A Rat-Tissue Aminotransferase Acting on L-Tyrosine O-Sulphate

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(Received 8 June 1965)

1. Rat tissues have been shown to possess an aminotransferase that is active towards L-tyrosine O-sulphate and dependent on 2-oxoglutarate and pyridoxal phosphate. 2. Kidney, liver and pancreas have the greatest activity and the enzyme is localized mainly in the mitochondrial fraction in the liver and kidney cell. 3. The enzyme was shown to be distinct from L-tyrosine-2-oxoglutarate aminotransferase but its true identity was not established. 4. A procedure for the assay of the enzyme in crude tissue preparations was developed.

In previous studies from these Laboratories (Dodgson, Powell, Rose & Tudball, 1961; Powell, Rose & Dodgson, 1963) it was found that L-tyrosine O-sulphate, a peptide-bound component of some mammalian fibrinogens (Doolittle & Blombäck, 1964) and present in the free state in mammalian urines (Tallan, Bella, Stein & Moore, 1955), is rapidly converted into two major metabolites when injected intraperitoneally into the rat. These metabolites appear in the urine and have been identified as the sulphate esters of *p*-hydroxyphenylpyruvic acid and *p*-hydroxyphenylacetic acid respectively. The latter sulphate ester appeared to be the end product of L-tyrosine O-sulphate metabolism in these animals (Dodgson *et al.* 1961) and the former was considered to be an intermediate.

The present work concerns the transformation of L-tyrosine O-sulphate into *p*-hydroxyphenylpyruvic acid sulphate and establishes the existence in rat tissues of an aminotransferase that can accomplish the process. The enzyme appears to be distinct from L-tyrosine-2-oxoglutarate aminotransferase and L-phenylalanine-pyruvate aminotransferase.

EXPERIMENTAL

Materials and experimental animals. The ^{35}S -labelled and non-labelled sulphate esters of L-tyrosine and *p*-hydroxyphenylpyruvic acid were prepared as potassium salts by the methods described by Dodgson *et al.* (1961) and Rose & Powell (1963). Female M.R.C. hooded rats, aged 2–4 months, were used in all experiments.

Preliminary studies. In preliminary experiments aqueous suspensions (20%, w/v) of rat liver and kidney, prepared with the aid of a glass homogenizer, were incubated with L-tyrosine O- ^{35}S -sulphate under a variety of experimental conditions and in the presence of various added cofactors.

Subsequently attempts were made to detect the formation of *p*-hydroxyphenylpyruvic acid [^{35}S]sulphate by subjecting samples of incubation mixtures to the paper-electrophoresis procedure described by Rose & Powell (1963). These experiments showed that *p*-hydroxyphenylpyruvic acid [^{35}S]sulphate was formed only if 2-oxoglutarate was present in the incubation mixture, suggesting that an aminotransferase enzyme was involved. By varying the concentration of L-tyrosine O- ^{35}S -sulphate and 2-oxoglutarate in the reaction mixture within the pH range 4–9, approximately optimum conditions for the enzyme were established qualitatively. These conditions were then used as a guide for the quantitative studies described below.

Estimation of enzyme activity. L-Tyrosine-2-oxoglutarate aminotransferase and L-phenylalanine-pyruvate aminotransferase (EC 2.6.1.5) can be assayed spectrophotometrically (Lin, Pitt, Civen & Knox, 1958). The method depends on the characteristic ultraviolet-absorption properties of the product obtained when boric acid is allowed to form a complex with the aromatic oxo acid formed as a result of transamination. *p*-Hydroxyphenylpyruvic acid sulphate also forms a boric acid complex with a characteristic absorption spectrum (λ_{max} , 302 m μ ; Rose & Powell, 1963) and the possibility that this property might be employed to assay the enzyme acting on L-tyrosine O-sulphate was therefore examined. It soon became apparent that the procedure of Lin *et al.* (1958) could not be applied directly to the crude enzyme preparations under investigation. First, the high concentrations of protein interfered with the spectrophotometric measurement; secondly, even when this problem had been surmounted, recoveries of *p*-hydroxyphenylpyruvic acid sulphate were incomplete. A similar failure to obtain complete recovery of liberated oxo acid was experienced by Lin *et al.* (1958) in measuring the activity of L-tyrosine-2-oxoglutarate aminotransferase. This was traced to the presence of tissue oxo acid oxidase in the enzyme extracts and could be avoided by incorporating diethyldithiocarbamate (an inhibitor of these oxidases) in the reaction mixture. However, this material was ineffective in preventing the losses of *p*-hydroxyphenylpyruvic acid sulphate that occurred consistently with the crude enzyme preparation used in the present work.

