A Possible Route to the Production of Free L-Tyrosine O-Sulphate by the Rat

By J. M. BASFORD, J. G. JONES, F. A. ROSE AND K. S. DODGSON
Department of Biochemistry, University College, St Andrew's Place, Cardiff

(Received 26 October 1965)

1. L-Tyrosylglycine $O[^{35}\text{S}]$ -sulphate is metabolized by the rat to yield the $O[^{35}\text{S}]$ -sulphate esters of L-tyrosine, *p*-hydroxyphenylpyruvic acid and *p*-hydroxyphenylacetic acid. 2. The proportion of the administered peptide which is excreted as L-tyrosine $O[^{35}\text{S}]$ -sulphate is greater at a higher dose. 3. An enzyme capable of hydrolysing the peptide bond of L-tyrosylglycine $O[^{35}\text{S}]$ -sulphate to yield L-tyrosine $O[^{35}\text{S}]$ -sulphate has been detected in rat liver and kidney. 4. The activity of this enzyme is completely inhibited by a large excess of L-tyrosylglycine.

L-Tyrosine *O*-sulphate was first discovered as a component of bovine fibrinopeptide B (Bettelheim, 1954). Subsequently this amino acid has been found in many mammalian fibrinogens (see Doolittle & Blombäck, 1964), in gastrin II isolated from hog antral mucosa (Gregory, Hardy, Jones, Kenner & Sheppard, 1964) and, in the free state, in mammalian urines (see John, Rose, Wusteman & Dodgson, 1965). The metabolism of administered L-tyrosine *O*-sulphate by the rat has been reported and the results show that the ester sulphate group is metabolically inert (see also Dodgson, Rose & Tudball, 1959) but the amino acid is metabolized to yield the *O*-sulphate esters of *p*-hydroxyphenylpyruvic acid and *p*-hydroxyphenylacetic acid (Dodgson, Powell, Rose & Tudball, 1961; Powell, Rose & Dodgson, 1963). In contrast, the mechanism of the biosynthesis of free L-tyrosine *O*-sulphate remains obscure and all attempts to transfer sulphate to free L-tyrosine using the usual 3'-phosphoadenylyl sulphate-phenol-sulphotransferase system (EC 2.8.2.1) have failed (see Jones & Dodgson, 1965). However, free L-tyrosine *O*-sulphate is produced in a rat-liver sulphating system when L-tyrosine methyl (or ethyl) ester is used as acceptor of sulphate (Jones & Dodgson, 1965). L-Tyrosine methyl ester *O*-sulphate is first formed and subsequently demethylated by a hydrolytic enzyme present in the relatively crude enzyme preparations (Jones & Dodgson, 1965). It has recently been shown that similar enzyme preparations can transfer sulphate to L-tyrosylglycine and L-tyrosyl-L-alanine but the resultant *O*-sulphate esters, unlike the *O*-sulphate ester of L-tyrosine methyl ester, are not subsequently degraded to yield free L-tyrosine *O*-sulphate (Jones, Scotland & Dodgson, 1966). The present work

provides an explanation for the failure to produce L-tyrosine *O*-sulphate in these experiments and shows that chemically synthesized L-tyrosylglycine $O[^{35}\text{S}]$ -sulphate is metabolized by the rat *in vivo* and also *in vitro* to yield free L-tyrosine $O[^{35}\text{S}]$ -sulphate. A preliminary account of this work has already been given (BASFORD, JONES, ROSE & DODGSON, 1965).

MATERIALS AND METHODS

Sulphate esters. Potassium L-tyrosine *O*-sulphate was prepared by the method described by Dodgson *et al.* (1959), and the ^{35}S -labelled ester was prepared as described by Dodgson *et al.* (1961). Potassium L-tyrosylglycine $O[^{35}\text{S}]$ -sulphate (2.2 $\mu\text{C}/\mu\text{mole}$) was prepared from L-tyrosylglycine (Yeda Research and Development Co. Ltd., Rehovoth, Israel) by the method described by Dodgson *et al.* (1961) for the sulphation of L-tyrosine. Ultraviolet-absorption spectra and chromatographic and electrophoretic mobilities of L-tyrosylglycine $O[^{35}\text{S}]$ -sulphate agreed with those reported for the unlabelled ester (Jones *et al.* 1966). Potassium *p*-hydroxyphenylacetic acid *O*-sulphate was prepared by the reaction of *p*-hydroxyphenylacetic acid with chlorosulphonic acid (N. Tudball, unpublished work) and the ^{35}S -labelled ester was kindly provided by Dr N. Tudball.

