Studies on L-Tyrosine *O*-Sulphate

1. PREPARATION AND PROPERTIES OF POTASSIUM *p*-HYDROXYPHENYLPYRUVIC ACID [^{35}S]SULPHATE

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It has been shown that L-tyrosine *O*-sulphate, a component of mammalian fibrinogens, is not desulphated to any appreciable extent by the rat (Dodgson, Powell & Tudball, 1960). However, the ester is metabolized to yield two major products, one of which has been identified as the sulphate ester of *p*-hydroxyphenylacetic acid (Dodgson, Powell, Rose & Tudball, 1961). The latter compound was eliminated in an unchanged form when injected into rats and the possibility therefore existed that the unidentified metabolite was the sulphate ester of *p*-hydroxyphenylpyruvic acid, a probable intermediate on the pathway between L-tyrosine *O*-sulphate and *p*-hydroxyphenylacetic acid sulphate. However, this could not be confirmed because an authentic sample of the ester could not be prepared.

p-Hydroxyphenylpyruvic acid readily undergoes spontaneous conversion into *p*-hydroxyphenylacetic acid (Bloxam, Day, Gibbs & Woolf, 1960) and *p*-hydroxybenzaldehyde (Doy, 1960) but the sulphate ester has been prepared by modifying the standard procedure (Burkhardt & Lapworth, 1926) so that mildly acidic conditions were maintained throughout. Under such conditions the ester sulphate linkage and the labile α-oxo acid side chain were reasonably stable. The present work describes this preparation and also shows that the sulphate ester exhibits behaviour similar to the parent phenol with respect to the lability of the α-oxo acid side chain.

MATERIALS AND METHODS

Potassium p-*hydroxyphenylpyruvic acid* [^{35}S]*sulphate.* Throughout the preparation, whenever the experimental procedure had to be interrupted the material was stored at −20° to minimize the possibility of hydrolysis of the ester sulphate linkage under the slightly acidic conditions prevailing.

p-Hydroxyphenylpyruvic acid (A. G. Fluka, Buchs, Switzerland) was recrystallized three times from 0·1N-HCl–ethanol (4:1, v/v) immediately before use. A mixture of carbon disulphide (2 ml.) and chloro[^{35}S]sulphonic acid (770 μc; 0·34 ml.) was cooled to −5°. Diethylaniline (1·7 ml.) was added dropwise and with constant stirring over 10 min., care being taken to keep the temperature between 0° and 5°. The temperature of the mixture (which separated into two layers on standing) was then allowed to rise to 15–20°, when *p*-hydroxyphenylpyruvic acid (0·7 g.) suspended in 2 ml. of carbon disulphide was added in one portion. The mixture was stirred for 1 hr. at room temperature before keeping for 16 hr. at 2°. Carbon disulphide was poured off from the viscous syrup and the latter was then liquefied by warming to 40° before adding to a mixture of 10 g. of crushed ice and 20 ml. of 0·3M-barium acetate solution that had been adjusted to pH 4 with acetic acid. The mixture was stirred thoroughly with the addition from time to time of ethanol (total vol. added, 10 ml.), which helped to disperse the syrup. Unchanged parent phenol was removed by three successive extractions with 50 ml. portions of ether. The $Ba^{35}SO_4$ precipitate was removed by centrifuging and washed with 10 ml. of water. The washings and supernatant were combined and excess of Ba^{2+} ions and diethylaniline were removed by passage through a column (10 cm. × 2 cm.) of Dowex 50 ion-exchange resin (200–400 mesh; H^+ form; Dow Chemical Co., Mich., U.S.A.). The column was finally washed with 40 ml. of water and the eluate and washings were combined. The strongly acidic solution was concentrated by freeze-drying at an ambient temperature of −15°. Under these conditions, acetic acid and hydrochloric acid were removed and the residue of the sulphate ester in the highly labile free acid form was obtained. Ice-cold water (5 ml.) was quickly added to the residue and the pH of the solution was raised to 4 by the cautious addition of 0·2N-KOH, the solution being kept ice-cold throughout. Local excesses of alkali were avoided. The potassium salt of the ester was precipitated by the addition of 50 ml. of ethanol and 30 ml. of ether. The whole was kept overnight at 0° and the material separated by centrifuging and dried *in vacuo* over $CaCl_2$ at room temperature. Yield of crude product, 400 mg.

