The Sulphation of *p*-Hydroxyphenylpyruvic Acid and Related Compounds by the Rat Liver Cytosol

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Cytosol preparations of rat liver and kidney were examined for their ability to transfer sulphate from adenosine 3'-phosphate 5'-sulphatophosphate to *p*-hydroxyphenylpyruvic acid. Little activity towards this substrate was observed, and the main product detected in the reaction mixtures was identified as *p*-hydroxybenzyl alcohol *O*-sulphate. This was formed from *p*-hydroxybenzaldehyde, a spontaneous oxidation product of *p*-hydroxyphenylpyruvic acid, by sulphation followed by a rapid enzyme-catalysed reduction of the intermediate *p*-hydroxybenzaldehyde *O*-sulphate. The product was formed mainly by this sequence of reactions, but the reverse, reduction followed by sulphation, also appeared possible. *p*-Hydroxybenzyl alcohol itself was very readily sulphated by both preparations, and the liver also produced a sulpho-conjugate of homogentisic acid. These observations are important in calculating the turnover of L-tyrosine *O*-sulphate in the mammalian system, and establish that *p*-hydroxyphenylpyruvic acid *O*-sulphate is an end product of its metabolism, rather than an intermediate in its synthesis by reversed transamination.

The normal urine of several mammals, including man, is known to contain the free *O*-sulphate ester of tyrosine (Tallan *et al.*, 1955; John *et al.*, 1966). The origin and metabolic significance of this compound, which is excreted in appreciable quantities (28 mg/24 h in humans), is obscure, since free tyrosine does not undergo sulphation in the biological sulphating system of the cytosol (Segal & Mologne, 1959; Grimes, 1959).

The ester occurs as a constituent of various mammalian fibrinogens (Blombäck, 1960; Blombäck & Doolittle, 1963; Blombäck *et al.*, 1965) and the polypeptide hormones gastrin II (Gregory *et al.*, 1964) and cholecystokinin-pancreozymin (Mutt & Jorpes, 1968), and it has been concluded that, since these molecules are constantly being turned over *in vivo*, tyrosine *O*-sulphate might be an end product of such processes and be excreted in the urine. Although studies on the metabolic fate of fibrinopeptide B containing ³⁵S-labelled L-tyrosine *O*-sulphate residues (Jones *et al.*, 1963) have shown that this is possible, calculations based on the accepted rate of turnover of molecules known to contain the ester indicate that such molecules cannot be the only source of this urinary component.

The possibility that the ester could arise in rat tissues from peptide materials containing *N*-terminal tyrosine residues by the sequential action of sulphotransferase and peptidase enzymes has also been demonstrated (Jones *et al.*, 1963, 1966; Jones & Dodgson, 1965; Basford *et al.*, 1966), but the contribution, if any, to the urinary pool from this source is not known.

A further route for the production of free L-tyrosine *O*-sulphate in mammals is indicated by the existence of an aminotransferase enzyme which in the presence of 2-oxoglutarate is capable of converting tyrosine *O*-sulphate into *p*-hydroxyphenylpyruvic acid *O*-sulphate and glutamic acid (Rose *et al.*, 1966). Since *p*-hydroxyphenylpyruvic acid is an intermediate in the normal metabolism of L-tyrosine it therefore seemed possible that this might undergo sulphation to yield *p*-hydroxyphenylpyruvic acid *O*-sulphate, which, in the presence of glutamate, might be then converted into tyrosine *O*-sulphate by the aminotransferase acting in the reverse direction. Preliminary studies (John, 1966) have shown that this reaction is freely reversible *in vitro*, and the present study was therefore concerned with determining whether the first step, namely the sulphation of *p*-hydroxyphenylpyruvic acid, could also take place.