An approximate measure of the losses was obtained by

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comparing the recoveries of added *p*-hydroxyphenylpyruvic acid sulphate from incubated (2 hr. at 37°) reaction mixtures with those obtained for non-incubated mixtures. A loss of approx. 20% of the added ester occurred and this was independent of its concentration in the incubation mixture. Similar results were obtained with a number of tissues examined. In all determinations losses of *p*-hydroxyphenylpyruvic acid sulphate were assumed to be constant and were corrected for approximately by using a calibration curve with the assay procedure described below.

Assay procedure. The reaction mixture consisted of 0.3 ml. of an aq. 10% (w/v) tissue suspension, 0.4 ml. of 0.1 M-phosphate buffer, pH 7.3, 0.1 ml. of aq. 0.22 M-L-tyrosine O-sulphate and 0.1 ml. of a solution containing 30 μ g. of pyridoxal phosphate and 6.0 μ moles of 2-oxoglutarate in 0.1 M-phosphate buffer, pH 7.3. After incubation for 2 hr. at 37° the reaction was stopped and protein precipitated by the addition of 0.2 ml. of 30% (w/v) trichloroacetic acid. The mixture was cooled at 0° for 1 hr. and precipitated protein was removed by centrifuging at 1500*g* for 15 min. A 60 μ l. portion of the clear supernatant was mixed with 200 μ l. of 2.0 M-sodium arsenate solution that contained boric acid (1.0 M) and that had been adjusted to pH 6.4 with HCl (test enol solution). A further 60 μ l. portion of the deproteinized incubation mixture was mixed with 200 μ l. of 2.0 M-sodium arsenate that had been adjusted to pH 6.4 with HCl (test keto solution). After standing for 20 min. at room temperature, the extinction of the test enol solution at 302 m μ was determined in 1 cm. micro-cells (Hilger Uvispek), the test keto solution being placed in the blank cell. Control determinations were made in an identical manner except that L-tyrosine O-sulphate was added only after incubation and immediately before the addition of trichloroacetic acid. The spectrum of the product formed in the test determination was identical with that of pure *p*-hydroxyphenylpyruvic acid sulphate in the wavelength range 250–330 m μ . An approximate measure of the amount of *p*-hydroxyphenylpyruvic acid sulphate liberated was obtained by subtracting the reading for the control determination from that for the test determination. The resultant value was then applied to the calibration curve constructed by incubating various amounts of the ester (range 0–200 μ g.) with a suspension of rat liver under the conditions of assay. One unit of activity is that amount of enzyme which liberates 1 μ g. of *p*-hydroxyphenylpyruvic acid sulphate under these conditions.

RESULTS

Optimum conditions for enzyme activity. The enzyme of rat tissues had optimum activity at pH 7.3 and in the presence of a substrate concentration of 24 mM (Figs. 1 and 2). The K_m for the reaction (1.68×10^{-2} M) was calculated by the method (case III) of Lineweaver & Burk (1934). The amount of *p*-hydroxyphenylpyruvic acid sulphate formed under the optimum conditions increased proportionately with the reaction time up to 2 hr. at 37°.

The preliminary qualitative examination of rat liver for the enzyme showed that it was dependent on 2-oxoglutarate for activity. Maximum activity was obtained in the presence of 6 mM-2-oxoglutarate

Fig. 1. pH-activity curve for the enzyme of rat-liver mitochondria acting on 22 mM-L-tyrosine O-sulphate in the presence of 6 mM-2-oxoglutaric acid, 68 μ M-pyridoxal phosphate and 0.1 M-phosphate buffer.

