The Metabolism of Glycyl-L-serine O^[35S]-Sulphate in the Rat

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It is now well established that mammalian tissues contain an enzyme system that can desulphate L-serine O-sulphate both *in vivo* (Tudball, 1962) and *in vitro* (Dodgson & Tudball, 1961). Information about the precise nature of the system is very limited and it is still not clear whether a simple hydrolase is involved. However, the localization of the enzyme system in the high-speed supernatant fractions of tissue homogenates shows that it cannot be identified with any of the known mammalian sulphotase enzymes. To obtain further information on this desulphating system, studies have been extended to include simple peptides containing sulphated L-serine residues. The present paper deals with the preparation and the metabolism in the rat of glycyl-L-serine O^[35S]-sulphate. A preliminary account of some of this work has already been published (Noda, Tudball & Dodgson, 1963).

MATERIALS AND METHODS

Potassium glycyl-L-serine O-sulphate. Before preparation of ³⁵S-labelled glycyl-L-serine O-sulphate, the unlabelled compound was prepared and its authenticity established. The method of preparation was based on that described for L-serine O-sulphate by Dodgson, Lloyd & Tudball (1961).

A portion (0.4 g.) of glycyl-L-serine (Sigma Chemical Co., St Louis, Mo., U.S.A.) was added at room temperature to 0.8 ml. of H₂SO₄ (sp.gr. 1.84), and the mixture stirred for 10 min. and then kept *in vacuo* for 50 min. before being poured into a suspension of Ba(OH)₂.8H₂O (5.5 g.) in 60 ml. of ice-cold water. The BaSO₄ was removed by centrifuging and the supernatant concentrated to 5 ml. *in vacuo* at 38°. The concentrate was then applied to a column (1 cm. × 7 cm.) of Dowex 50 ion-exchange resin (20–50 mesh; H⁺ form) (Dow Chemical Co., Mich., U.S.A.). The column was washed with 20 ml. of water and the combined eluate and washings were adjusted to pH 7.8 with aq. 5% (w/v) KOH before being evaporated to dryness *in vacuo* at 38°. The residue was dissolved in the minimum amount of water and precipitated with acetone. The white crystalline precipitate was separated, washed with acetone followed by ether and dried *in vacuo* over CaCl₂ at room temperature. The yield was 200 mg., with $[\alpha]_D^{25} - 0.4^\circ$ (c 3.7 in water) (Found: ester SO₄²⁻ ion, 33.0; amino N, 5.1; K, 13.9; C₆H₅KN₂O₇, requires ester SO₄²⁻ ion, 34.3; amino N, 5.0; K, 13.9%).

The infrared-absorption spectrum was characteristic of an O-sulphate ester (Lloyd, Tudball & Dodgson, 1961), and

determination of amino N by formol titration showed that N-sulphation had not occurred. The stability of the ester sulphate link in acid and alkali was similar to that of L-serine O-sulphate (Dodgson *et al.* 1961).

The homogeneity of the preparation was checked by descending paper chromatography on Whatman no. 1 paper in butan-1-ol-acetic acid-water (50:12:25, by vol.) and 2-methylpropan-2-ol-formic acid-water (8:3:4, by vol.). Spots were detected with ninhydrin. The preparation moved as a single spot in both solvent systems (*R_F* 0.12 and *R_F* 0.36 respectively). The preparation also moved as a single spot when subjected to horizontal paper electrophoresis on Whatman no. 1 paper in the presence of 0.25 N-acetic acid, 0.1 M-ammonium acetate or 0.1 M-veronal buffer, pH 8.0.

Potassium glycyl-L-serine O^[35S]-sulphate. This was prepared by treating 0.578 g. of glycyl-L-serine with 0.75 ml. of H₂³⁵SO₄ (sp.gr. 1.84; specific activity 2.4 mc/m-mole) under the conditions described for the unlabelled ester. The yield was 488 mg. (specific activity 8.65 μC/mg.). The chromatographic and electrophoretic mobilities of the product were identical with those of authentic potassium glycyl-L-serine O-sulphate. The material was homogeneous and contained no free inorganic ³⁵S-sulphate.