A knowledge of the ability of mammalian tissues to synthesize *p*-hydroxyphenylpyruvic acid *O*-sulphate was also required to explain the precise significance of its presence in normal human urine (Hext, 1972). In the rat, the ester is known to be one of the major products in the urine after the administration of L-tyrosine *O*-sulphate, so that in normal urine it might arise, in part at least, from the normal turnover of tyrosine *O*-sulphate. Such considerations are vital to any precise determination of tyrosine *O*-sulphate excretion.

The results indicate that the sulphation of *p*-hydroxyphenylpyruvic acid is only a minor process in rat tissues. The formation of L-tyrosine *O*-sulphate

by reversed transamination is therefore likely to be only a minor pathway and any *p*-hydroxyphenylpyruvic acid *O*-sulphate in the urine can be concluded to be only an end product of tyrosine *O*-sulphate metabolism. However, during these investigations, it was observed that the spontaneous degradation products of *p*-hydroxyphenylpyruvic acid readily formed a number of sulpho-conjugates which could be significant products of tyrosine metabolism in the urine.

Materials and Methods

Materials

Chemicals. *p*-Hydroxyphenylacetic acid was obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K., and *p*-hydroxybenzaldehyde from BDH Chemicals Ltd., Poole, Dorset, U.K. *p*-Hydroxyphenylpyruvic acid was obtained from A.G. Fluka, Buchs, Switzerland. Radioactively labelled chemicals were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Potassium *p*-hydroxyphenylacetic acid *O*-[^{35}S]sulphate (specific radioactivity 0.55 $\mu\text{Ci/mg}$) was prepared by the method of Dodgson *et al.* (1961). Potassium *p*-hydroxybenzaldehyde *O*-sulphate and the ^{35}S -labelled ester (specific radioactivity 0.12 $\mu\text{Ci/mg}$) were prepared by the method of Flanagan (1963). Potassium *p*-hydroxyphenylpyruvic acid *O*-[^{35}S]sulphate (specific radioactivity 15.4 $\mu\text{Ci/mg}$) was prepared by the method of Rose & Powell (1963), but with chloro[^{35}S]sulphonic acid of a much higher specific radioactivity (5.2 mCi/mmol). Adenosine 3'-phosphate 5'-[^{35}S]sulphatophosphate was prepared by the method of Mattock & Jones (1970).

***p*-Hydroxybenzyl alcohol.** *p*-Hydroxybenzaldehyde was recrystallized four times from water, and 770 mg of the material was dissolved in water (20 ml) and treated with 50 mg of NaBH_4 . After being kept for 1 h at room temperature (16°C), the solution was adjusted to pH 4 with 2M-HCl, and then extracted with three 20 ml portions of ether. The combined extracts were evaporated to dryness by warming to 40°C in a stream of N_2 , and the residue was dissolved in the smallest volume of water at 90°C. On cooling, crystals of *p*-hydroxybenzyl alcohol separated. These were collected, recrystallized twice more from water and stored dry at -10°C. The yield was 218 mg (Found: C, 68.0; H, 6.5. $\text{C}_7\text{H}_8\text{O}_2$ requires C, 67.8; H, 6.5%).

Potassium *p*-hydroxybenzyl alcohol *O*-sulphate. Unlabelled *p*-hydroxybenzaldehyde *O*-sulphate (430 mg) was dissolved in 5 ml of water, and 15 mg of NaBH_4 was added. The solution was left for 3 h at room temperature and was adjusted to pH 7.0 with 1M-acetic acid. The solution was then passed through a column (6 cm $\times$ 2.2 cm) of Dowex 50 [X8, 200-400 mesh, H^+ form; Sigma (London) Chemical Co.,