Fig. 2. Substrate concentration-activity curve for the enzyme of rat-liver mitochondria acting on L-tyrosine O-sulphate in the presence of 6 mM-2-oxoglutaric acid and 68 μ M-pyridoxal phosphate. Incubation was carried out in 0.1 M-phosphate buffer, pH 7.3.

(Fig. 3). Glutamic acid was identified as a product of the reaction by paper chromatography as described by Lin *et al.* (1958).

Enzyme activity was detectable in crude tissue preparations without the need for prior addition of pyridoxal phosphate. Moreover, the addition of increasing amounts of pyridoxal phosphate to the reaction mixture did not markedly increase the enzyme activity of crude liver and kidney preparations. However, dialysis of liver preparations at 0° for 16 hr. against 0.1 M-phosphate buffer, pH 7.3,

Fig. 3. Acceptor (2-oxoglutaric acid) concentration-activity curve for the enzyme of rat-liver mitochondria acting on 22mM-L-tyrosine *O*-sulphate in the presence of 68 μ M-pyridoxal phosphate. Incubation was carried out in 0.1M-phosphate buffer, pH7.3.

Table 1. *Distribution of L-tyrosine O-sulphate aminotransferase activity in rat tissues*

Details of the assay procedure are given in the text.

Tissue	No. of expts.	Activity (units/g. wet wt.)	
		Mean	Range
Liver	6	3600	3000-3900
Kidney	4	4900	4100-5200
Pancreas	2	3270	2280-4260
Heart	2	1837	1804-1870
Lung	2	223	220-260
Brain	2	766	690-842
Spleen	2	333	300-366
Ovaries	2	500	420-580
Blood	2	165	152-178
Gut	2	478	400-556
Thyroid	2	652	640-664

completely inactivated the enzyme but activity could be restored by adding pyridoxal phosphate (5 μ g.) to the reaction mixture. These results indicated that the enzyme was dependent on pyridoxal phosphate.

Distribution of enzyme activity in rat tissues. The amount of enzyme in various tissues of the rat is shown in Table 1. Suspensions of the tissues (10%, w/v) were prepared in 0.25M-sucrose with the aid of a glass homogenizer. Whole organs were used, except for liver and intestine, where samples were taken. The intestine was opened and well washed with ice-cold water before homogenization. The results show that the enzyme is widely distri-

buted in the rat. Kidney, pancreas and liver, however, appear to possess the greatest activity.

Intracellular localization of enzyme activity. The distribution of enzyme activity in the liver and kidney cell was compared with that of cytochrome oxidase (cytochrome *c*-oxygen oxidoreductase, EC 1.9.3.1), a typical liver mitochondrial enzyme, and lactate dehydrogenase (L-lactate-NAD oxidoreductase, EC 1.1.1.27), a supernatant enzyme. Cytochrome oxidase was determined by the method of Cooperstein & Lazarow (1951) and lactate dehydrogenase by the method of Delbruck, Schimmassek, Bartsch & Bücher (1959). Tissue fractionation was carried out in iso-osmotic (0.25M) sucrose solution according to the scheme described by de Duve, Pressman, Gianetto, Wattiaux & Appelmans (1955). Preliminary experiments showed that sucrose did not interfere with the assay method. Uncontaminated cell nuclei were obtained by method 1 of Roodyn (1956).

The results (Table 2a) show that the bulk of the enzyme activity is associated with the mitochondrial fraction of both the liver and kidney cell. The non-particulate fraction of the kidney cell also possesses appreciable activity. This was shown to represent a true distribution of the enzyme by preparing a suspension of kidney in 0.25M-sucrose with the aid of the Dounce-type homogenizer (see Allfrey, 1959), centrifuging for 30 min. at 100000g and measuring the distribution of activity between the debris and supernatant fractions obtained. By this treatment, artifacts that may have been introduced previously by rupturing the cell particles during homogenization and by the subsequent washing procedure were minimized. The results (Table 2b) indicate that the enzyme has a bimodal distribution in the kidney cell.

