneal injection of potassium* p-*nitrophenyl* [^{35}S]*sulphate*

Each rat received 5 μmoles of $NP^{35}S$, the radioactivity of the dose being $6{\cdot}2 \times 10^5$ counts/min., measured after hydrolysis as an infinitely thick plate of $Ba^{35}SO_4$.

Tissue	Total wt. (from two rats)	^{35}S content (counts/min./g. wet wt. of tissue)
Spleen	2·1	110
Pancreas	1·9	110
Kidney	5·4	160
Liver	25·1	100
Heart and aorta	2·3	250
Blood	—	58*

* Counts/min./ml.

to one-tenth of its volume *in vacuo* at 37°. The concentrated extract was stirred for 30 min. with potassium acetate, acetic acid and kaolin (1 g., 1 ml. and 1 g. respectively/50 ml. of concentrate). The supernatant separated by centrifuging at 5000 *g* for 15 min. was kept at 0° while the residue was washed three times by suspending it in 50 ml. portions of water and re-centrifuging. All the supernatants were combined and concentrated to low bulk *in vacuo* at 37°. Ethanol (4 vol. of 95%, v/v) was added and the whole kept overnight at 0°. The precipitated material was separated by centrifuging and washed well with ethanol followed by ether. The material was dried *in vacuo* at 2° over P_2O_5. The yield of crude mucopolysaccharide from skin was 785 mg., and from cartilage, 73 mg.

The crude mucopolysaccharide was further purified by paper electrophoresis. The cartilage preparation (34 mg.) and that from skin (120 mg.) were dissolved in 0·1 M-ammonium acetate–acetic acid buffer, pH 6·0 (5 μl./mg. of mucopolysaccharide) and subjected to horizontal paper electrophoresis in the same buffer, on Whatman no. 17 paper for 16 hr. at a potential gradient of 6 v/cm. Control preparations of bovine cartilage chondroitin sulphate (Dodgson, Lloyd & Spencer, 1957) and hyaluronic acid (Wyeth, Philadelphia, Pa., U.S.A.) were run simultaneously. Acid mucopolysaccharides were detected by pressing a strip of Whatman no. 1 paper on the wet no. 17 paper at the end of the run and subsequently staining the 'print' with the Alcian blue technique of Heremans & Vaerman (1958). With the skin preparation two staining zones corresponding to hyaluronic acid and chondroitin sulphate were detected, whereas with the cartilage preparation only the chondroitin sulphate zone was observed. The appropriate areas of the electrophoresis strip were then eluted with water and the eluates concentrated to a syrup *in vacuo* at 37°. Acid mucopolysaccharide was then precipitated by the addition of 50 ml. of 95% ethanol containing 0·5 g. of potassium acetate. The precipitated material was separated by filtration through weighed filter-paper disks. The disks were subsequently dried, reweighed and counted for radioactivity. The hyaluronic acid fraction from skin, as expected, contained no radioactivity. The chondroitin sulphate fraction from skin showed 36 counts/min./mg. and that from cartilage 146.

Treatment of liver. Taurine was isolated from the liver by a procedure based on that of Boström & Åqvist (1952). The livers (64·6 g.) were macerated in 200 ml. of ethanol. The whole was filtered, the filtrate being retained and the residue was then re-extracted with a further 100 ml. of ethanol. The pooled extracts were stored at 4° whilst the residue was refluxed for 2 hr. with ethanol–ether (3:1, v/v). All extracts were combined and concentrated to dryness *in vacuo*. The concentrate was dissolved in the smallest possible volume of $CHCl_3$ and re-precipitated by pouring into a large excess of acetone. The precipitated material was separated, re-dissolved in $CHCl_3$ and re-precipitated with acetone. The precipitate was separated and hydrolysed by boiling under reflux with 100 ml. of 6 N-HCl for 24 hr. After cooling, the solidified fatty acids were removed and the solution was concentrated to dryness *in vacuo* at 37°. The residue was dissolved in water and then re-concentrated as before to remove excess of HCl. This procedure was repeated several times. The residue was finally dissolved in 4 ml. of water and a portion (2 ml.) was applied to a column (15 cm. × 1 cm.) of Dowex-50 ion-exchange resin (H^+ form, × 8, mesh 200–400) which had been equilibrated with 0·1 M-sodium citrate–HCl buffer, pH 3·41, as described by Moore & Stein (1951). The column was eluted with the same buffer and the first 50 ml. of eluate was collected in 25 equal fractions. Horizontal paper electrophoresis on Whatman no. 1 paper in 0·1 M-ammonium acetate–acetic acid buffer, pH 4·5, with a potential gradient of 12 v/cm. for 2 hr., showed that fractions 10–15 contained one ninhydrin-positive component only. The electrophoretic mobility of the component was identical with that of an authentic sample of taurine. These fractions were pooled, concentrated to dryness and the residue was dissolved in the minimum volume of water and subjected to paper electrophoresis as described above but with Whatman no. 17 paper. The taurine was located on a 'print' by spraying with ninhydrin and the appropriate zone was eluted with water. The concentrated elute was counted for radioactivity. The total yield (from 64·6 g. of liver) gave 312 counts/min. No quantitative significance could be attached to the count since the material was contaminated with appreciable amounts of the citrate buffer which was used to elute the taurine from the ion-exchange column.