At this stage the preparation was contaminated with traces of chloride and inorganic sulphate. Paper electrophoresis revealed the presence of appreciable amounts of a ^{35}S-labelled organic contaminant that was not identified but which could not be removed by recrystallization. Purification was achieved by cellulose-column chromatography. ECTEOLA-cellulose (Brown Co., Berlin, New Hampshire, U.S.A.) (50 g.) was freed from impurities by washing successively with 5 l. of 0·5N-NaOH, 5 l. of 0·3M-NaCl and 3 l. of 0·1M-NaCl–0·1N-HCl (1:1, v/v) as described by Ringertz & Reichard (1960). A slurry of the material in 0·1M-NaCl–0·1N-HCl (1:1, v/v) was de-aerated under reduced pressure at 15° and was packed into a glass tube under gravity flow. The column was then washed free from Cl^- ions with water immediately before use. The crude sulphate ester (0·4 g.), dissolved in 2 ml. of water, was

applied to the column (32 cm. × 2 cm.) and allowed to drain in. The column was eluted with 0·01 M-K_2SO_4 at a flow rate of approx. 2 ml./min. until the eluate no longer contained Cl^- ions (110 ml. collected). The sulphate ester was then eluted with 0·1 M-K_2SO_4. The presence of the ester in the eluate was detected with Phenistix paper reagent for aromatic pyruvic acids (Ames Co., London) and the first 150 ml. of positively reacting eluate was collected. Inorganic sulphate was precipitated as $BaSO_4$ by the addition of a slight excess of M-barium acetate that had been adjusted to pH 4 with acetic acid. The precipitate was separated by centrifuging, washed with 20 ml. of water and the clear supernatant and washings were passed through a column of Dowex 50 resin as described above. The acid eluate was concentrated to dryness by freeze-drying at −15° and the residue dissolved in 5 ml. of ice-cold water. The potassium salt of the ester was prepared and separated as described for the crude material. The product contained a small quantity of inorganic sulphate, which was liberated during freeze-drying. This was removed by dissolving the product in 2 ml. of water and adding slowly 8 ml. of ethanol at 15°. The small precipitate of K_2SO_4 was removed by centrifuging at 2000*g* and 15° for 5 min. The ester was then precipitated by adding a large excess of ethanol (50 ml.) and leaving overnight at 0°. After separation and being washed twice with ethanol, the material was dried *in vacuo* over $CaCl_2$ at room temperature and was stored in a sealed tube at −20°. Yield, 220 mg.; specific activity, 0·15 μc/mg. (Found: C, 32·2; H, 1·8; K, 23·5; ester SO_4^{2-} ion, 29·2. $C_9H_6K_2O_7S$ requires C, 32·1; H, 1·8; K, 23·3; SO_4, 28·5%).

Other sulphate esters. Potassium *p*-hydroxyphenylacetic acid [^{35}S]sulphate was prepared by the method of Dodgson *et al.* (1961) and potassium *p*-hydroxybenzaldehyde [^{35}S]-sulphate was kindly provided by Mr T. Flanagan.

Paper chromatography and paper electrophoresis. Whatman no. 1 filter paper was used throughout. Development (descending) of chromatograms was with butan-1-ol–acetic acid–water (50:12:25, by vol.) or *tert.*-butyl alcohol–formic acid–water (8:3:4, by vol.) at 15°. In electrophoresis experiments, compounds were run horizontally as spots (approx. 5 mm. diameter) in the presence of 0·1 M-sodium acetate–acetic acid buffer, pH 4·5, for 2·5 hr. at 15° and at a potential gradient of 8 v/cm. Radioactive materials were located on papers by radioautography (see Dodgson & Tudball, 1960); the compounds could also be detected by viewing under ultraviolet light.

Spectrophotometry. Ultraviolet-absorption spectra were determined in 1 cm. cells either manually with the Hilger Uvispek or automatically with the Unicam SP. 700 recording spectrophotometer.