Experimental animals. Male M.R.C. hooded rats (3 months old) were used except where otherwise stated. Animals were fed and housed, and urine and faeces collected, as described by Dodgson & Tudball (1960).

Measurement of radioactivity in urine, faeces and bile. The procedures for assaying ³⁵S in urine and faeces described by Tudball (1962) were used. Total bile samples were diluted to 15 ml. with water and treated in the same way as urine.

Detection of radioactivity on paper chromatograms and paper-electrophoresis strips. The procedures described by Dodgson & Tudball (1960, 1961) were employed.

EXPERIMENTAL AND RESULTS

Animals under light ether anaesthesia were injected intraperitoneally with 10 μmoles of potassium glycyl-L-serine O^[35S]-sulphate dissolved in 0.5 ml. of water. Samples of urine and faeces were collected after 24 and 48 hr. Preliminary experiments had shown that most of the radioactivity appeared in the urine within 48 hr. after injection, and animals were killed after this time and the residual ³⁵S in the carcass was determined as described by Dodgson & Tudball (1960). The results show that about 48% of the ³⁵S recovered in the urine was present as inorganic ³⁵S-sulphate. A small percentage (5%) of the activity appeared in the faeces (Table 1).

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Table 1. *Distribution of ^{35}S in the urine and carcass of rats injected intraperitoneally with glycyl-L-serine $\text{O}[^{35}\text{S}]$ -sulphate*

Eight animals (body wt. range 210–220 g.) were used. The radioactivity of the injected glycyl-L-serine $\text{O}[^{35}\text{S}]$ -sulphate was 75.3×10^3 counts/min. (as BaSO_4 in infinitely thick layer). The results are given as average values with the ranges in parentheses.

	^{35}S recovered in 48 hr. (% of ^{35}S injected)		Distribution of ^{35}S in urine (% of ^{35}S recovered in urine)	
	Inorganic sulphate fraction	Total sulphate fraction	Inorganic sulphate fraction	Total sulphate fraction
Urine (0–24 hr.)	30.7 (24.0–40.1)	75.4 (62.9–86.3)	42.6 (35.1–47.5)	92.3 (87.2–95.6)
Urine (24–48 hr.)	3.8 (2.9–5.2)	5.0 (3.8–7.2)	5.6 (3.5–9.5)	7.4 (4.3–12.8)
Carcass	—	4.5 (3.8–6.2)*	—	—
Faeces	—	5.2 (4.9–5.5)*	—	—
Total	—	93.0 (88.7–99.8)	—	—

* Values after oxidation.

Dohlman (1956) and Lloyd (1961) have shown that micro-organisms present in the intestinal flora of rats can desulphate some carbohydrate sulphate esters. Homogenates of rat faeces can also liberate inorganic sulphate from L-serine O-sulphate (Tudball, 1962). The possibility therefore existed that the inorganic ^{35}S sulphate present in rat urine after the injection of glycyl-L-serine $\text{O}[^{35}\text{S}]$ -sulphate could have arisen from contamination of urine with faeces or, alternatively, by a more circuitous route involving excretion of the labelled ester into the gut via the bile, desulphation by the gut flora and subsequent reabsorption of inorganic ^{35}S sulphate into the circulation. Rat-faecal homogenates were therefore tested for their ability to desulphate the labelled ester by the procedure described by Tudball (1962), except that $10\text{ }\mu\text{moles}$ of the ester were present in the incubation mixture. During incubation for 72 hr., ^{35}S appeared in inorganic sulphate at a linear rate of 13 % per 24 hr. When rat urine was substituted for faeces, no desulphation was observed.