Paper chromatography and electrophoresis. Urine samples (20 $\mu\text{l.}$) were applied to Whatman no. 1 chromatography paper and subjected to descending chromatography for 16 hr. at room temp. with butan-1-ol-acetic acid-water (50:12:25, by vol.). Samples (20 $\mu\text{l.}$) were also examined by electrophoresis on Whatman no. 1 paper for 6 hr. in 0.1M-sodium acetate-acetic acid buffer, pH 4.5, and a potential gradient of 10 v/cm. Under these conditions L-tyrosylglycine $O[^{35}\text{S}]$ -sulphate moved 2.7 cm., L-tyrosine $O[^{35}\text{S}]$ -sulphate moved 3.8 cm. and *p*-hydroxyphenylacetic acid $O[^{35}\text{S}]$ -sulphate moved 7.8 cm.

Detection of radioactivity. Radioactive areas were located on dried chromatograms and electrophoresis strips by radioautography or scanning with the C100 Actigraph

automatic strip-scanner (Nuclear-Chicago Corp., Des Plaines, Ill., U.S.A.).

Experimental animals and collection of urine and faeces. The experimental animals were mature M.R.C. hooded rats, each weighing approx. 120g. The procedures for injection of the animals and for collection of urine and faeces were as described by Lloyd (1961).

Measurement of radioactivity. The ^{35}S -labelled inorganic and ester sulphate content of 24hr. and 48hr. samples of urine and the total ^{35}S content of faeces (48hr. samples) and the remaining carcasses were estimated by the procedures described by Lloyd (1961).

Preparation of tissue suspensions. M.R.C. hooded rats (approx. 150g.) were killed by a blow on the head and the livers and kidneys removed, blotted and chilled to 0° . Suspensions (20%, w/v) of the tissues were then prepared in 0.15M-KCl with the aid of a glass homogenizer. The suspensions were then centrifuged at $500g_{av}$ for 10min. and the supernatants adjusted to pH 8.0 with 0.1N-NaOH and then stored in 3.0ml. portions at -8° .

In other experiments a high-speed supernatants of rat liver was prepared as described by Jones & Dodgson (1965).

EXPERIMENTAL AND RESULTS

Rats were injected intraperitoneally with L-tyrosylglycine $\text{O}[^{35}\text{S}]$ -sulphate (3.7 μmoles or 13.8 μmoles) dissolved in 0.5ml. of water. Urine and faeces were collected separately and samples taken for determination of ^{35}S . After 48hr. the animals were killed with chloroform and the whole carcass taken for the determination of ^{35}S . The results (Table 1) show that the bulk of the injected ^{35}S was eliminated as ester sulphate within 24hr., only a small amount appearing as inorganic sulphate. The ^{35}S content of 48hr. samples of faeces and the remaining carcasses accounted for 4–7% of the injected dose.

Qualitative analysis of urines of rats receiving 3.7 μmoles of L-tyrosylglycine $\text{O}[^{35}\text{S}]$ -sulphate. Analysis of 24hr. urine samples by paper chromatography and paper electrophoresis followed by radioautography showed the presence of two radio-

active metabolites. The chromatographic mobility of one of the metabolites (R_f 0.47) was identical with that of authentic *p*-hydroxyphenylacetic acid $\text{O}[^{35}\text{S}]$ -sulphate. Similar agreement was obtained between the electrophoretic mobilities of these two compounds. This radioactive metabolite was separated from 2ml. of a 24hr. urine sample by paper chromatography and the appropriate areas were eluted with water. To the combined eluates (approx. 150ml.) was added 150mg. of unlabelled *p*-hydroxyphenylacetic acid *O*-sulphate and the whole evaporated *in vacuo* at 40° to approx. 0.5ml. and then added to 10ml. of ethanol. The white precipitate which separated was washed with 5ml. of ethanol followed by 5ml. of ether and finally dried *in vacuo* over phosphorus pentoxide. A sample (15mg.) of the dry product was retained and the remainder precipitated a further two times as described above, a 15mg. sample being retained after each precipitation. The ^{35}S sulphate content of each sample was then estimated by the method described by Dodgson *et al.* (1961). Material of constant specific activity (270 counts/min./15mg.) was obtained at each precipitation. The ultra-violet-absorption spectrum of the final product in 0.1N-sodium hydroxide was as expected for *p*-hydroxyphenylacetic acid *O*-sulphate (λ_{max} at 265m μ).