Kingston-upon-Thames, Surrey KT2 7BH, U.K.]. The total acid effluent was collected, adjusted to pH 7 with 2M-KOH, and evaporated to dryness *in vacuo* at 40°C. The residue was dissolved in the smallest possible volume of water at 35°C and the potassium salt of *p*-hydroxybenzyl alcohol *O*-sulphate was precipitated by addition of ethanol-ether (1:1, v/v). The precipitate was removed by centrifuging, washed three times with 100 ml portions of ethanol, recrystallized from hot water and dried *in vacuo* over P_2O_5 . The yield was 300 mg (Found: C, 34.7; H, 3.0; K, 16.0; ester SO_4^{2-} , 39.9; $\text{C}_7\text{H}_7\text{O}_5\text{SK}$ requires C, 34.7; H, 2.8; K, 16.1; ester SO_4^{2-} , 39.7%). A ^{35}S -labelled sample was also prepared from potassium *p*-hydroxybenzaldehyde *O*-[^{35}S]sulphate (specific radioactivity 0.10 $\mu\text{Ci/mg}$).

All other chemicals were AnalaR grade.

Animals. Young male hooded rats (Medical Research Council; 200-250 g body wt.) were used throughout.

Methods

Sulphotransferase preparations from liver and kidney. Rats were killed by a blow on the head, and the livers and kidneys were quickly excised and chilled. After passage through a fine wire mesh to remove connective tissue, the pulp was weighed and homogenized as a 20% (w/v) suspension in 0.25M-sucrose with the aid of a Tri-R homogenizer (Tri-R Instrument Co. Ltd., Rockville Centre, N.Y., U.S.A.; clearance between tube and Teflon pestle, 0.19 mm) rotating at 4000 rev./min. The homogenate was then centrifuged at 100000 g_{av} for 1 h in a Spinco model L preparative ultracentrifuge in a no. 40 rotor. The clear supernatant (including the surface lipid layer) was poured off, buffered to pH 7.5 by addition of 1M-Tris-HCl buffer, pH 7.5 (1 vol. of buffer to 40 vol. of supernatant preparation), and stored at -10°C in 1 ml portions until required. Such preparations retained activity for up to 4 weeks.

Paper chromatography and paper electrophoresis. One-dimensional descending chromatography was performed on Whatman no. 1 or 3MM paper for 16 h at room temperature (16°C) with butan-1-ol-acetic acid-water (25:6:12, by vol.). Two-dimensional chromatography was performed on Whatman no. 1 paper. Chromatograms were developed first with the upper layer of a mixture of butan-1-ol-acetic acid-water (4:1:5, by vol.) for 16 h at room temperature, and after drying at 35°C for 1 h, with 20% (w/v) KCl for 4 h in the second direction. Paper electrophoresis was performed on Whatman no. 1 paper and run for 2 h in 0.1M-sodium acetate-acetic acid buffer, pH 4.5, at a potential gradient of 10V/cm.

Detection and measurement of radioactivity on chromatograms and electrophoretograms. On one-dimensional chromatograms and electrophoretograms

grams this was achieved by radioautography on X-ray film (Ilford Industrial B) for periods of up to 4 weeks and also by scanning with a Packard radiochromatogram scanner (model 7200). The relative amount of radioactivity associated with each spot was measured from the record of the scanner as described by Dodgson & Tudball (1961). Radioactivity on two-dimensional chromatograms was also located by radioautography and by scanning with a two-dimensional radiochromatogram scanner (Baird and Tatlock Instruments Ltd., Chadwell Heath, Essex, U.K.).

Gas-liquid chromatography. This was carried out on a column of polyethylene glycol adipate (150 cm $\times$ 0.5 cm), 10% (w/v) on Diatomite C (100–200 mesh), maintained at 132°C in a Pye model 104 gas chromatograph fitted with an identical reference column and dual flame-ionization detectors. Volatile trimethylsilyl derivatives of phenols were prepared by shaking the phenols for 2 min with 0.1 ml of a mixture of pyridine – trimethylchlorosilane – hexamethyldisilazane (5:1:1, by vol). Silane reagents were purchased from Kodak Ltd., Kirkby, Liverpool L33 7UF, U.K.