To eliminate unequivocally the possibility that faecal micro-organisms might be responsible for some of the desulphation that was observed *in vivo*, the following experiment was performed. Catheters were inserted into the bile ducts of three rats (7 months old) under Nembutal anaesthesia and $10\text{ }\mu\text{moles}$ of the labelled ester in 0.5 ml. of water were administered to each animal via the jugular vein. The whole of the bile produced by each rat in 4.5 hr. was then collected. Each rat was killed, the urine removed from the bladder with a syringe and the carcass analysed. Less than 1.5 % of the administered activity appeared in the bile during the experimental period; almost half was excreted in the urine, 37 % of which was present as inorganic ^{35}S sulphate (Table 2). It was therefore concluded that the extensive desulphation of glycyl-L-serine $\text{O}[^{35}\text{S}]$ -sulphate that occurs *in vivo* cannot be attributed to the activity of faecal micro-organisms.

Table 2. *Excretion and distribution of ^{35}S in bile, urine and carcass of rats 4.5 hr. after the administration of glycyl-L-serine $\text{O}[^{35}\text{S}]$ -sulphate*

Three animals (body wt. range 330–350 g.) were used. The radioactivity of the injected glycyl-L-serine $\text{O}[^{35}\text{S}]$ -sulphate was 37.7×10^3 counts/min. (as BaSO_4 in infinitely thick layer). The results are given as average values with the ranges in parentheses.

	Recovery of activity (% of ^{35}S injected)	
	Inorganic sulphate fraction	Total sulphate fraction
Urine	18.0 (13.2–23.1)	48.5 (41.0–53.9)
Bile	0.32 (0.27–0.4)	1.5 (1.3–1.8)
Carcass	—	40.5 (38.5–42.5)*

* Values after oxidation.

The small amount of the injected ester that entered the gut via the bile presumably accounted for the ^{35}S detected in faeces (Table 1).

Paper chromatography of the urine of rats receiving glycyl-L-serine $\text{O}[^{35}\text{S}]$ -sulphate. In preliminary experiments, the labelled ester was added to normal rat urine before chromatography; no exchange of ^{35}S took place with other sulphate esters present. Samples ($30\text{ }\mu\text{l.}$) of the 24 hr. urine of rats that had been injected with the labelled ester were subjected to paper chromatography in butan-1-ol-acetic acid-water (50:12:25, by vol.). Radioactive zones with R_f values of 0.09, 0.16, 0.29 and 0.63 were detected, the mobilities of the first two zones being identical with those of inorganic ^{35}S sulphate and glycyl-L-serine $\text{O}[^{35}\text{S}]$ -sulphate respectively. The remaining zones, which were of relatively weak intensity, probably reflect the utilization of some of the liberated inorganic ^{35}S sulphate for the synthesis of normal urinary sulphate esters (see Dodgson & Tudball, 1960; Tudball, 1962).

Activity of the desulphating enzyme towards glycyl-L-serine $\text{O}[^{35}\text{S}]$ -sulphate. The extent of the desul-

phation of glycyl-L-serine $O[^{35}\text{S}]$ -sulphate that occurred *in vivo* suggested that the desulphating enzyme possessed appreciable activity towards the ester. However, the dipeptide derivative was a relatively poor substrate for partially purified preparations of the desulphating enzymes of sheep and rat livers compared with L-serine O-sulphate. A 10% homogenate of the fresh livers of sheep or rat in ice-cold water was centrifuged at 10500g for 1 hr. The clear supernatant was adjusted to pH 5 with N-acetic acid, allowed to stand for 30 min. at 0° and centrifuged at 3000g for 15 min., and the clear supernatant was readjusted to pH 7 with N-sodium hydroxide. Ammonium sulphate was added at 0°, and the material that was precipitated between 50% and 60% saturation was dissolved in 15 ml. of ice-cold water and dialysed for 36 hr. at 2° against several changes of water.