The second radioactive metabolite, detected in 24hr. urines of animals receiving 3.7 μmoles of L-tyrosylglycine $\text{O}[^{35}\text{S}]$ -sulphate, exhibited an R_f (0.28) identical with that recorded for *p*-hydroxyphenylpyruvic acid $\text{O}[^{35}\text{S}]$ -sulphate (Powell *et al.* 1963). Chromatograms of the same urines after keeping at room temperature for 2 days showed no trace of this second metabolite but showed an intensified spot corresponding to *p*-hydroxyphenylacetic acid $\text{O}[^{35}\text{S}]$ -sulphate. The spontaneous conversion of *p*-hydroxyphenylpyruvic acid $\text{O}[^{35}\text{S}]$ -sulphate into *p*-hydroxyphenylacetic acid $\text{O}[^{35}\text{S}]$ -sulphate when kept in rat urine has already been

Table 1. *Distribution of ^{35}S in the urine, faeces and carcasses of female rats (approx. 120g.) injected intraperitoneally with L-tyrosylglycine $\text{O}[^{35}\text{S}]$ -sulphate*

Results are average values with the ranges in parentheses. ^{35}S was precipitated and counted as $\text{Ba}^{35}\text{SO}_4$ as described by Lloyd (1961). The radioactivity of the various doses corresponded to approx. 7×10^3 counts/min./ μmole , measured after hydrolysis as an infinitely thick plate of $\text{Ba}^{35}\text{SO}_4$.

Dose (μ moles)	No. of animals	Distribution of ^{35}S in urine (% of ^{35}S injected)				Total ^{35}S content of faeces (% 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			
		24 hr.	48 hr.	24 hr.	48 hr.		
3.7	3	5.0 (4.0-5.6)	4.1 (3.3-5.2)	84.3 (75.9-93.5)	6.4 (4.5-9.0)	6.7 (5.1-8.4)	None detected
13.8	2	6.2 (5.7-6.6)	0.9 (0.8-1.0)	81.4 (75.3-87.5)	3.7 (2.5-4.9)	2.7 (1.7-3.7)	1.6 (1.4-1.8)

described (Powell *et al.* 1963). Finally the electrophoretic mobility of this second metabolite (the metabolite moved 10.4 cm. towards the anode) is in accord with the conclusion that the metabolite is *p*-hydroxyphenylpyruvic acid $O[^{35}\text{S}]$ -sulphate (see Powell *et al.* 1963).

In one experiment an additional radioactive metabolite was detected with mobilities identical with those of authentic L-tyrosine $O[^{35}\text{S}]$ -sulphate (R_F 0.23; distance travelled on electrophoresis 3.8 cm.). This metabolite accounted for 10% of the total radioactivity detected in the urine (estimated from the area of the peaks on the recording chart from the chromatogram scanner). No trace of unchanged L-tyrosylglycine $O[^{35}\text{S}]$ -sulphate could be detected in any experiment.

Qualitative analysis of urine of rats receiving 13.8 μmoles of L-tyrosylglycine $O[^{35}\text{S}]$ -sulphate. After the injection of the larger doses of the sulphate ester, chromatographic and electrophoretic analysis of 24 hr. urines as described earlier revealed the presence of three radioactive metabolites. Two of these metabolites had the same mobilities as, and were therefore assumed to be, the $O[^{35}\text{S}]$ -sulphate esters of *p*-hydroxyphenylacetic acid and *p*-hydroxyphenylpyruvic acid. The third radioactive metabolite possessed mobilities identical with those of L-tyrosine $O[^{35}\text{S}]$ -sulphate, and accounted for 47% of the total radioactivity detected in the urine sample. This metabolite was then isolated from 2 ml. of urine by paper chromatography, mixed with 160 mg. of unlabelled L-tyrosine *O*-sulphate and precipitated four times from a small volume of water by the procedures described above for the identification of *p*-hydroxyphenylacetic acid $O[^{35}\text{S}]$ -sulphate. After each precipitation a 20 mg. sample was retained and assayed for ^{35}S as described earlier. Material of constant specific activity (150 counts/min./20 mg.) was obtained at each precipitation. The ultraviolet-absorption spectrum of the final product in 0.1N-sodium hydroxide was as expected for L-tyrosine *O*-sulphate (λ_{max} , 263 m μ).

