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# The Metabolism of L-Serylglycine *O*[$^{35}$S]-Sulphate in the Rat

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1. The preparation of potassium L-serylglycine *O*-sulphate and the corresponding $^{35}$S-labelled ester is described. 2. Intraperitoneal injection of potassium L-serylglycine *O*[$^{35}$S]-sulphate to rats results in about 75% of the radioactivity of the dose appearing in the urine within 48hr. Almost 72% of the radioactivity recovered in the urine was in the form of inorganic [$^{35}$S]sulphate. 3. Analysis of urines by paper chromatography showed the presence of unchanged L-serylglycine *O*[$^{35}$S]-sulphate and several other unidentified $^{35}$S-labelled materials. 4. It has been established that micro-organisms of the gastrointestinal tract do not play any significant role in the production of inorganic [$^{35}$S]sulphate from the injected ester. 5. L-Serylglycine *O*-sulphate was hydrolysed by crude dipeptidase preparations from rat kidney and intestine to yield L-serine *O*-sulphate and glycine as the sole products.

Evidence has accumulated that shows that L-serine *O*-sulphate, both in the free and peptide-bound form, undergoes desulphation *in vivo* and *in vitro* (Dodgson, Lloyd & Tudball, 1961; Dodgson & Tudball, 1961; Tudball, 1962; Tudball, Noda & Dodgson, 1964). The enzyme system responsible for the desulphation of L-serine *O*-sulphate is widely distributed amongst higher animals but its presence in micro-organisms has thus far been limited to its demonstration in *Pseudomonas aeruginosa* (Harada, 1964). The liberation of inorganic sulphate from both free and peptide-bound L-serine *O*-sulphate by suspensions of rat faeces (Tudball, 1962; Tudball *et al.* 1964) presumably also reflects the activity of faecal micro-organisms.

As an extension of investigations into the mammalian enzyme system responsible for the desulphation of L-serine *O*-sulphate, both in the free and peptide-bound form, the metabolism of L-serylglycine *O*[$^{35}$S]sulphate in the rat has now been examined.

## MATERIALS AND METHODS

*Potassium L-serylglycine O-sulphate.* L-Serylglycine, the starting material employed in the synthesis, was prepared by the methods of Fruton (1942) and Sheehan & Hess (1955). The method of Dodgson *et al.* (1961) for the sulphation of hydroxylated amino acids was then adapted to the preparation of L-serylglycine *O*-sulphate, and the authenticity of the product was established before attempting to prepare $^{35}$S-labelled material.

L-Serylglycine (400mg.) was added at room temperature to 0·5ml. of $H_2SO_4$ (sp.gr. 1·84) and the mixture was stirred for 5min. before keeping *in vacuo* over $CaCl_2$ for 1hr. The reaction mixture was poured into a suspension of $Ba(OH)_2,8H_2O$ (3·5g.) in 75ml. of ice-cold water to neutralize the excess of $SO_4^{2-}$ ions. Precipitated $BaSO_4$ was removed by centrifuging and the clear supernatant applied to a column (1cm. × 12cm.) of Dowex 50 ion-exchange resin ($H^+$ form; 20–50 mesh) (Dow Chemical Co., Midland, Mich., U.S.A.). The column was washed with water until the eluate was no longer acid, and the eluate and washings were combined and adjusted to pH7–8 with aq. 5% (w/v) KOH before concentrating to small volume (0·5ml.) *in vacuo* at 38°. Crystallization was induced by the dropwise addition of ethanol, and the crystals were separated in the centrifuge and washed with ethanol followed by ether. The material was finally dried *in vacuo* over $CaCl_2$. The yield was 331mg.; $[\alpha]_D^{20}$ 1·7° (*c* 0·64 in water) (Found: ester $SO_4^{2-}$ ion, 33·5; K, 14·4. $C_5H_9KN_2O_7S$ requires ester $SO_4^{2-}$ ion, 34·3; K, 13·9%).