EXPERIMENTAL AND RESULTS

Keto–enol tautomerism of potassium p-*hydroxyphenylpyruvic acid* [^{35}S]*sulphate*

p-Hydroxyphenylpyruvic acid, in common with other aromatic α-oxo acids, undergoes a slow spontaneous keto–enol tautomerization (Bücher & Kirbirger, 1952; Knox & Pitt, 1957; Schwarz, 1961). When freshly crystallized from ethanol, the acid appears to exist mainly in the enol form. Aqueous solutions, however, readily yield an equilibrium mixture containing large amounts of the keto form.

The ultraviolet-absorption spectra of the keto and enol forms are markedly different. Thus the enol tautomer in solution at pH 6 shows a single peak at 300 mμ ($\epsilon_{max.}$ 25 120). Equilibration to the keto tautomer occurs at this pH and is accompanied by a shift in the absorption maximum to a lower wavelength (276 mμ) and a decrease in absorption ($\epsilon_{max.}$ 21 870) (Knox & Pitt, 1957).

Unfortunately, the corresponding sulphate ester could not be crystallized from ethanol and aqueous ethanol had to be used. When crystallized in this way, the ester showed evidence of keto–enol transformation, but the wavelength shifts and changes in intensity of absorption contrasted with those shown by the ethanol-crystallized parent phenol. Thus the absorption spectrum of the ester in solution at pH 6 showed two weak maxima (Fig. 1, curve *A*, ϵ_{276} 871; ϵ_{290} 785), which did not, however, change appreciably at room temperature within 24 hr. At pH 2·9 the same two absorption peaks were present, but the absorption at 290 mμ rapidly increased with time for about 2 hr., when

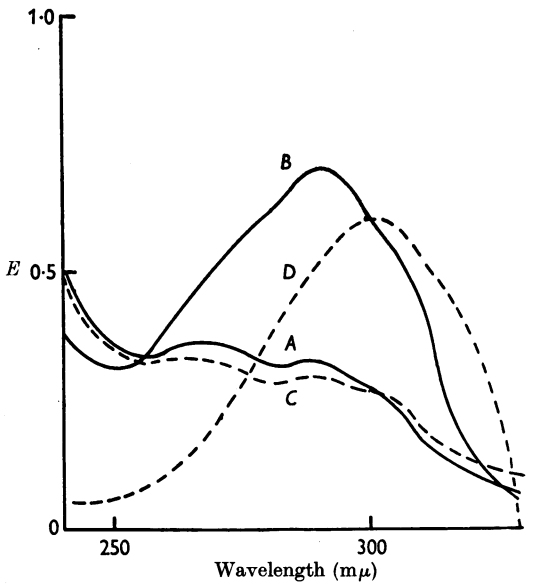

Fig. 1. Changes in absorption spectrum associated with keto–enol tautomerism of potassium *p*-hydroxyphenylpyruvic acid [^{35}S]sulphate. Absorption spectra of the ester (0·42 mM): *A*, in 0·1 M-sodium acetate–acetic acid buffer, pH 6; *B*, 2 hr. after solution in 1% (v/v) acetic acid (pH 2·9); *C*, after keeping in 1% (v/v) acetic acid for 2 hr. (see curve *B*) followed by adjusting to pH 6 with 10 N-NaOH and keeping for a further 1 hr. *D*, Enol–borate spectrum of potassium *p*-hydroxyphenylpyruvic acid [^{35}S]sulphate (55·7 μM) 2 hr. after solution in 0·17 M-phosphate buffer containing 0·34 M-boric acid (pH 6·4).

a steady state was reached. The spectrum then showed only a single peak at 290 mμ (curve *B*, $\epsilon_{max.}$ 1700). This peak decreased in intensity when the pH was subsequently raised to 6 (curve *C*). These results suggest that the keto form predominates when the sulphate ester is recrystallized from aqueous ethanol.

A more precise spectrophotometric method for following the interconversion of the keto and enol forms of aromatic α-oxo acids has been developed by Knox & Pitt (1957) and depends on the formation of a complex of the enol tautomer with boric acid. The absorption spectrum of the enol–borate complex of freshly crystallized free *p*-hydroxyphenylpyruvic acid (enol form) is similar to that of the pure enol form, except that the absorption maximum is at a higher wavelength ($\lambda_{max.}$ enol form 300 mμ; $\lambda_{max.}$ enol–borate complex 308 mμ). On the other hand, when boric acid is added to the keto form, the spectrum is not immediately altered but the absorption at 308 mμ increases slowly with time, reaching equilibrium after 24 hr. These workers have also shown that the spontaneous rate of formation of the enol–borate complex is considerably increased in the presence of arsenate buffer.