Attempts to release the enzyme from liver mitochondria. Several recognized procedures used for disrupting mitochondria were studied in attempts to obtain the enzyme in a soluble form. The effect of the treatment on the stability and solubility of the enzyme was determined from assays on the whole mitochondrial suspension before and after treatment and on the clear supernatant obtained by centrifuging the treated suspension at 25000g for 30 min.

Alternate freezing (with acetone-solid carbon dioxide mixtures at -40°) and thawing (37°) of rat-liver mitochondria, isolated as described above and suspended in water, 0.15M-potassium chloride or 0.25M-sucrose, was ineffective in releasing the enzyme into solution and resulted in a 10-15% inactivation. Treatment of the mitochondria with ice-cold acetone, followed by extraction with water, was similarly of no value. Ultrasonic disintegration of mitochondria suspended in water or 0.15M-potassium chloride by treatment at 18-20kc./sec.

Table 2. *Distribution of L-tyrosine O-sulphate-2-oxoglutarate aminotransferase in the rat liver and kidney cell compared with the distribution of cytochrome oxidase and lactate dehydrogenase*

Details of the tissue fractionation procedure used and enzyme assays are given in the text. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951). Results are the averages of three experiments, with the ranges in parentheses.

		Activity (% of the whole suspension)			Protein (% of whole suspension)
		L-Tyrosine O-sulphate aminotransferase	Cytochrome oxidase	Lactate dehydrogenase	
(a) Liver	Nuclei	8 (5-15)	5 (4-6)	9 (8-9)	12.5
	Mitochondria	53 (45-57)	62 (52-80)	7 (6-8)	15.5
	Lysosomes	13 (7-18)	2 (8-18)	4 (4-5)	15.5
	Microsomes	7 (2-14)	7 (4-9)	13 (2-21)	13.5
	Supernatant	25 (25-27)	1 (0-2)	62 (48-75)	40.5
	Recovery	109	86	95	97.5
Kidney	Nuclei	8 (4-11)	5 (3-7)	5 (3-8)	10.0
	Mitochondria	35 (21-45)	44 (27-52)	4 (0-9)	19.0
	Lysosomes	19 (10-31)	17 (6-30)	4 (1-5)	10.0
	Microsomes	3 (2-5)	11 (6-14)	6 (5-6)	19.0
	Supernatant	32 (18-44)	3 (3-4)	62 (56-75)	47.0
	Recovery	97	80	81	105.0
(b) Liver	Particulate	89 (87-90)	61 (55-66)	23 (19-26)	
	Non-particulate	15 (14-16)	4 (0-8)	63 (49-82)	
Kidney	Particulate	61 (59-63)	74 (67-80)	13 (6-17)	
	Non-particulate	36 (35-37)	3 (1-5)	66 (50-72)	

for 5 min. at 5° by using the MSE ultrasonic disintegrator released little or no activity into solution. When mitochondria were suspended in 0.25M-sucrose and similarly disintegrated, however, a 75% solubilization of the enzyme without loss of activity was obtained.

Attempts to distinguish the enzyme from L-tyrosine-2-oxoglutarate aminotransferase. Kenney (1962) and Litwack, Sears & Diamondstone (1963) have shown that L-tyrosine-2-oxoglutarate aminotransferase is localized in the cell sap of the liver cell. This would apparently distinguish the enzyme from the L-tyrosine O-sulphate-2-oxoglutarate aminotransferase, which is localized mainly in the mitochondrial fraction (see Table 2) of this cell.