Experimental and Results

Sulphation of p-hydroxyphenylpyruvic acid

Reaction mixtures consisting of 10 μ l of *p*-hydroxyphenylpyruvic acid (30 mM) in dimethyl sulfoxide, 10 μ l of 1 M-Tris-HCl buffer, pH 7.5, 40 μ l of 0.3 mM-adenosine 3'-phosphate 5'-[³⁵S]sulphatophosphate and 100 μ l of liver or kidney high-speed supernatant were incubated for 1 h at 37°C. The reaction was terminated by immersing the tubes in boiling water for 1 min. Control determinations were made similarly except that 10 μ l of dimethyl sulfoxide was substituted for the *p*-hydroxyphenylpyruvic acid solution. Samples (10 μ l and 15 μ l) of the incubation mixtures were subjected to paper electrophoresis or chromatography respectively, and the radioactive products were detected as described. Incubations with liver and kidney produced three and two main radioactive products respectively. Of these products, one possessing mobilities which were identical with those of authentic *p*-hydroxyphenylpyruvic acid *O*-[³⁵S]sulphate (R_F 0.32; m 8.0 cm) was detected in the test incubations. Further proof of the identity of this compound was obtained by converting it into *p*-hydroxybenzaldehyde *O*-[³⁵S]sulphate under alkaline conditions (see Rose & Powell, 1963). Chromatographic and electrophoretic examination of mixtures which had been made alkaline for 24 h showed a marked decrease in the component ascribed to *p*-hydroxyphenylpyruvic acid *O*-[³⁵S]sulphate (up to 90%) and the concomitant appearance of a new component identifiable as *p*-hydroxybenzaldehyde *O*-[³⁵S]sulphate.

The rate of formation of *p*-hydroxyphenylpyruvic acid *O*-[³⁵S]sulphate over a range of acceptor concentrations (30 μ M–1.9 mM) was measured for the liver preparation by this assay procedure. The results were expressed as double-reciprocal plots (Lineweaver & Burk, 1934) and the K_m was found to be 1 mM for the liver preparation. The activity of kidney preparations was too low for accurate determinations of K_m . The addition of dithiothreitol (0.48 μ mol) to the reaction mixture had no effect on the activity of either tissue.

Identification of the 'unknown products'

(a) *Major product formed by liver and kidney.* Chromatography and electrophoresis of the reaction products obtained in the above experiments revealed the presence of a major component possessing a slightly greater chromatographic mobility (R_F = 0.4) but considerably lower electrophoretic mobility (m = 5.3 cm) than *p*-hydroxyphenylpyruvic acid *O*-[³⁵S]sulphate. Measurement of the relative intensity of the two spots by scanning indicated that the ratio of the 'unknown' to *p*-hydroxyphenylpyruvic acid *O*-[³⁵S]sulphate was approx. 3:1. In a series of preliminary experiments designed to determine its origin, it was clearly established that it arose either directly or indirectly from the acceptor *p*-hydroxyphenylpyruvic acid and not from the other components of the reaction mixture. Since *p*-hydroxyphenylpyruvic acid was previously shown to undergo spontaneous oxidation (Doy, 1960), attempts were made to identify the 'unknown' by examining the ability of the two main degradation products of *p*-hydroxyphenylpyruvic acid, namely *p*-hydroxybenzaldehyde and *p*-hydroxyphenylacetic acid, to act as sulphate acceptors in the system. The addition of *p*-hydroxybenzaldehyde, the primary spontaneous degradation product, yielded two components, one of which had chromatographic and electrophoretic mobility identical with those of authentic *p*-hydroxybenzaldehyde *O*-[³⁵S]sulphate (Powell *et al.*, 1963) when added to the reaction mixture. The second component had mobilities quite different from those of *p*-hydroxyphenylacetic acid *O*-[³⁵S]sulphate, but identical with those of the 'unknown'. When *p*-hydroxybenzyl alcohol, a possible product of the reduction of *p*-hydroxybenzaldehyde, was added to the incubation mixture, a single component, with mobilities identical with those of the 'unknown', was produced. The addition of authentic synthetic *p*-hydroxybenzyl alcohol *O*-[³⁵S]sulphate to the reaction mixture before chromatography and electrophoresis increased the radioactivity of the area occupied by the 'unknown' product.