The activity of the dialysed material towards L-serine O-sulphate and glycyl-L-serine O-sulphate was followed by determining liberated SO_4^{2-} ions by method B of Dodgson (1961). A portion (100 $\mu\text{l.}$) of a 0.1 M substrate solution in 0.5 M-tris-acetic acid buffer, pH 7.0, was incubated with 100 $\mu\text{l.}$ of enzyme solution for 1 hr. at 38°. Enzyme action was stopped by the addition of 25% (w/v) trichloroacetic acid (30 $\mu\text{l.}$). Appropriate control experiments were made. Both enzyme sources exhibited relatively little activity towards glycyl-L-serine O-sulphate when compared with that shown towards L-serine O-sulphate, the ratios of the activities being 1:36 and 1:18 for rat and sheep preparations respectively.

Action of dipeptidase preparations on glycyl-L-serine $O[^{35}\text{S}]$ -sulphate. The results of the experiments *in vitro* described above seemed to be incompatible with the findings *in vivo*. However, the appearance of inorganic $[^{35}\text{S}]$ sulphate in urine after the injection of glycyl-L-serine $O[^{35}\text{S}]$ -sulphate may have arisen from hydrolysis of the peptide bond by a dipeptidase to give L-serine $O[^{35}\text{S}]$ -sulphate, which is known to be almost completely desulphated *in vivo* (Tudball, 1962). The possibility of the presence of an appropriate dipeptidase in rat tissues was investigated. Crude preparations of rat-intestine and rat-kidney enzymes were obtained by a method that was essentially that of Harris & Fruton (1951). Finely chopped intestine or whole kidney (2 g.) was stirred at 4° with 20 ml. of water for 30 min. and the whole was dialysed against 10 l. of water at 4° for 16 hr. The dialysed material was filtered through glass wool and the cloudy filtrate used as the enzyme source. Samples (100 $\mu\text{l.}$) of the enzyme preparations were incubated with glycyl-L-serine $O[^{35}\text{S}]$ -sulphate (100 $\mu\text{l.}$ of a 0.1 M solution in 0.08 M-tris-acetic acid buffer, pH 7.9) at 38° for 2 hr. Enzyme activity was stopped by immersing the reaction tubes in boiling

water for 2 min. Appropriate control determinations were made in which enzyme and substrate were incubated separately and then mixed immediately before the heat treatment. Precipitated protein was removed by centrifuging at 3000g for 15 min. and samples (15 $\mu\text{l.}$) of the clear supernatants were subjected to two-way ascending paper chromatography on Whatman 3MM paper with pyridine-water (13:7, v/v) and butan-1-ol-acetic acid-water (50:12:25, by vol.) as first and second solvents respectively. Similar samples were subjected to paper electrophoresis on Whatman 3MM paper at a potential gradient of 22 v/cm. for 3 hr. in the presence of 0.25 N-acetic acid. Spots were detected with ninhydrin. Markers of glycine, L-serine, L-serine O-sulphate, glycyl-L-serine and glycyl-L-serine O-sulphate were run. Similar experiments were carried out with glycyl-L-serine as substrate. The results showed that glycyl-L-serine $O[^{35}\text{S}]$ -sulphate and glycyl-L-serine were hydrolysed by the intestine preparations to give glycine and L-serine $O[^{35}\text{S}]$ -sulphate on the one hand and glycine and L-serine on the other. However, the results with rat-kidney preparations and glycyl-L-serine $O[^{35}\text{S}]$ -sulphate showed in addition small amounts of inorganic $[^{35}\text{S}]$ sulphate. To avoid this complication rat-intestine preparations were examined since this tissue is known to be a rich source of dipeptidase enzymes; moreover, preliminary experiments showed that intestine preparations were unable to desulphate either glycyl-L-serine $O[^{35}\text{S}]$ -sulphate or L-serine $O[^{35}\text{S}]$ -sulphate (see also Dodgson & Tudball, 1961). The procedures used were identical with those described for rat kidney. Preparations of rat intestine hydrolysed glycyl-L-serine $O[^{35}\text{S}]$ -sulphate, yielding glycine and L-serine $O[^{35}\text{S}]$ -sulphate as the sole products.