No unchanged L-tyrosylglycine $O[^{35}\text{S}]$ -sulphate could be detected in any experiment.

Metabolism of L-tyrosylglycine $O[^{35}\text{S}]$ -sulphate in vitro. L-Tyrosylglycine $O[^{35}\text{S}]$ -sulphate was incubated with liver or kidney suspensions in the following reaction mixture: 250 $\mu\text{l.}$ of tissue suspension, 200 $\mu\text{l.}$ of 0.1M-tris-hydrochloric acid buffer (pH 8.0) and 40 $\mu\text{l.}$ of an aqueous solution of L-tyrosylglycine $O[^{35}\text{S}]$ -sulphate adjusted to pH 8.0 with N-sodium hydroxide (the final concentration of the peptide in the reaction mixture was 1 mM). After incubating at 37° for 2 hr. protein was removed by heating for 15 min. at 100° followed by centrifuging. Samples (40 $\mu\text{l.}$) of the deproteinized incubation mixtures were then examined by paper electrophoresis followed by radioautography. The

results showed that both tissues achieved the complete hydrolysis of the dipeptide to produce free L-tyrosine $O[^{35}\text{S}]$ -sulphate. In control experiments, when the tissue suspensions were heated at 100° for 15 min. before incubation with the substrate, no breakdown of L-tyrosylglycine $O[^{35}\text{S}]$ -sulphate was detected.

Identical results were obtained when rat-liver high-speed supernatant was used as source of enzyme. However, the hydrolysis of L-tyrosylglycine $O[^{35}\text{S}]$ -sulphate by this enzyme preparation was completely prevented by the addition of L-tyrosylglycine (final concentration 68 mM) to the reaction mixture.

DISCUSSION

The present work establishes that L-tyrosylglycine $O[^{35}\text{S}]$ -sulphate is metabolized by the rat *in vivo* to yield the $O[^{35}\text{S}]$ -sulphate esters of L-tyrosine, *p*-hydroxyphenylpyruvic acid and *p*-hydroxyphenylacetic acid. The sequence of reactions leading to the excretion of these products almost certainly begins with the hydrolysis of the peptide bond to yield free L-tyrosine $O[^{35}\text{S}]$ -sulphate and an enzyme capable of achieving this has been detected in preparations of rat liver and kidney. The liberated L-tyrosine $O[^{35}\text{S}]$ -sulphate is then partially deaminated to produce *p*-hydroxyphenylpyruvic acid $O[^{35}\text{S}]$ -sulphate and the responsible enzyme can be detected in liver and kidney preparations if such preparations are suitably fortified with pyridoxal phosphate and 2-oxoglutarate (Flanagan & Rose, 1964; Rose, Flanagan & John, 1966). Finally, *p*-hydroxyphenylacetic acid $O[^{35}\text{S}]$ -sulphate is formed from *p*-hydroxyphenylpyruvic acid $O[^{35}\text{S}]$ -sulphate, probably by the spontaneous oxidative decarboxylation which is known to occur in the presence of urine (Powell *et al.* 1963).

When high doses of L-tyrosylglycine $O[^{35}\text{S}]$ -sulphate are given, large amounts of L-tyrosine $O[^{35}\text{S}]$ -sulphate are excreted, together with relatively small amounts of the *O*-sulphate esters of *p*-hydroxyphenylpyruvate and *p*-hydroxyphenylacetate. This is in accord with the observations of Flanagan, Rose & Powell (1964) on the metabolism of L-tyrosine *O*-sulphate at various doses.

It is worth stressing that the hydrolysis of the peptide linkage of L-tyrosylglycine $O[^{35}\text{S}]$ -sulphate by rat-liver preparations is completely inhibited by an excess of L-tyrosylglycine, and this probably explains why Jones *et al.* (1966) did not detect free L-tyrosine $O[^{35}\text{S}]$ -sulphate during the sulphation of L-tyrosylglycine by a sulphating system in which rat-liver supernatant was used as source of enzyme.