The absorption spectrum of freshly crystallized potassium *p*-hydroxyphenylpyruvic acid [^{35}S]-sulphate (55·7 μM) in the presence of 0·17 M-phosphate buffer containing 0·34 M-boric acid (the whole at pH 6·4) is shown in Fig. 1, curve *D*. A characteristic enol–borate spectrum was not obtained immediately but developed with time, equilibrium being attained after about 2 hr., when a single peak at 302 mμ was established. This observation is consistent with the conclusions drawn from the preliminary spectroscopic studies that the ester crystallizes from aqueous ethanol in the keto form.

When 0·17 M-arsenate buffer was substituted for the phosphate buffer virtually the same spectrum was obtained but the time taken to attain equilibrium was reduced to 10 min.

Lin, Pitt, Civen & Knox (1958) have used the keto–enol difference spectrum of *p*-hydroxyphenylpyruvic acid (i.e. the spectrum obtained when a solution of the keto acid in the presence of borate–arsenate buffer is measured against the same concentration of keto acid in the presence of arsenate buffer alone) to identify and assay aromatic α-oxo acids in biological materials. The keto–enol difference spectrum of the sulphate ester was identical with that of the enol–borate complex.

Conversion into potassium p-*hydroxybenzaldehyde* [^{35}S]*sulphate*

The occurrence of *p*-hydroxybenzaldehyde in 'highly purified' preparations of *p*-hydroxyphenylpyruvic acid has been known for some time and it is now clear that the former compound can arise from the spontaneous transformation of the latter, particularly under mildly alkaline conditions (Doy, 1960). Cavallini & Stirpe (1957) have noted that an analogous transformation of phenylpyruvic acid into benzaldehyde occurs, oxidatively, under similar alkaline conditions. These conversions can be studied by observing the instantaneous and irreversible spectroscopic changes that occur when the pH of a solution of the acid is raised momentarily.

It has now been shown that the sulphate ester of *p*-hydroxyphenylpyruvic acid behaves in a similar way. A solution (0·134 mM) of the ester in 0·1 M-phosphate buffer, pH 7·7, absorbed only weakly in the region 240–300 mμ (Fig. 2, curve *A*). Upon raising the pH of the solution above 11 momentarily, followed by re-adjustment to 7·7, the spectrum was markedly altered and showed a single peak at 256 mμ (curve *B*), characteristic of authentic *p*-hydroxybenzaldehyde sulphate (curve *C*). Subsequent lowering of the pH of the solution to 2·9 was accompanied by only a minor change in the wavelength of maximum absorption. When the

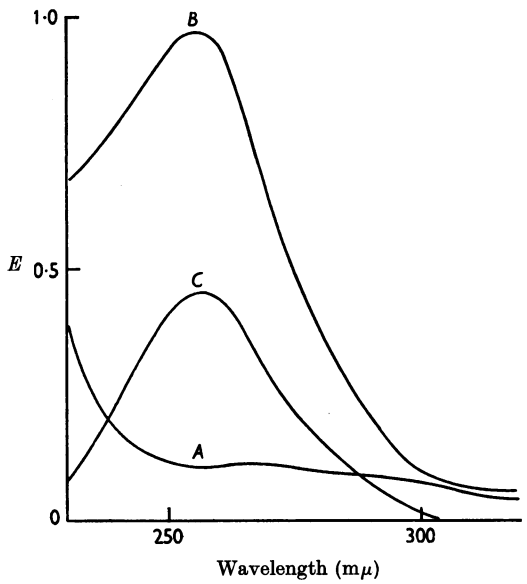

Fig. 2. Spectroscopic changes associated with the spontaneous conversion of potassium *p*-hydroxyphenylpyruvic acid [^{35}S]sulphate into *p*-hydroxybenzaldehyde [^{35}S]-sulphate under mildly alkaline conditions. *A*, spectrum of *p*-hydroxyphenylpyruvic acid [^{35}S]sulphate (0·134 mM) in 0·1 M-phosphate buffer, pH 7·7; *B*, spectrum obtained when the pH of the solution used for *A* was raised momentarily to approx. 11·5 with 10 N-NaOH and re-adjusted to 7·7 with 10 N-HCl; *C*, spectrum of *p*-hydroxybenzaldehyde [^{35}S]sulphate (22·5 μM) in 0·1 M-phosphate buffer, pH 7·7.

