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THE SITE OF METABOLISM OF POTASSIUM L-TYROSINE *O*-^{35}S-SULPHATE IN THE RAT

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Abstract—The fate of potassium L-tyrosine *O*-^{35}S-sulphate was studied in mice and young rats. The ester was excreted unchanged in the urine of mice but was metabolized in the rat. The inability of the mouse to metabolize potassium L-tyrosine *O*-^{35}S-sulphate is explained by the failure of the ester to penetrate to the appropriate enzyme sites. Autoradiograms obtained following injection of the labelled ester to rats showed that radioisotope accumulated in the renal sub-cortex. The kidney was identified as the site of metabolic conversion in the rat and it was shown that the renal sub-cortex is particularly rich in L-tyrosine *O*-^{35}S-sulphate-2-oxoglutarate aminotransferase activity. The implications of these findings are discussed in relation to studies on arylsulphate esters in general and in relation to the origin of L-tyrosine *O*-sulphate residues in fibrinogen.

THE SULPHATE ester of the amino acid L-tyrosine is the only amino acid sulphate known to occur naturally. It is found in peptide-bound form in fibrinogen (see for example, Doolittle and Blombäck[1]), in gastrin II, one of the hormones of antral mucosa (Gregory Hardy, Jones, Kenner and Sheppard[2]), in phyllokinin, a bradykinin-like polypeptide isolated by Anastasi, Bertaccini and Erspamer[3] from the skin of the tree frog, and in caerulein, a decapeptide isolated from the skins of various Australian amphibia of the genus *Hyla* (Anastasi, Erspamer and Enden[4]). The ester also occurs in the free state in a number of mammalian urines (John, Rose, Wusteman and Dodgson[5]).

Injected potassium L-tyrosine *O*-^{35}S-sulphate (TO^{35}S) is metabolized by the rat to yield two major urinary metabolites which have been identified as the ^{35}S-labelled sulphate esters of *p*-hydroxyphenylpyruvic acid and *p*-hydroxyphenylacetic acid (Dodgson, Powell, Rose, and Tudball;[6] Powell, Rose and Dodgson[7]). An aminotransferase enzyme capable of initiating the metabolism by producing *p*-hydroxyphenylpyruvic acid *O*-^{35}S-sulphate from TO^{35}S is widely distributed in mouse and rat tissues, liver, kidney and pancreas being the richest sources (Rose, Flanagan and John[8]).

The present investigation offers an explanation of why the mouse, in spite of the presence of the aminotransferase enzyme in its tissues, is unable to metabolize TO^{35}S and also pinpoints the site of the metabolic breakdown of the ester in the rat.

MATERIALS AND METHODS

Aryl sulphate esters

The ^{35}S-labelled and unlabelled sulphate ester of L-tyrosine and the ^{35}S-labelled sulphate ester of *p*-hydroxyphenylacetic acid were prepared by the methods described by Dodgson *et al.*[6] *p*-Hydroxyphenylpyruvic acid *O*-^{35}S-sulphate was prepared according to the method of Rose and Powell.[9]

L-*Tyrosine-*^{14}C *(U)*

^{14}C-labelled L-tyrosine (as the hydrochloride) was obtained from The Radiochemical Centre, Amersham, Bucks, as an aqueous solution (225 mc/mM) containing 2% ethanol. Water was added so that the final solution contained 5 μc/0·2 ml.

Experimental animals

These included adult white mice (20–40 g body wt.), young (4–6 weeks) MRC hooded rats (30–50 g body wt.) and adult MRC hooded rats (200–250 g body wt.). In some experiments, animals received i.p. injections of materials while under general ether anaesthesia and were subsequently placed in metabolism cages designed to permit separate collection of urine and faeces. In experiments on rats in which urine was collected via ureter cannulae, animals were first lightly anaesthetized with ether, the jugular vein was cannulated and phenobarbitone (Nembutal, veterinary grade) injected via the cannula (see Flynn, Dodgson, Powell and Rose[10]).

Thin-layer chromatography (TLC)

Samples were applied to silica gel plates and chromatograms were developed in a saturation chamber of the type described by Davies[11] with l-butanol : acetic acid : water (3 : 1 : 1 by volume).