The infrared-absorption spectrum of the ester was marked by the appearance of strong absorption bands in the ranges 1210–1250 and 770–810cm.$^{-1}$, characteristic of the spectra obtained with the *O*-sulphate esters of serine, threonine and hydroxyproline (see Lloyd, Tudball & Dodgson, 1961). There was no absorption at 3320cm.$^{-1}$, a region ascribed to vibrations involving the unsubstituted hydroxyl group. These observations confirm that the method of preparation yields *O*- rather than *N*-sulphates.

Descending paper chromatography on Whatman no. 1 paper in butan-1-ol–acetic acid–water (50:12:25, by vol.) and ethanol–urea–water (80:0·5:20, v/w/v) yielded a single ninhydrin-positive spot in each solvent system ($R_F$ values 0·18 and 0·16 respectively). The preparation was also homogeneous when subjected to horizontal paper electrophoresis on Whatman no. 1 paper in the presence of aq. 0·1M-ammonium acetate or 0·05M-veronal buffer, pH8·0.

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*Potassium* L-*serylglycine* O[$^{35}$S]-*sulphate*. The method of preparation was that described above for the unlabelled ester except that 0·5ml. of $H_2{}^{35}SO_4$ (sp.gr. 1·84; specific activity 50·6mc/g.) was used. The yield was 418mg., specific activity 17 $\mu$c/mg. The material moved as a single ninhydrin-positive zone having the mobility of authentic L-serylglycine *O*-sulphate when subjected to two-way ascending paper chromatography on Whatman no. 1 paper with 2-methylpropan-2-ol–formic acid–water (8:3:4, by vol.) as the first solvent and propan-1-ol–ammonia (20% soln.)–water (6:3:1, by vol.) as the second solvent. Paper electrophoresis on Whatman no. 1 paper in 0·1M-ammonium acetate showed the material to be homogeneous and free from inorganic [$^{35}$S]sulphate.

During the experimental period over which the labelled ester was used, some inorganic [$^{35}$S]sulphate was liberated when the material was stored in the solid form at low temperatures. This effect could be effectively minimized by storing the labelled ester in frozen aqueous solution at −20°.

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

*Measurement of* $^{35}$S *radioactivity*. The $^{35}$S content of urine, faeces and carcass was assayed by the procedure of Dogdson & Tudball (1960). Bile samples were treated as described by Tudball *et al.* (1964).

*Detection of* $^{35}$S *radioactivity on paper chromatograms and paper-electrophoresis strips*. The procedures of Dodgson & Tudball (1960, 1961) were employed.

## EXPERIMENTAL AND RESULTS

Rats were injected intraperitoneally under light ether anaesthesia with 10 $\mu$moles of potassium L-serylglycine *O*[$^{35}$S]-sulphate in 0·5ml. of water. Total urine and faeces samples were collected 24 and 48hr. after the injection. After 48hr. the animals were killed by a blow on the back of the head and the whole carcass was assayed for $^{35}$S radioactivity. Preliminary experiments showed that after 48hr. negligible amounts of $^{35}$S appeared in the excreta.

With male rats approx. 54% of the dose, and with female rats approx. 39% of the dose, appeared in the urine as inorganic [$^{35}$S]sulphate (Table 1). It is not possible to say whether these values reflect a sex difference because of the small number of animals tested. Moreover, the female animals were of an age group (6 weeks old) that was different from that of the males (12 weeks).

Micro-organisms present in the gastrointestinal tract of the rat are known to be capable of desulphating carbohydrate sulphates (Dohlman, 1956; Lloyd, 1961) and amino acid and peptide sulphates (Tudball, 1962; Tudball *et al.* 1964). It is possible that injected L-serylglycine *O*[$^{35}$S]-sulphate might pass into the gastrointestinal tract, via the bile, and then be desulphated by micro-organisms to yield inorganic [$^{35}$S]sulphate, which could be reabsorbed into the circulation and subsequently appear in the urine. To check this possibility, catheters were inserted into the bile duct and bladders of three female rats (5 months old) maintained under Nembutal anaesthesia and the labelled ester (10 $\mu$moles in 0·5ml. of water) was administered to each animal via the jugular vein. The rats were kept under Nembutal anaesthesia with periodic infusions of glucose for 5hr. Samples of bile and urine were collected at 30min. intervals for the whole of the experimental period and the inorganic and total