(b) *Co-crystallization of major product with p-hydroxybenzyl alcohol O-sulphate.* A mixture consisting of 100 μ l of 30 mM-*p*-hydroxyphenylpyruvic acid in dimethyl sulfoxide, 100 μ l of 1 M-Tris-HCl

buffer, pH 7.5, 400 μ l of 0.3 mM-adenosine 3'-phosphate 5'-[35 S]sulphatophosphate and 1 ml of liver high-speed supernatant preparation was incubated at 37°C for 30 min. The reaction was stopped by the addition of 0.1 ml of aq. 30% (w/v) trichloroacetic acid, and precipitated protein was removed by centrifuging. The supernatant was applied (as 10 μ l spots) to two sheets of Whatman 3MM chromatography paper (57 cm $\times$ 27 cm) and was subjected to descending chromatography in butan-1-ol-acetic acid-water (25:6:12, by vol.) for 16 h. After drying the sheets at 40°C, the radioactive areas corresponding to the *p*-hydroxybenzyl alcohol *O*-[35 S]sulphate were removed, and the radioactivity was eluted with water. The combined eluates were decreased in volume to 0.1 ml by freeze-drying in a silicon-treated flask and then applied (as a thin streak) across a sheet of Whatman 3MM chromatography paper (45 cm $\times$ 12 cm), 10 cm from one end. The sheet was subjected to horizontal electrophoresis for 3 h under the conditions described above. The radioactive area corresponding to *p*-hydroxybenzyl alcohol *O*-[35 S]sulphate was removed from the dried electrophoretogram, and the radioactivity was eluted as before. The eluate was passed through a small column (2 cm $\times$ 1 cm) of Dowex 50 (X8; 200-400 mesh; H⁺ form) and washed through with 2 ml of water before freeze-drying. The residue, dissolved in 0.1 ml of water, was adjusted to pH 7 by the addition of 2 μ l portions of 0.5 M-NaOH. Chromatography and electrophoresis of this solution showed the presence of one radioactive component with mobilities identical with authentic *p*-hydroxybenzyl alcohol *O*-[35 S]sulphate.

A sample (80 μ l) of the solution obtained above was added to 0.2 ml of an aqueous solution of 54 mg of unlabelled *p*-hydroxybenzyl alcohol *O*-sulphate and the whole was recrystallized by dropwise addition of ethanol-ether (4:1, v/v) with constant stirring. The crystals were separated by centrifuging and were dried *in vacuo* over P₂O₅. A weighed sample was removed and the remainder was recrystallized a further five times, a weighed sample being removed after each recrystallization.

Each sample was dissolved in 0.1 ml of water, and 30 μ l of the solution was added to 10 ml of a scintillation mixture consisting of 2,5-diphenyloxazole (5 g) dissolved in toluene-Triton X-100 (2:1, v/v; 1.5 litres) which was then counted for radioactivity for 20 min in a Beckman model LS-100 liquid-scintillation spectrometer. Within the limits of experimental error, material of constant specific radioactivity was obtained after the first recrystallization.