The separate identity of the two enzymes was also confirmed by a study of the amounts of the enzymes in the livers of rats that were receiving intraperitoneal injections of L-tyrosine, L-tyrosine O-sulphate and hydrocortisone. The rats were killed 4 hr. after injection and the amounts of the two enzymes determined in the excised livers. The results (Table 3) showed that the amount of L-tyrosine-2-oxoglutarate aminotransferase was elevated approximately threefold in the hydrocortisone-treated animals, in agreement with the observations of Kenney & Flora (1961), whereas the amount of the other enzyme remained unchanged. Injection of L-tyrosine O-sulphate had no significant effect

Table 3. *Effect of intraperitoneal injections of hydrocortisone, L-tyrosine and L-tyrosine O-sulphate on the L-tyrosine aminotransferase and L-tyrosine O-sulphate aminotransferase of rat liver*

Animals were injected with 1 ml. of 0.9% NaCl (control) or with 1 ml. of 0.9% NaCl containing 10 mg. of hydrocortisone, 100 mg. of L-tyrosine or 5 mg. of L-tyrosine O-sulphate/200 g. body wt. 4 hr. before enzyme assay. L-Tyrosine O-sulphate aminotransferase was assayed as described in the text. L-Tyrosine aminotransferase was assayed by a modified Briggs reaction as described by Litwack (1962). Results are the means $\pm$ s.e.m. of six experiments.

	Aminotransferase activity (units/g. wet wt.)	
	L-Tyrosine O-sulphate	L-Tyrosine
None	3130 $\pm$ 470	3.6 $\pm$ 0.4
0.9% NaCl	3330 $\pm$ 960	3.7 $\pm$ 0.7
Hydrocortisone	3370 $\pm$ 600	10.4 $\pm$ 0.3
L-Tyrosine	4960 $\pm$ 1020	7.5 $\pm$ 0.9
L-Tyrosine O-sulphate	3530 $\pm$ 590	4.2 $\pm$ 0.6

on the amount of either enzyme, whereas injection of L-tyrosine elevated the L-tyrosine-2-oxoglutarate-aminotransferase activity to a greater extent than that of L-tyrosine O-sulphate-2-oxoglutarate aminotransferase.

Attempts to distinguish the enzyme from L-phenylalanine-pyruvate aminotransferase. Although it seemed unlikely that L-phenylalanine-pyruvate aminotransferase could be identical with the aminotransferase acting on L-tyrosine *O*-sulphate, preparations of the former enzyme were obtained according to the procedure of Lin *et al.* (1958) and tested for activity towards the two compounds. The preparations exhibited considerable activity towards L-phenylalanine but were completely inactive towards L-tyrosine *O*-sulphate under a wide variety of experimental conditions.

DISCUSSION

The widespread distribution in rat tissues of an enzyme that deaminates L-tyrosine *O*-sulphate to produce *p*-hydroxyphenylpyruvic acid sulphate has been clearly shown. The enzyme is presumably responsible for the rapid and efficient deamination of L-tyrosine *O*-sulphate that occurs when the ester is injected intraperitoneally into the rat. Although the affinity for substrate is low ($K_m 1.68 \times 10^{-2} M$) the widespread distribution of the enzyme may account for the observed efficiency of the system *in vivo*. The enzyme might tentatively be called L-tyrosine *O*-sulphate-2-oxoglutarate aminotransferase, and its distinction from L-tyrosine-2-oxoglutarate aminotransferase and the L-phenylalanine-pyruvate aminotransferase described by Lin *et al.* (1958) seems certain. However, it has not been established whether the enzyme shows absolute specificity towards L-tyrosine *O*-sulphate. The enzyme has some characteristics in common with a 2-oxoglutarate-dependent aminotransferase already recognized (Nakano, Tsuchiya & Danowski, 1963) that deaminates di-iodo-L-tyrosine. Further, the tissue distribution of the two enzymes and their localization in the kidney cell are very similar. Nakano & Danowski (1964) have shown that rat-kidney mitochondria possess a number of enzymes that deaminate derivatives of L-tyrosine, and the identification of L-tyrosine *O*-sulphate aminotrans-

ferase as a separate enzyme must therefore await its purification.

This work was supported by the Royal Society and by U.S. Public Health Service Research Grant no. AM01982-07 from the Arthritis and Metabolic Diseases Division. T. H. F. and R. A. J. are grateful to the Medical Research Council and the Department of Scientific and Industrial Research respectively for studentships.

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