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

Five male animals (body wt. range 260–320g.) and three female animals (body wt. range 75–80g.) were used. The specific radioactivity of the injected L-serylglycine *O*[$^{35}$S]-sulphate was $4{\cdot}27 \times 10^4$ counts/min./$\mu$mole (measured as $Ba^{35}SO_4$ in infinitely thick layer). The results given are 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 |
| Male | Urine (0–24 hr.) | 50·9 (47·9–59·0) | 71·4 (67·4–74·7) | 70·2 (64·7–79·2) | 94·1 (93·2–94·6) |
| | Urine (24–48hr.) | 3·6 (3·6) | 4·2 (4·2) | 4·9 (4·5–5·8) | 5·8 (5·3–6·8) |
| | Carcass | — | 4·9 (3·4–7·3)* | — | — |
| | Faeces | — | 8·0 (5·5–9·3)* | — | — |
| | Total | — | 88·0 (85·2–90·6) | — | — |
| Female | Urine (0–24hr.) | 40·3 (35·3–45·3) | 61·1 (56·0–66·3) | 59·3 (57·5–62·3) | 91·5 (91·2–91·9) |
| | Urine (24–48hr.) | 4·5 (3·3–5·5) | 4·9 (3·2–6·4) | 7·8 (7·5–8·3) | 8·5 (8·3–8·8) |
| | Carcass | — | 10·7 (9·2–12·1) | — | — |
| | Faeces | — | 9·0 (7·4–10·6)* | — | — |
| | Total | — | 86·6 (84·0–89·3) | — | — |

* Values after oxidation.

Table 2. *Excretion and distribution of* $^{35}S$ *in bile, urine and carcass of rats 5 hr. after the administration of* L-*serylglycine O*[$^{35}S$]-*sulphate*

Three female rats (body wt. range 145–203 g.) were used. The specific radioactivity of the injected L-serylglycine *O*[$^{35}S$]-sulphate was $1{\cdot}69 \times 10^4$ counts/min./$\mu$mole (as $BaSO_4$ in infinitely thick layer). The results are given as average values with the ranges in parentheses.

| | Recovery of radioactivity (% of $^{35}S$ injected) | |
|---|---|---|
| | Inorganic sulphate fraction | Total sulphate fraction |
| Urine | 34·17 (25·75–40·77) | 42·12 (35·57–47·7) |
| Bile | 1·08 (0·8–1·33) | 5·3 (4·6–6·19) |
| Carcass | — | 40·3* |

* Value after oxidation.

sulphate fractions assayed for $^{35}S$ content. At the end of the experimental period the animals were killed and the $^{35}S$ remaining in the whole carcass was determined. Table 2 shows that, over a period of 5 hr., 42% of the administered dose appeared in the urine and that 81% of this was inorganic [$^{35}S$]sulphate. During this time only 5% of the dose was excreted via the bile. Though this small biliary excretion presumably accounts for the appearance of $^{35}S$ in faeces in experiments with intact animals (see Table 1) it could not account for the rapid appearance of large amounts of inorganic sulphate in the urine.

Nevertheless, micro-organisms of the gastrointestinal tract are able to desulphate L-serylglycine *O*[$^{35}S$]-sulphate. When examined by the procedure of Tudball *et al.* (1964), faecal suspensions (2%) incubated with 0·6 mM solutions of substrate for 24, 48 and 72 hr. at 20° liberated 13, 38 and 45% respectively of the available $^{35}S$ in the form of inorganic [$^{35}S$]sulphate. When rat urine was similarly tested, no liberation of sulphate occurred.