(c) *Minor product formed by liver.* In the preliminary attempts to identify the products by testing various compounds as sulphate acceptors, it was noted that when homogentisic acid was used as the

substrate, a product (*R_f* 0.35) identical with the component produced only by the liver preparation was obtained. The possibility that the product formed from *p*-hydroxyphenylpyruvic acid was therefore a sulpho-conjugate of its hydroxylation product, homogentisic acid, was explored further by adding 10 μ l of 3.5 mM-diethyldithiocarbamic acid to a liver test incubation mixture to inhibit *p*-hydroxyphenylpyruvate hydroxylase (EC 1.13.11.27). The product, considered to be homogentisic acid sulphate, was not produced in this system. The inhibitor was shown to be without effect on the sulphotransferase reaction, since the product was formed in a similar incubation mixture to which homogentisic acid had been added before incubation. The component therefore appeared to be a sulpho-conjugate of homogentisic acid, but its absolute identity was not established, since it was a relatively minor product.

Route of formation of p-hydroxybenzyl alcohol O-sulphate from p-hydroxyphenylpyruvic acid

The formation of *p*-hydroxybenzyl alcohol *O*-[35 S]sulphate in the above experiments, when *p*-hydroxybenzaldehyde was being used as the sulphate acceptor, indicated that the latter was an intermediate in the production of the alcohol sulphate from *p*-hydroxyphenylpyruvic acid. The possible routes by which this might occur are shown in Fig. 1. The likelihood of these processes taking place in the reaction mixture was examined as follows.

Conversion of p-hydroxybenzaldehyde into p-hydroxybenzyl alcohol. A reaction mixture of 50 μ l of 30 mM-*p*-hydroxybenzaldehyde in water, 50 μ l of 1 M-Tris-HCl buffer, pH 7.5, 200 μ l of water (in place of adenosine 3'-phosphate 5'-[35 S]sulphatophosphate) and 500 μ l of liver high-speed supernatant preparation was incubated for 1 h at 37°C. Two controls were prepared similarly, except that in one the substrate solution was replaced by water, and in the other the tissue preparation was replaced by sucrose. These were used to correct for any endogenous source of *p*-hydroxybenzyl alcohol, or its formation by a non-enzymic route. After incubation, the reactions were stopped by the addition of 20 μ l of 30% (w/v) trichloroacetic acid, and the precipitated protein was removed by centrifuging. The supernatant was then extracted with three successive 1 ml portions of ether, which were combined and evaporated to dryness in a small tube in a stream of dry air. Trimethylsilyl derivatives of the dried extracts from the incubation mixtures and of samples (0.1 mg) of *p*-hydroxybenzaldehyde and *p*-hydroxybenzyl alcohol were prepared as described earlier, and 2.5 μ l samples of each were subjected to g.l.c.

A component having a retention time identical with that of the trimethylsilyl derivative of *p*-hydroxybenzyl alcohol was detected in extracts of the test

Fig. 1. Possible routes of formation of *p*-hydroxybenzyl alcohol *O*-sulphate from *p*-hydroxyphenylpyruvic acid

reaction mixture. Addition of authentic trimethylsilyl-*p*-hydroxybenzyl alcohol to the test extract increased the height of the appropriate recording peak, and manipulation of the column temperature produced identical changes in the mobility of synthesized and authentic materials. These observations established with reasonable certainty that *p*-hydroxybenzyl alcohol could be produced enzymically from *p*-hydroxybenzaldehyde in the incubation mixture, although the conversion did not appear to be extensive (1–2%).

Sulphation of *p*-hydroxybenzyl alcohol. The remarkable ability of *p*-hydroxybenzyl alcohol to accept sulphate had been demonstrated in the preliminary experiments. The rate of the reaction over a range of

acceptor concentrations (30 μM –1.9 mM) was examined by using the liver preparation and the assay procedure described above. The results were expressed as double-reciprocal plots (Lineweaver & Burk, 1934) and the K_m was found to be 18 μM . Although the substrate possessed an alcohol group and a phenolic group, both of which might possibly have been sulphated with the crude enzyme preparation used, only the latter was involved.