These results suggest that the inorganic $[^{35}\text{S}]$ -sulphate that appears in the urine after the injection of glycyl-L-serine $O[^{35}\text{S}]$ -sulphate could arise, at least in part, by preliminary fragmentation of the sulphated dipeptide in either the intestine or the kidney to yield L-serine $O[^{35}\text{S}]$ -sulphate, which then acts as the substrate for the desulphating enzyme.

DISCUSSION

After the intraperitoneal injection of glycyl-L-serine $O[^{35}\text{S}]$ -sulphate into rats almost 50% of the ^{35}S recovered in the urine within the next 48 hr. was in the form of inorganic $[^{35}\text{S}]$ sulphate. Most of this liberated sulphate can clearly be attributed to enzyme action in the tissues rather than to the activity of gut micro-organisms. Some ester-bound ^{35}S does find its way into the intestine via the bile and this, no doubt, accounts for the traces of ^{35}S that can be detected in faecal pellets.

It is not possible to say whether the liberation of inorganic sulphate that occurs in the living animal is due to the direct hydrolysis of the dipeptide sulphate or of L-serine O[³⁵S]-sulphate liberated as a result of dipeptidase activity. Possibly both routes are involved. Chromatography showed that some of the injected material appears in the urine in an unchanged form, but no free L-serine O[³⁵S]-sulphate was detected although an exhaustive search for this compound was not made. This need not necessarily mean that dipeptidases are without effect on the sulphated dipeptide *in vivo*, since any L-serine O[³⁵S]-sulphate liberated as a result of dipeptidase might well undergo immediate desulphation.

The physiological significance of the desulphation process is still obscure. Sulphated L-serine residues have never been detected in proteins, although the corresponding phosphorylated derivatives are of fairly widespread occurrence.

SUMMARY

1. Potassium glycyl-L-serine O-sulphate and the corresponding ³⁵S-labelled ester were prepared.

2. Partially purified preparations of rat and sheep livers, capable of desulphating L-serine O-sulphate, exhibited feeble activity towards glycyl-L-serine O-sulphate. However, after the intraperitoneal administration of potassium glycyl-L-serine O[³⁵S]-sulphate to rats, about 83 % of the radioactivity of the dose was recovered in the urine. Almost 50 % of this recovered radioactivity was in the form of inorganic [³⁵S]sulphate.

3. Analysis of urines showed the presence of at least four radioactive materials, two of which moved with the mobilities of inorganic [³⁵S]sulphate and glycyl-L-serine O[³⁵S]-sulphate.

4. Faecal preparations were capable of desulphating the ³⁵S-labelled ester. Bile catheter experiments showed that faecal micro-organisms did not contribute appreciably to the formation of inorganic [³⁵S]sulphate found in rat urine after injection of the ester.

5. Crude preparations of rat kidney and rat intestine were capable of cleaving the sulphated peptide to yield glycine and L-serine O-sulphate.

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Polybasic and Polyacidic Substances or Aggregates and the Excitability of Cerebral Tissues, Electrically Stimulated *in vitro*

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Lysine polymers and some basic proteins render cerebral tissues unresponsive to electrical excitation; certain acidic substances of molecular weight about 1000 or more restore excitability (McIlwain, 1959, 1961). The changes in excitability were concluded to be due to the basic peptides hindering the re-establishment of membrane potentials, and this to be dependent on an action of the peptides in diminishing particular ion movements at the tissue

(Hillman, 1961; McIlwain, Woodman & Cummins, 1961); each of these actions is antagonized by the acidic substances. Substances of both categories become attached to the tissues whose excitability they alter (Thomson & McIlwain, 1961; Balakrishnan & McIlwain, 1961).

Many simple acidic substances do not restore excitability, but the few substances known to restore it are very disparate in structure. They in-