pH of the original solution was lowered to 2·9 without intermediate elevation to 11, as expected, a single peak at 290 mμ, characteristic of the parent ester at this pH, was obtained (cf. curve *B*).

Further proof of the conversion of *p*-hydroxyphenylpyruvic acid [^{35}S]sulphate into the corresponding aldehyde came from chromatographic and electrophoretic experiments. Higher concentrations (7·4 mM) of starting material had to be used in these experiments and under these circumstances the conversion, as measured by changes in the absorption spectrum, did not occur so readily. However, the process could be accelerated by bubbling air through the alkaline solution for 30 min. The chromatographic mobility (in two solvent systems) and electrophoretic mobility of the material produced were identical with those of authentic *p*-hydroxybenzaldehyde [^{35}S]sulphate.

Conversion into potassium p-*hydroxyphenylacetic acid* [^{35}S]*sulphate*

Bloxam *et al.* (1960) have shown by paper chromatography that *p*-hydroxyphenylpyruvic acid can undergo spontaneous conversion into *p*-hydroxyphenylacetic acid in aqueous solution in the range pH 6–9. During preliminary chromatographic studies on the [^{35}S]sulphate ester of *p*-hydroxyphenylpyruvic acid, traces of a contaminant having a mobility identical with that of *p*-hydroxyphenylacetic acid [^{35}S]sulphate were sometimes detectable, suggesting that a similar conversion was occurring. However, when attempts were made to confirm this possibility by spectrophotometric means, it became apparent that the sulphate ester was less susceptible to spontaneous conversion into the acetic acid derivative than was the parent phenol. It was possible, however, to demonstrate that the conversion could occur on paper chromatograms. Chromatograms of potassium *p*-hydroxyphenylpyruvic acid [^{35}S]sulphate were run in one direction with butan-1-ol–acetic acid–water and were then kept in air at room temperature for 5, 25 or 52 hr. before running in a second direction in the same solvent system.

Increasing quantities of a material having a mobility identical with that of authentic *p*-hydroxyphenylacetic acid [^{35}S]sulphate were formed as the period of standing in air was increased. This material was eluted with water from the chromatograms and, after addition of sufficient 10N-sodium hydroxide to give a final concentration of 0·1N-sodium hydroxide, the absorption spectrum of the eluate was measured against that of an eluate from an adjacent and equal area of the paper. The resultant spectrum was similar to that of authentic *p*-hydroxyphenylacetic acid [^{35}S]sulphate ($\lambda_{max.}$ 265 mμ) in 0·1N-sodium hydroxide.

DISCUSSION

The successful preparation of unlabelled and ^{35}S-labelled potassium *p*-hydroxyphenylpyruvic acid sulphate depends on strict adherence to the experimental details given. In particular it is important to recrystallize the parent phenol, especially if it has been stored for some time. The time allowed for the sulphation step should not be decreased and the precautions that are taken to protect the labile α-oxo acid side chain should be observed. It is important to store the material at low temperature in sealed containers.

The conversion of the ester into the *p*-hydroxybenzaldehyde derivative presumably proceeds by an oxidative mechanism similar to that proposed by Cavallini & Stirpe (1957) for the conversion of phenylpyruvic acid into benzaldehyde. In contrast, little is known of the mechanisms involved in the spontaneous conversion of aromatic α-oxo acids into the corresponding acetic acid derivatives. The conversion of *p*-hydroxyphenylpyruvic acid [^{35}S]sulphate into *p*-hydroxyphenylacetic acid [^{35}S]sulphate did not proceed readily in ordinary aqueous solution. Powell, Rose & Dodgson (1963), however, have noted that the change occurs relatively rapidly in the presence of rat urine. The present work re-emphasizes the need for care in interpreting the results of metabolic studies on the aromatic α-oxo acids and their derivatives (cf. Powell *et al.* 1963).