Paper chromatography

Samples were applied to Whatman No. 1 chromatography paper and chromatograms were developed with l-butanol : acetic acid : water (50 : 12 : 25 by volume).

Paper electrophoresis

Samples were subjected to electrophoresis on Whatman No. 1 paper for 2·5 hr in 0·1M sodium acetate : acetic acid (pH 4·5) with a potential gradient of 8V/cm.

Detection of radioactive areas on chromatograms and electrophoretograms

These were located using a Packard Model 7200 Radiochromatogram Scanner and the relative amount of radioactivity associated with each spot was estimated from the record of the scanner as described by Jones and Dodgson.[12] Alternatively, radioactive areas were located by autoradiography. Chromatograms or electrophoretograms were kept in contact with X-ray film (Ilford Industrial B) for periods of up to 10 days.

Whole-body and microautoradiography

Whole-body autoradiography was carried out as described by Powell, Curtis and Dodgson[13] and microautoradiography according to the method of Hammerström, Appelgren and Ullberg.[14]

Preparation of pancreatectomized animals

Adult rats were anaesthetized with Nembutal and ureters and the common bile duct cannulated through a midline abdominal incision. The duodenal loop was exposed and each artery and vein within the loop was ligated and divided as close as possible to the duodenum. The pancreatico-duodenal vessels were ligated and pancreatic tissue was then removed. The remainder of the pancreatic tissue, distributed in the greater omentum, together with the spleen, was removed after ligating and dividing blood vessels as described above.

Liver perfusion

Isolated rat livers were perfused according to the method described by Flynn *et al.*[10]

Kidney perfusion

The apparatus was essentially that used for perfusing isolated rat livers except that an additional peristaltic pump was included in the system. This pump supplied perfusate directly to the kidney at a pressure of 100–120 mm mercury. The perfusate (100 ml) was prepared as described by Flynn *et al.*[10]

The rat donating the kidney was anaesthetized (Nembutal) and following cannulation of the left ureter, received a continuous infusion (0·1 ml/min) of saline via the jugular vein. The superior mesenteric and coeliac arteries, suprarenal vessels and iliolumbar vessels were ligated and divided. The right renal artery and dorsal aorta were also ligated. The abdominal aorta and the renal vein were cannulated. Heparinized saline (0·2–0·3 ml) was introduced into the abdominal aorta cannula and the whole preparation was then transferred to the perfusion apparatus and the kidney perfused at an average rate of 0·8 ml/min. During initial experiments it was not possible to maintain this flow rate for more than 5 min after attaching the kidney to the apparatus and it seemed likely that this was due to the presence of vasoconstrictor substances (Lustinec[15]). It has been reported (Brauer, Leong and Pessotti[16]) that hepatic vasoconstrictors in freshly heparinized blood can be eliminated by perfusing the blood through an isolated liver. Thus, in all subsequent experiments a rat liver was first perfused for approximately 1·5 hr after which time it was replaced by the rat kidney preparation.

Assay of L-*tyrosine* O-*sulphate-2-oxoglutarate aminotransferase activity*

Enzyme activity was assayed according to the method of Rose *et al.*[8] and the nitrogen content of tissue homogenates was determined by a micro-Kjeldahl method (see Markham[17]).

EXPERIMENTAL AND RESULTS

Distribution of radioactivity in urine of mice and young rats following the administration of TO^{35}S

Mice and young rats were injected intraperitoneally with TO^{35}S (1 mg/20g body wt., dissolved in 0·2 ml of water). Animals were placed in metabolism cages and urine was collected over 6 hr into vessels immersed in an ice/salt mixture. Portions (5–10 μl) of the urine samples were examined by thin-layer chromatography and paper electrophoresis, authentic TO^{35}S, *p*-hydroxyphenylpyruvic acid O-^{35}S sulphate and *p*-hydroxyphenylacetic acid O-^{35}S-sulphate being used as markers.