*Enzymic desulphation of* L-*serylglycine* O-*sulphate.* It has been previously demonstrated that the enzyme system that desulphates L-serine *O*-sulphate exhibited relatively little activity *in vitro* towards glycyl-L-serine *O*-sulphate (Tudball *et al.* 1964). Since appreciable desulphation of L-serylglycine *O*[$^{35}S$]-sulphate occurred *in vivo* it was conceivable that it might prove an efficient substrate for the desulphating enzyme. A partially purified (20-fold) preparation of the rat-liver enzyme (N. Tudball & J. H. Thomas, unpublished work) was used to test this possibility. The activity of the preparation towards L-serine *O*-sulphate and L-serylglycine *O*-sulphate was followed by estimating liberated $SO_4^{2-}$ ions according to the procedure of Dodgson (1961; method A). Substrate (200 $\mu$l. of a 0·1 M solution in 0·5 M-sodium acetate–acetic acid solution, pH 7·0) was incubated with 100 $\mu$l. of a 12 mM solution of mercaptoethanol in the same buffer and 100 $\mu$l. of enzyme preparation for 3 hr. at 38°. At the end of this time, enzyme action was stopped by the addition of aq. 25% (w/v) trichloroacetic acid (60 $\mu$l.). Appropriate control experiments were carried out simultaneously. The ratio of enzyme activities towards L-serine *O*-sulphate and L-serylglycine *O*-sulphate was 27:1. This ratio is similar to that obtained by Tudball *et al.* (1964) when glycyl-L-serine *O*-sulphate was compared with L-serine *O*-sulphate.

*Dipeptidase activity towards* L-*serylglycine* O-*sulphate.* Glycyl-L-serine *O*-sulphate is capable of acting as a substrate for dipeptidase preparations from rat kidney and intestine (Tudball *et al.* 1964). It follows that part of the desulphation observed *in vivo* in the present investigation after the administration of the $^{35}S$-labelled dipeptide sulphate ester to rats could arise from serine *O*[$^{35}S$]-sulphate liberated from the dipeptide sulphate by a dipeptidase. Consequently, rat kidney and intestine were examined for dipeptidase action, with L-serylglycine *O*-sulphate as substrate.

The preparation and assay of tissue extracts was carried out as described by Tudball *et al.* (1964), except that L-serylglycine and L-serylglycine *O*-sulphate were used as substrates. Both substrates were hydrolysed by kidney and intestine dipeptidase preparations. The parent dipeptide was degraded to yield glycine and L-serine, and the sole products resulting from the breakdown of the dipeptide sulphate were glycine and L-serine *O*-sulphate. There is thus a real possibility that the liberation of free L-serine *O*[$^{35}S$]-sulphate *in vivo* plays a part in the appearance of inorganic [$^{35}S$]sulphate in urine after the administration of L-serylglycine *O*[$^{35}S$]-sulphate.

*Attempted sulphation of serine residues in dipeptides.* It has recently been demonstrated that carboxyl-blocked L-tyrosine residues but not free L-tyrosine may be sulphated by the particle-free supernatant fraction of rat-liver preparations (Jones & Dodgson, 1964). Free L-serine does not undergo biological sulphation in the usual liver sulphating system (Spencer, 1960; Dodgson *et al.* 1961), though, by analogy with L-tyrosine, sulphation of blocked L-serine derivatives could occur. L-Serylglycine and glycyl-L-serine (Sigma Chemical Co., St Louis, Mo., U.S.A.) were used as acceptors and conditions similar to those used for the sulphation of L-tyrosine residues were employed.

To 30 $\mu$l. of a particle-free rat-liver supernatant in iso-osmotic potassium chloride (see Spencer, 1960) were added 5 $\mu$c of carrier-free $Na_2{}^{35}SO_4$, 10 $\mu$l. of an aq. 0·33 M solution of acceptor adjusted to pH 7·2 with N-sodium hydroxide and 10 $\mu$l. of a solution (adjusted to pH 7·2) containing 1 $\mu$mole of ATP,

1 $\mu$mole of potassium dihydrogen phosphate, 0·3 $\mu$mole of magnesium chloride and 0·3 $\mu$mole of cysteine hydrochloride. As a control, 10 $\mu$l. of water was substituted for the acceptor solution. Incubation was for 1 hr. at 38°. Enzyme action was stopped by immersing the reaction tubes in boiling water for 30 sec. and coagulated protein removed by centrifuging at 2000*g* for 15 min. The clear supernatant (10 $\mu$l.) was then subjected to two-way chromatography as described above for L-serylglycine O[$^{35}$S]-sulphate. Marker chromatograms of L-serylglycine O[$^{35}$S]-sulphate and glycyl L-serine O[$^{35}$S]-sulphate were run simultaneously. Dried chromatograms were placed in contact with Ilford Industrial B X-ray film for 7 days to locate the radioactive zones. The presence of a radioactive zone on the test chromatogram having the same mobility as the marker material was taken as the criterion of acceptor sulphation. No radioactive zones other than those due to endogenous acceptors present in the liver preparations were observed, indicating that the added acceptors were not sulphated under the experimental conditions employed.