To determine the effect of the presence of *p*-hydroxybenzyl alcohol on the sulphation of *p*-hydroxyphenylpyruvic acid in the same reaction mixture, mixed-substrate experiments were performed. On adding *p*-hydroxybenzyl alcohol at various concentrations (0–2 mM) to a reaction

Fig. 2. Rates of formation of various products by the rat liver cytosol

The sulphating system with *p*-hydroxyphenylpyruvic acid as the substrate and conditions as described in the text were used. The products, *p*-hydroxyphenylpyruvic acid O-[³⁵S]sulphate (○), *p*-hydroxybenzaldehyde O-[³⁵S]sulphate (●) and *p*-hydroxybenzyl alcohol O-[³⁵S]sulphate (▲) were determined after electrophoretic separation as described in the text.

mixture containing 30mM-*p*-hydroxyphenylpyruvic acid, sulphation of the latter was unaffected up to 0.1 mM, but above this it was progressively inhibited, to 100% at 1.5mM. Conversely, the sulphation of low concentrations of *p*-hydroxybenzyl alcohol (<1 mM) was inhibited by approx. 10% by the presence of 30mM-*p*-hydroxyphenylpyruvic acid in the reaction mixture.

Sulphation of *p*-hydroxybenzaldehyde. With the use of the assay procedure already described, the rate of formation of *p*-hydroxybenzaldehyde O-[³⁵S]sulphate in the reaction mixture containing the liver preparation was examined over a range of *p*-hydroxybenzaldehyde concentrations up to 30mM. However, it was not possible to obtain a value for K_m for the reaction under these conditions, since at concentrations of acceptor up to 0.5mM, only *p*-hydroxybenzyl alcohol O-[³⁵S]sulphate could be detected as the product. Above this concentration, the latter compound was produced in constant amounts (4nmol/h), presumably indicating that some cofactor for the reductive processes had been used up, and unchanged *p*-hydroxybenzaldehyde O-[³⁵S]sulphate was then detectable in increasing quantities with increasing *p*-hydroxybenzaldehyde concentration.

Conversion of *p*-hydroxybenzaldehyde O-[³⁵S]sulphate into *p*-hydroxybenzyl alcohol O-[³⁵S]sulphate. The reaction mixture used for the determination of *p*-hydroxybenzyl alcohol formation, as described above, was used, but with 30mM-*p*-hydroxybenzaldehyde O-[³⁵S]sulphate in the place of the *p*-hydroxybenzaldehyde. A control containing sucrose in place of tissue preparation was also used. After incubation, 15 μl samples were subjected to chromatography and

electrophoresis under the conditions already described. Quantitative examination of the results showed that approx. 50% of the *p*-hydroxybenzaldehyde O-[³⁵S]sulphate in the test incubation had been converted, whereas in the control it remained unchanged.

These results indicate that *p*-hydroxybenzyl alcohol sulphate could be derived from *p*-hydroxybenzaldehyde by either or both of the routes, although the second, i.e. sulphation followed by reduction, seems the more efficient. The occurrence of an aldehyde derivative in the reaction mixtures was more difficult to explain. It seemed likely that it could have already existed in the *p*-hydroxyphenylpyruvic acid sample before mixing, since although freshly recrystallized keto acid was used, the spontaneous and rapid oxidation of the enol tautomer, which is always present to some extent, occurs very readily (Schwarz, 1961). This could not be established, since the amount of aldehyde in question was not readily detectable. Alternatively, the conversion might have occurred in the reaction mixture, either by the same spontaneous oxidation or enzymically, although in separate experiments the progressive formation of greater quantities of *p*-hydroxybenzaldehyde could not be demonstrated.

The remaining possible route for the formation of *p*-hydroxybenzyl alcohol O-sulphate, namely by oxidation of *p*-hydroxyphenylpyruvic acid O-sulphate to *p*-hydroxybenzaldehyde O-sulphate, followed by reduction of the latter, seemed unlikely to occur in the same reaction mixture. Indeed, *p*-hydroxyphenylpyruvic acid O-[³⁵S]sulphate remained unchanged when incubated in the reaction mixture for 1 h at various concentrations.