Chromatographic behaviour of ^{35}S*-labelled compounds in urine*

The undiluted urine of male and female rats which had each received 15 mg. of NP^{35}S as described above was subjected to paper chromatography on Whatman no. 1 paper with butan-1-ol–acetic acid–water (50:12:25) with downward irrigation. Where practical, sufficient urine was applied to the paper to give approx. 1000 counts/min. (measured on the paper with a Panax monitor). Urines from animals which had received lower doses (1·28 mg.) of NP^{35}S were treated in a similar fashion. In addition, urines from male and female rats which had each been injected with a mixture of 0·1 ml. of a carrier-free solution of $Na_2{}^{35}SO_4$ (0·5 mc) and 0·4 ml. of 0·15 M-Na_2SO_4 solution, were examined simultaneously. Chromatograms were run for 16 hr. at room temperature and dried at the same temperature before being examined for radioactivity. Preliminary experiments in which freshly voided normal rat urine (15 ml.) was kept with 15 mg. of NP^{35}S at room temperature for 24 hr. before subjecting it to paper chromatography showed that NP^{35}S was stable under these conditions and that no exchange of sulphate occurred between the compound and other sulphate esters present in the urine.

Table 3 records the chromatographic mobilities (in relation to that of NP^{35}S) of the various radioactive spots which were detected on chromatograms of the urine of female rats receiving either $Na_2{}^{35}SO_4$ or NP^{35}S. After administration of $Na_2{}^{35}SO_4$, 11 radioactive spots were obtained in addition to inorganic [^{35}S]sulphate. After administration of NP^{35}S, ten radioactive spots in addition to inorganic [^{35}S]sulphate and NP^{35}S were detected. Spot 7 was not present on chromatograms of the $Na_2{}^{35}SO_4$ urines and had the same chromatographic mobility as that of an authentic specimen of *p*-aminophenyl sulphate, a possible metabolite of NP^{35}S. Other differences observed were the absence of spots 2 and 13 from the NP^{35}S urine and the absence of spot 11 (possibly a second metabolite of NP^{35}S) from the $Na_2{}^{35}SO_4$ urines. Spot 15 could not be detected in the NP^{35}S urine because of the overlap of the very dense

Table 3. *Chromatographic mobilities in butan-1-ol–acetic acid–water* (50:12:25) *of radioactive compounds present in the urine of female rats receiving potassium* p-*nitrophenyl* [^{35}S]*sulphate or* $Na_2{}^{35}SO_4$

Experimental details are given in the text. The mobilities quoted are average values obtained from several different experiments and are expressed (R_{NPS}) relative to that of NP^{35}S, which is taken as unity.

	R_{NPS}	
Spot no.	$Na_2{}^{35}SO_4$ urine	NP^{35}S urine
1 ($Na_2{}^{35}SO_4$)	0·12	0·12
2	0·19	—
3	0·23	0·23
4	0·33	0·33
5	0·41	0·41
6	0·45	0·45
7	—	0·55
8	0·59	0·59
9	0·65	0·65
10	0·71	0·71
11	—	0·75
12	0·83	0·83
13	0·92	—
14 (NP^{35}S)	—	1·00
15	1·04	?

spot associated with NP^{35}S. The chromatographic pattern of the urine of male rats receiving $Na_2{}^{35}SO_4$ was virtually identical with that of female rats. On the other hand, with the NP^{35}S urines of male rats only spots 1, 3, 6, 7, 8, 10, 12 and 14 (see Table 3) could be detected with certainty.

Similar chromatographic experiments were run with NP^{35}S and $Na_2{}^{35}SO_4$ urines which had been hydrolysed at 95° with N-HCl for 90 min. or, alternatively, which had been incubated for 4 hr. at 37° and pH 6·5 with the digestive juice of *Helix pomatia* (the richest known source of arylsulphatase and a potent source of steroid sulphatase; see Henry & Thevenet, 1952). In these cases, only inorganic [^{35}S]sulphate could be detected on chromatograms, indicating that the radioactive compounds present in the unhydrolysed urines were all ester sulphates (either aryl- or 3β-sulphates of 5α- and Δ^5-steroids).

DISCUSSION

The results obtained provide strong evidence that hydrolysis of arylsulphates by the arylsulphatase enzymes does occur *in vivo*. Alternative mechanisms whereby inorganic sulphate could arise from NP^{35}S are, of course, possible. For example, excretion of NP^{35}S via the bile could be followed by degradation of the ester by the bacterial flora of the gut with subsequent reabsorption of inorganic sulphate from the large intestine. A similar effect has been observed in rats by Dohlman (1956) after the injection of ^{35}S-labelled chondroitin sulphate. However, it is doubtful whether a biliary circulation of this sort could account for the amounts of inorganic [^{35}S]sulphate which were found in the 24 hr. urines in the present work. The negligible amounts of radioactivity which were observed in faeces also support this conclusion. It is perhaps significant that Hawkins & Young (1954) failed to obtain any marked increase in the output of inorganic [^{35}S]sulphate in the urine when phenyl [^{35}S]sulphate was administered orally rather than by intraperitoneal injection.