The results of the present investigation should now be considered in relation to the observation that relatively large amounts of L-tyrosine *O*-sul-

phate are normally found in mammalian urines (see John *et al.* 1965). The fact that free L-tyrosine cannot be sulphated by the normal liver sulphating mechanism is now well established and it is therefore necessary to consider other possibilities. One such possibility is that urinary L-tyrosine O-sulphate arises as a result of the metabolism of fibrinogen and, in this connexion, Jones, Dodgson, Powell & Rose (1963) have shown that ^{35}S -labelled rabbit fibrinopeptide B is degraded by the rabbit to yield free L-tyrosine O[^{35}S]-sulphate, which is then excreted partially as such, the remainder being eliminated as *p*-hydroxyphenylacetic acid O[^{35}S]-sulphate. However, calculations based on the accepted values for fibrinogen turnover rates (see Blombäck, Böstrom & Vestermark, 1960), and on the assumption that a fibrinogen molecule (mol. wt. approx. 340 000) contains no more than four L-tyrosine O-sulphate residues (Jevons, 1963), make it clear that fibrinogen turnover could not account for more than a small proportion of the urinary L-tyrosine O-sulphate.

A second possibility is that peptides containing N-terminal tyrosine residues which arise as a result of normal protein metabolism might be sulphated in the liver and other tissues and then subsequently undergo proteolysis to yield free L-tyrosine O-sulphate. The present work, taken into consideration with that of Jones *et al.* (1963, 1966), has provided strong support for this possibility.

Under these circumstances there would arise a pool of L-tyrosine O-sulphate in the tissues which might be regarded as the source of supply of the ester for incorporation into fibrinogen. However, little is known about the origin of the L-tyrosine O-sulphate residues of fibrinogen and a number of experiments designed to test the possibility that the free ester can be incorporated into fibrinogen

have been attempted without success in these Laboratories. It seems more likely that sulphation of the L-tyrosine residues of fibrinogen may occur after the formation of the polypeptide chain, and this and other possibilities are now being tested.

This work was supported by U.S. Public Health Service Research Grant no. AM01982 from the Arthritis and Metabolic Diseases Division.

REFERENCES

- Basford, J. M., Jones, J. G., Rose, F. A. & Dodgson, K. S. (1965). *Biochem. J.* **97**, 33 p.
- Bettelheim, F. R. (1954). *J. Amer. chem. Soc.* **76**, 2838.
- Blombäck, B., Böstrom, H. & Vestermark, A. (1960). *Biochim. biophys. Acta*, **38**, 502.
- Dodgson, K. S., Powell, G. M., Rose, F. A. & Tudball, N. (1961). *Biochem. J.* **79**, 209.
- Dodgson, K. S., Rose, F. A. & Tudball, N. (1959). *Biochem. J.* **71**, 10.
- Doolittle, R. F. & Blombäck, B. (1964). *Nature, Lond.*, **202**, 147.
- Flanagan, T. H. & Rose, F. A. (1964). *Biochem. J.* **90**, 13 p.
- Flanagan, T. H., Rose, F. A. & Powell, G. M. (1964). *Biochem. J.* **92**, 24 p.
- Gregory, H., Hardy, P. M., Jones, D. S., Kenner, G. W. & Sheppard, R. C. (1964). *Nature, Lond.*, **204**, 931.
- Jevons, F. R. (1963). *Biochem. J.* **89**, 621.
- John, R. A., Rose, F. A., Wusteman, F. S. & Dodgson, K. S. (1965). *Biochem. J.* **94**, 37 p.
- Jones, J. G. & Dodgson, K. S. (1965). *Biochem. J.* **94**, 331.
- Jones, J. G., Dodgson, K. S., Powell, G. M. & Rose, F. A. (1963). *Biochem. J.* **87**, 548.
- Jones, J. G., Scotland, S. M. & Dodgson, K. S. (1966). *Biochem. J.* **98**, 138.
- Lloyd, A. G. (1961). *Biochem. J.* **80**, 572.
- Powell, G. M., Rose, F. A. & Dodgson, K. S. (1963). *Biochem. J.* **87**, 545.
- Rose, F. A., Flanagan, T. H. & John, R. A. (1966). *Biochem. J.* **98**, 168.