Autoradiograms of mouse urines showed the presence of a single radioactive spot which corresponded in mobility to TO^{35}S, showing that the ester is not metabolized in the mouse under these conditions. In contrast, autoradiograms of chromatograms and electrophoretograms of rat urines showed three radioactive spots corresponding in mobility with TO^{35}S, *p*-hydroxyphenylpyruvic acid O-^{35}S-sulphate and *p*-hydroxyphenylacetic acid O-^{35}S-sulphate. The extent of metabolism of TO^{35}S in young rats was quantitated by scanning the electrophoretograms when two radioactive peaks were obtained, one corresponding to TO^{35}S and the other to the metabolites of TO^{35}S (*p*-hydroxyphenylpyruvic acid O-^{35}S-sulphate and *p*-hydroxyphenylacetic acid O-^{35}S-

sulphate). Metabolites of TO^{35}S were detected in the urine of each rat although the relative concentration of metabolites with respect to unchanged TO^{35}S varied from one animal to another (see Table 1). The extent of metabolism of TO^{35}S by small rats (at a dose level of 1 mg/20 g body wt.) is considerably less than that observed following the administration of the ester to adult rats (at a dose level of 1·5 mg/200 g body wt.) when only traces of unchanged TO^{35}S are detected in the urine (Dodgson *et al.*[6]).

TABLE 1. THE RELATIVE AMOUNTS OF UNCHANGED TO^{35}S AND TO^{35}S METABOLITES EXCRETED IN THE URINES OF YOUNG RATS FOLLOWING THE ADMINISTRATION OF TO^{35}S (FOR DETAILS SEE TEXT)

Rat No.	Sex	TO^{35}S in urine (% ^{35}S excreted)	TO^{35}S metabolites in urine (% ^{35}S excreted)
1	male	76	24
2	male	86	14
3	male	51	49
4	female	73	27
5	female	69	31
6	female	66	34

Distribution of radioactivity in urine of young rats receiving either dipotassium p-*hydroxyphenylpyruvic acid* O-^{35}*S-sulphate or potassium* p-*hydroxyphenylacetic acid* O-^{35}S-*sulphate*

Young rats were injected intraperitoneally with the appropriate ester (1 mg/20 g body wt., dissolved in 0·2 ml of water). Urines were collected over 6 hr into vessels immersed in an ice/salt mixture, and portions (5–10 μl) were examined by thin-layer and paper chromatography and paper electrophoresis, together with appropriate markers. Urines from rats receiving potassium *p*-hydroxyphenylacetic acid *O*-^{35}S-sulphate contained one radioactive component corresponding to the injected ester.

Analysis of urines obtained from animals receiving dipotassium *p*-hydroxyphenylpyruvic acid *O*-^{35}S-sulphate showed the presence of one major radioactive component corresponding to unchanged ester, together with trace amounts of *p*-hydroxyphenylacetic acid *O*-^{35}S-sulphate. Traces of the latter ester were also detected in control urines (normal rat urine to which dipotassium *p*-hydroxyphenylpyruvic acid *O*-^{35}S-sulphate had been added and allowed to stand for 6 hr immersed in an ice/salt mixture).

Whole-body autoradiography

Mice and young rats were injected intraperitoneally with TO^{35}S (1 mg/20g body wt., dissolved in 0·2 ml of water) and killed at time intervals ranging from 5 min to 2 hr after injection. Whole-body autoradiograms of mice and young rats were similar in a number of respects, and were characterized by accumulation of isotope in kidneys and bladder, and to a much lesser extent in pancreas. The concentration of isotope in other organs of both species did not exceed that of the blood. However, marked differences were observed in the distribution of isotope in the kidneys of the two species. In the mouse the most intense radioactive zone was associated with the inner medulla and pelvis, whereas in the rat, radioisotope was located primarily in a sub-cortical zone (see Fig 1). Accumulation of isotope was also observed in this region in kidneys of adult rats which had been injected with TO^{35}S (1·5 mg/200 g body wt.). No accumulation of radioactivity was noted in this region following the administration of either

dipotassium *p*-hydroxyphenylpyruvic acid O-^{35}S-sulphate or potassium *p*-hydroxyphenylacetic acid O-^{35}S-sulphate, at a dose level of 1 mg/20 g body wt.

In other experiments mice and young rats were injected intraperitoneally with L-tyrosine-^{14}C (U)-hydrochloride (0·2 ml) and killed 10 min and 30 min after injection. Whole-body autoradiograms showed accumulation of radioisotope in practically all tissues (see Fig. 1). Areas showing the greatest concentration of isotope corresponded to pancreas, liver, salivary glands, Harderian gland, skin, gut wall, renal cortex (in the mouse) and renal sub-cortex (in the rat).