The results indicate that the prominent route is via sulphation of the impurity *p*-hydroxybenzaldehyde, followed by reduction of the product. This was confirmed in an experiment (Fig. 2) in which the main products formed in an incubation of the liver preparation with *p*-hydroxyphenylpyruvic acid, under the conditions described above, were measured at various time-intervals.

Discussion

The main objective of the present study was to determine whether *p*-hydroxyphenylpyruvic acid could undergo biological sulphation, the first stage required for the production of free L-tyrosine O-sulphate by a reversed transamination route. Although the results indicate that the sulphation step is possible, they also establish that the yield of product *in vivo* is likely to be very poor. It was shown that this was not due to the breakdown of *p*-hydroxyphenylpyruvic acid O-sulphate into *p*-hydroxybenzyl alcohol sulphate, the major product, since this was formed by an independent sequence of reactions

from an endogenous and persistent impurity, *p*-hydroxybenzaldehyde. However, the finding that *p*-hydroxybenzyl alcohol was so readily sulphated raised the possibility that, if only one sulphotransferase was involved in the production of *p*-hydroxybenzyl alcohol *O*-sulphate and *p*-hydroxyphenylpyruvic acid *O*-sulphate, then competition for the two substrates might have diminished the yield of the latter compound. It was not possible to explore this reaction fully in the experiments, since only a crude sulphotransferase preparation was used, but competition did seem possible. However, since the *p*-hydroxybenzyl alcohol was only a trace impurity (less than 0.1 mM) in the reaction mixture, its presence would have had no effect on the result. The same considerations apply to the *p*-hydroxybenzaldehyde present. In any event, it is most likely that the *p*-hydroxyphenylpyruvic acid produced in the metabolism of L-tyrosine *in vivo* would be more readily oxidized further, rather than sulphated, and even sulphated homogentisic acid seems a more likely product. The production of free L-tyrosine *O*-sulphate by this novel route is therefore likely to be only trivial.

An important side issue of this study is that *p*-hydroxyphenylpyruvic acid *O*-sulphate, which is present in normal mammalian urine (Hext *et al.*, 1973), can safely be assumed to have arisen only from the metabolism of L-tyrosine *O*-sulphate, and can therefore be taken into account when calculating the turnover *in vivo* of the latter compound. Although *p*-hydroxyphenylpyruvic acid *O*-sulphate remained stable in the incubation mixture, its spontaneous oxidation to *p*-hydroxybenzaldehyde *O*-sulphate, under the strongly alkaline conditions which can obtain in certain mammalian urines, occurs readily (Rose & Powell, 1963). The latter compound can therefore also be a urinary end product of L-tyrosine *O*-sulphate metabolism. It is unlikely that it could undergo further change to the *p*-hydroxybenzyl alcohol *O*-sulphate, since this is a reductive process and is not spontaneous. Any of this product found in urine would therefore have arisen from *p*-hydroxybenzaldehyde *O*-sulphate formed directly in the tissues and would have no connexion with L-tyrosine *O*-sulphate metabolism. The reductase system detected, which is active towards *p*-hydroxybenzaldehyde *O*-sulphate, is worthy of further investigation.

p-Hydroxyphenylacetic acid *O*-sulphate was readily produced in the system by sulphation of the free phenol, but the compound was not produced by oxidation of *p*-hydroxyphenylpyruvic acid *O*-sulphate under the same conditions. This latter reaction readily occurs spontaneously in alkaline urine (Rose & Powell, 1963), so that in addition to the compounds already mentioned, *p*-hydroxyphenylacetic acid *O*-

sulphate also can represent a urinary end product of L-tyrosine *O*-sulphate metabolism. However, in view of its ease of formation from the free phenol, and also from tyramine (P. M. Hext & F. A. Rose, unpublished work), caution is needed in calculating its precise significance in urine.

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