If it is accepted that arylsulphatase activity is mainly responsible for the production of inorganic sulphate from NP^{35}S then it can tentatively be assumed, from specificity studies (see Dodgson *et al.* 1959), that arylsulphatase C is the responsible enzyme. Moreover, the fact that the degree of hydrolysis observed in the present work is greatly in excess of that noted for phenyl [^{35}S]sulphate (4% in 4 days) by Hawkins & Young (1954), suggests that the relative specificity of the enzyme in the living organism is similar to that shown *in vitro*.

The complex pattern of radioactivity in chromatograms of the urines of rats receiving $Na_2{}^{35}SO_4$ (see also Boström & Vestermark, 1959) or NP^{35}S is of interest. No attempt has yet been made to identify the unknown components but it seems clear that they are either aryl or steroid sulphate esters. For animals injected with $Na_2{}^{35}SO_4$ it is virtually certain from the work of Lipmann and others (see Dodgson, 1958) that these sulphate esters arise from the inorganic sulphate through the intermediate formation of 3′-phosphoadenosine 5′-phosphosulphate ('active sulphate'), and the subsequent transfer of sulphate from this compound under the influence of the appropriate sulphokinase. With animals receiving NP^{35}S, the urine ester [^{35}S]sulphate pattern is, in most respects, similar to that of the urine of animals receiving $Na_2{}^{35}SO_4$. There are two theoretically possible metabolic routes whereby the sulphate group of NP^{35}S could be made available to other phenols or steroids. Hydrolysis of NP^{35}S by arylsulphatase enzymes would provide inorganic sulphate which could subsequently be used for the sulphation of the appropriate phenolic and steroid acceptors by the usual 3′-phosphoadenosine 5′-phosphosulphate synthetic route. However, Gregory & Lipmann (1957) have shown that, in the presence of 3′:5′-diphosphoadenosine, liver phenolsulphokinase can also catalyse the direct transfer of sulphate from NPS to suitable phenolic acceptors, provided that the product has a sulphate group potential well below that of NPS. 3′:5′-Diphosphoadenosine acts as a coenzyme for this transfer. This direct type of mechanism may be contributing significantly to the production of some of the radioactive arylsulphate esters which were present in the NP^{35}S urines.

Whether, in the presence of the appropriate sulphokinases, NP^{35}S can transfer sulphate, via 3′:5′-diphosphoadenosine, to non-phenolic acceptors is

still not known. Attempts by Gregory & Lipmann (1957) to test this possibility with enzyme preparations containing both phenol and steroid sulphokinases were unsuccessful. In view of this it is also wise to assume, for the time being, that the ^{35}S which appears in skin and cartilage chondroitin sulphate after injection of NP^{35}S is not incorporated by direct transfer from the latter. More probably, inorganic sulphate derived from NP^{35}S by action of arylsulphatase is the starting material for this incorporation (see D'Abramo & Lipmann, 1957).

It is possible, of course, that the arylsulphatase enzymes might themselves be capable of transferring sulphate directly from NP^{35}S to a variety of acceptors. Attempts have been made in these Laboratories (unpublished) to transfer sulphate from NPS or dipotassium 2-hydroxy-5-nitrophenyl sulphate (nitrocatechol sulphate) to alcohols, phenols, simple sugars, amino sugars, *N*-acetylchondrosin and chondroitin, with purified arylsulphatases of mammalian and molluscan origin. No evidence of transfer has yet been obtained.

The occurrence of small amounts of ^{35}S in rat-liver taurine after the administration of NP^{35}S provides some support for the view that inorganic sulphate sulphur can give rise to taurine sulphur in the intact rat (Boström & Åqvist, 1952). A similar utilization of inorganic sulphate for taurine synthesis has been noted in the developing chick embryo (Machlin, Pearson & Denton, 1955; Lowe & Roberts, 1955). The process does not appear to involve the preliminary formation of sulphur-containing amino acids and the biosynthetic route involved is at present obscure.

SUMMARY

1. After intraperitoneal injection of potassium *p*-nitrophenyl [^{35}S]sulphate to male and female rats, the bulk of the ^{35}S appears in the urine within 24 hr.

2. Up to 30% of the ^{35}S of the original dose appears in the urine as inorganic [^{35}S]sulphate, most of the remainder appearing as ester [^{35}S]-sulphate.

3. Chromatographic examination of the urines shows the presence of at least ten radioactive sulphate esters in addition to potassium *p*-nitrophenyl [^{35}S]sulphate. The chromatographic pattern is very similar to that obtained with the urines of rats receiving $Na_2{}^{35}SO_4$ by intraperitoneal injection.

4. After 48 hr., ^{35}S is still present in the tissues. Incorporation of ^{35}S into the chondroitin sulphate of skin and cartilage and into liver taurine has been demonstrated.

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