Comparison of the autoradiograms obtained following the administration of L-tyrosine-^{14}C (U)-hydrochloride with those obtained with TO^{35}S, showed that the presence of the sulphate moiety has a marked effect on the behaviour of the amino acid *in vivo*. The sulphate ester appears to have a limited ability to penetrate various biological barriers, since cellular accumulation of radioisotope appears to be associated with pancreas and kidney only. However, the precise localization of radioisotope is not possible with whole-body autoradiography and, in order to confirm these initial observations, selected tissues were subjected to microautoradiography.

Microautoradiography

TO^{35}S was injected i.p. into mice (1 mg/20 g body wt., dissolved in 0·2 ml of water) and mature rats (1·5 mg/200 g body wt., dissolved in 0·4 ml of water) and after 20 min the animals were killed by a blow on the back of the head. Portions of liver, pancreas and kidney were immersed in an acetone–solid CO_2 mixture for 10 sec. Sections (6μ) were cut at $-20°$ and, after freeze-drying, were subjected to microautoradiography Microautoradiograms were allowed to develop for periods ranging from 2–35 days. Suitable controls were prepared from tissues of animals injected with unlabelled TOS.

The microautoradiograms confirmed the results obtained using whole-body autoradiography. Thus, no accumulation of radioisotope could be detected in mouse- or rat-liver cells even after 35 days exposure. Radioactivity was detectable in preparations of pancreas but was confined to areas corresponding to the interlobular septa, and was not detected in pancreatic cells. Microautoradiograms of mouse kidney confirmed the presence of radioisotope in the inner medulla and pelvis, and no cellular accumulation was recorded. In contrast, microautoradiograms of rat kidney (exposure time 2 days) showed accumulation of radioactivity in the cells of the distal portion of the proximal tubules (sub-cortex).

The collation of information from the distribution patterns of radioisotope, together with information on the metabolic fate of TO^{35}S in rats and mice, suggested that metabolism in the rat occurs in the kidney. Thus, although the pancreas, liver and kidney are rich in L-tyrosine O-sulphate-2-oxoglutarate aminotransferase activity, the collective observations suggest that only the kidney contributes to the metabolic conversion of the ester *in vivo*. In order to test this hypothesis experiments were designed to investigate the roles of pancreas liver and kidney in TO^{35}S metabolism.

Metabolism of TO^{35}S in pancreatectomized rats

Pancreatectomized rats (2 from the same litter) were injected intravenously with TO^{35}S (1·5 mg/200 g body wt., dissolved in 0·4 ml of water). Urine, collected over 3·5 hr, was subjected to paper electrophoresis; radioactive areas of the electrophoretograms were detected and measured by scanning. In these two experiments, 84 and 95

per cent of the radioactivity in urine was associated with the metabolites of $TO^{35}S$. These results strongly suggest that the pancreas is not the major site of $TO^{35}S$ metabolism *in vivo*.

Liver perfusion

Isolated rat livers (3) were perfused with rat blood containing 1·5 mg $TO^{35}S$/8ml of perfusate for 3·5 hr and samples (0·5 ml) of the perfusate were removed every 30 min during this period. Bile was also collected at 30 min intervals.

Perfusate samples were centrifuged, plasma was applied to Whatman No. 1 paper (to give approximately 500 cpm measured with a Panax monitor) and subjected to electrophoresis and paper chromatography. Bile samples were treated in a similar fashion. Radioactive areas were located by scanning and in all samples of plasma and bile only one radioactive spot was detectable. This spot corresponded in mobility to authentic $TO^{35}S$ showing that the ester is not metabolized by the isolated perfused rat liver.

Kidney perfusion

Rat kidneys (3) were perfused with rat blood containing 1·5 mg $TO^{35}S$/8 ml of perfusate. Urines were collected over a 3 hr perfusion period and samples were examined by electrophoresis. The results obtained showed that metabolism of $TO^{35}S$ had occurred in the cases of two of the three kidneys, and the metabolites represented 10 per cent of the total radioactive content of the urines.