SUMMARY

1. A method is described for the preparation of the [^{35}S]sulphate ester of *p*-hydroxyphenylpyruvic acid.

2. The sulphate ester, like the parent phenol, exhibits keto–enol tautomerism and undergoes spontaneous conversion into *p*-hydroxybenzaldehyde [^{35}S]sulphate at pH greater than 11.

3. Spontaneous conversion of the ester into *p*-hydroxyphenylacetic acid [^{35}S]sulphate occurs on paper chromatograms.

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Studies on L-Tyrosine *O*-Sulphate

2. IDENTIFICATION OF *p*-HYDROXYPHENYLPYRUVIC ACID [35S]SULPHATE AS A METABOLITE OF L-TYROSINE *O*[35S]-SULPHATE IN THE RAT

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Dodgson, Powell, Rose & Tudball (1961) have shown that *p*-hydroxyphenylacetic acid [35S]-sulphate and an unknown [35S]sulphate ester are present in the urine of rats that have been injected with L-tyrosine *O*[35S]-sulphate. The identity of the unknown ester has now been established as *p*-hydroxyphenylpyruvic acid [35S]sulphate. A preliminary account of this work has been given (Powell, Rose & Dodgson, 1962).

MATERIALS AND METHODS

Sulphate esters. Potassium L-tyrosine *O*[35S]-sulphate was prepared as described by Dodgson *et al.* (1961). Other sulphate esters were obtained as described by Rose & Powell (1963).

Paper chromatography. Descending chromatograms were run overnight on Whatman no. 1 paper with butan-1-ol–acetic acid–water (50:12:25, by vol.). Sufficient material was applied to the paper to give approx. 500 counts/min. (measured on the paper with a Panax monitor). Radioactive areas were located with the C 100 Actigraph automatic strip scanner (Nuclear-Chicago Corp., Ill., U.S.A.) or by radioautography.

Paper electrophoresis. The conditions described by Rose & Powell (1963) were used. Separation of inorganic sulphate and the sulphate esters of *p*-hydroxyphenylpyruvic acid, *p*-hydroxyphenylacetic acid, *p*-hydroxybenzaldehyde and L-tyrosine could be achieved, the rate of movement towards the anode decreasing in that order.

Experimental animals. Female M.R.C. hooded rats (aged 2–4 months) were used throughout. These animals were injected intraperitoneally under light ether anaesthesia and were subsequently placed in metabolism cages designed to permit separate collection of urine and faeces. The first voided urines were collected and either used immediately or stored at −20° until required.

EXPERIMENTAL AND RESULTS

Preliminary experiments in which the urine from rats that had been injected with L-tyrosine *O*[35S]-sulphate (1·5 mg./200 g. body wt.) was subjected to paper chromatography showed that the mobility of the unknown metabolite (R_F 0·28, see Dodgson *et al.* 1961) corresponded to that of the authentic sample of *p*-hydroxyphenylpyruvic acid [35S]-sulphate when the latter was chromatographed in the presence of normal rat urine. A similar agreement was obtained between electrophoretic mobility of metabolite and authentic sample. Both compounds could be detected on paper under ultraviolet light. These results could not consistently be repeated, however, and during these experiments, and others designed for the isolation of the metabolite, it became obvious that the material was subject to spontaneous changes similar to those undergone by the authentic sample of *p*-hydroxyphenylpyruvic acid [35S]sulphate, which at room temperature is converted into *p*-hydroxybenzaldehyde [35S]sulphate and *p*-hydroxyphenylacetic acid [35S]sulphate (Rose & Powell, 1963). In all subsequent experiments precautions were taken to collect urine immediately it was voided, and, as far as possible during subsequent manipulation, urine samples were kept at 2° and extremes of pH were avoided.

Keto–enol difference spectrum of the metabolite

Rose & Powell (1963) have shown that *p*-hydroxyphenylpyruvic acid [35S]sulphate gives a characteristic keto–enol difference spectrum analogous to that given by free *p*-hydroxyphenylpyruvic acid