*Paper chromatography of rat urine after the administration of* L-*serylglycine* O[$^{35}$S]-*sulphate.* Urines were collected for a period of 24 hr. after the intraperitoneal administration of 10 $\mu$moles of L-serylglycine O[$^{35}$S]-sulphate to three male rats. As a control, 10 $\mu$moles of the labelled ester in 0·5 ml. of water were placed in the collection vessel before collecting a 24 hr. urine sample from a normal male rat in the usual way. Test and control urines were treated with solid barium hydroxide octahydrate until precipitation was complete. The precipitated material was removed by centrifuging, the clear supernatant was added to a column (1 cm. × 20 cm.) of Dowex 50 ($H^+$ form; 200–400 mesh) and the acid eluate collected in 1 ml. portions. The fractions containing $^{35}$S radioactivity were combined, adjusted to neutrality with aq. 1% (w/v) potassium hydroxide and concentrated *in vacuo* at 38° to approx. 1 ml. Portions (10 $\mu$l.) of the concentrated solution were subjected to two-way ascending chromatography and radioactive areas located as described above. The pattern of the test chromatogram was complex, showing many radioactive zones. One of these zones could be associated with L-serylglycine O[$^{35}$S]-sulphate, the other zones probably arising from conjugation of liberated inorganic [$^{35}$S]sulphate *in vivo* with endogenous acceptors in the liver (cf. Dodgson & Tudball, 1960; Tudball, 1962).

## DISCUSSION

The results of this and previous investigations (Tudball *et al.* 1964) make it abundantly clear that L-serine O-sulphate residues bound in peptide form are extensively degraded *in vivo*. It is by no means certain how sulphate is released from such compounds, though the enzyme system that desulphates L-serine O-sulphate is almost certainly involved, either directly or indirectly. The presence both in rat kidney and intestine of a system capable of degrading the dipeptide sulphate to glycine and L-serine O-sulphate could indicate that such a cleavage occurs before desulphation. Possibly sulphate is liberated *in vivo* before and after cleavage of the peptide linkage, though which is the major sulphate-forming pathway is difficult to assess. Since the activity of the desulphating enzyme towards the dipeptide sulphate *in vitro* is much smaller than that observed towards L-serine O-sulphate, it is tempting to suggest that scission of the peptide bond followed by sulphate liberation from L-serine O-sulphate is the major sulphate-forming pathway. However, information about the affinity of the dipeptidase system for the dipeptide sulphate is not available and further conjecture should be reserved.

Chemically sulphated insulin in which the hydroxyl groups of L-serine and L-threonine had been esterified showed unimpaired hormonal activity *in vivo*, as demonstrated by the blood-sugar-lowering potential (Glendening, Greenberg & Fraenkel-Conrat, 1947). It was concluded that aliphatic hydroxyl groups played no role in determining the biological activity of insulin, since regeneration of insulin in the body was considered unlikely unless the occurrence of a specific enzyme system was postulated. It is now known that L-serine O-sulphate residues are extensively degraded *in vivo*. Moreover, L-threonine O-sulphate also undergoes appreciable desulphation *in vivo* (N. Tudball, unpublished work). The conclusions of Glendening *et al.* (1947) should thus be viewed with caution in the light of present knowledge.

Sulphated serine derivatives have never been found in Nature, and it is extremely doubtful if L-serine or its derivatives could undergo sulphation in the usual liver sulphating system. The energy requirements for such a reaction may well be prohibitively high, as appears to be the case for the analogous phosphorylation of L-serine (Dayan & Wilson, 1963).

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