Collectively, the results obtained using pancreatectomized animals, isolated liver experiments and isolated kidney experiments support the hypothesis that the selective permeability of the cells of the renal proximal tubules to $TO^{35}S$ pinpoints this area as the site of metabolic conversion *in vivo* in the rat. If such a hypothesis were correct then the possibility exists that the renal sub-cortex in rat is a rich source of L-tyrosine *O*-sulphate-2-oxoglutarate aminotransferase activity.

Distribution of L-*tyrosine* O-*sulphate-2-oxoglutarate aminotransferase activity in rat kidney*

The distribution of the enzyme was determined in four separate experiments. In each experiment, mature male rats (6) were killed by a blow on the back of the head; the kidneys were rapidly removed and placed in vessels at 0°. A sagittal section (approximately 2 mm thick) was obtained from the central region of each kidney and was dissected into three areas, viz: outer cortex, sub-cortex, and medulla. The dissection was carried out under a magnifying lens and was facilitated by the fact that in fresh rat kidneys the sub-cortex appears as a pale zone between the outer cortex and medulla. Aqueous homogenates (10 per cent, w/v) of each area were prepared and L-tyrosine *O*-sulphate-2-oxoglutarate aminotransferase activities and total nitrogen contents were determined. Similar analyses were made with homogenates of whole rat kidneys.

The experiments showed that the enzyme activity (expressed as mg of *p*-hydroxyphenylpyruvic acid sulphate liberated/mg of N/min) was considerably higher in the sub-cortex (range 0·75–1·06) than in the other two dissected zones (cortex, range 0·40–0·47; medulla, range 0·46–0·55). Furthermore, it was shown that the enzyme activity of the sub-cortex was significantly greater than that obtained with whole kidney (range 0·46–0·68).

DISCUSSION

It has been previously established that TO^{35}S undergoes transamination in the rat with the formation of *p*-hydroxyphenylpyruvic acid O-^{35}S-sulphate and *p*-hydroxyphenylacetic acid O-^{35}S-sulphate and further that the enzyme, L-tyrosine O-^{35}S-sulphate -2-oxoglutarate aminotransferase is widely distributed in mouse and rat tissues (Rose *et al.*[8]).

The results of the present investigation indicate the site of the metabolic conversion of TO^{35}S in the rat as the renal sub-cortex, a region where L-tyrosine O-^{35}S-sulphate-2-oxoglutarate aminotransferase activity is particularly concentrated. This finding is of considerable interest in view of the fact that, although the pancreas, liver and kidney are rich sources of aminotransferase activity only the kidney contributes significantly to the metabolic breakdown of TO^{35}S *in vivo*. This probably reflects the inability of the ester to penetrate the cells of the pancreas and liver. Support for this view comes from the results obtained with whole-body autoradiography and microautoradiography since radioactivity could not be detected in the cells of pancreas or liver following the administration of TO^{35}S to rats.

The inability of the mouse to metabolize the ester can be explained in the same way. Whole-body autoradiograms and microautoradiograms showed that there was no cellular accumulation of radioactivity in pancreas, liver or kidney following the administration of TO^{35}S. Thus, although mouse tissues are rich with respect to the aminotransferase, the activity of the enzyme is not apparent since the substrate is not able to penetrate to the enzyme sites.

The identification of the renal sub-cortex as the site of TO^{35}S breakdown has a number of wider implications. In the first instance, it is clear that a full understanding of the metabolic fate of a substance can only be attained when considered in the light of permeability phenomena. This principle may be usefully applied to investigations designed to elucidate the biological importance of arylsulphate esters as natural substrates for the arylsulphatase enzymes. Such investigations have been directed towards determining the metabolic fate of arylsulphate esters following their administration to experimental animals. It is clear from the present studies that the results may depend largely on the ability or inability of such esters to cross various biological barriers. If permeability phenomena are limiting in this respect then the inability of an experimental animal to degrade a particular arylsulphate ester may be explained solely on the grounds that the ester does not penetrate to the enzyme site. This line of reasoning is supported by the findings of Flynn *et al.*[10]

A further implication of the present investigation concerns the origin of the tyrosine O-sulphate residues in fibrinogen. Experiments designed to test the possibility that free TO^{35}S can be incorporated into fibrinogen have been attempted in these laboratories without success. However, the validity of such experiments must now be open to doubt in the light of the present study since it has been shown that exogenous TO^{35}S does not readily penetrate the liver cells, the site of fibrinogen formation.

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