The Detection and Determination of L-Tyrosine O-Sulphate in Rabbit and other Mammalian Urine

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A sensitive method using the Technicon Amino Acid Autoanalyzer was developed for the determination of L-tyrosine O-sulphate in mammalian urine. Normal rabbit urine was shown to contain a material that has properties identical with L-tyrosine O-sulphate. The material is also a normal constituent of rat, sheep, calf and mouse urine and is excreted by these animals in quantities ranging from 0.1 to 0.8 mg./day/kg. body wt.

The O-sulphate ester of L-tyrosine occurs in a bound form in the mammalian system as a component of the fibrinopeptides B and fibrinogens of a wide variety of species (see Doolittle & Blombäck, 1964). Its presence in gastrin II isolated from hog antral mucosa has also been reported (Gregory, Hardy, Jones, Kenner & Sheppard, 1964). The ester, in a free state, is found as a constituent of normal human urine (Tallan, Bella, Stein & Moore, 1955) and this has been assumed by some workers to arise partly from fibrinogen turnover. Grimes (1959), however, failed to detect tyrosine O-sulphate in the urine of rat, mouse, guinea pig and rabbit but confirmed that it was present in human urine.

The rapid deamination of L-tyrosine O-sulphate when injected into rats that was observed by Dodgson, Powell, Rose & Tudball (1961) provided a possible explanation for the absence of the ester from rat urine. However, the later finding of L-tyrosine O-[³⁵S]-sulphate in the urine of rabbits that had been injected with rabbit fibrinopeptide B containing ³⁵S-labelled L-tyrosine O-sulphate residues (Jones, Dodgson, Powell & Rose, 1963) indicated the necessity to re-examine rabbit urine for the presence of the ester. By chromatography on a cation-exchange resin by using a special elution procedure and the Technicon Amino Acid Autoanalyzer, L-tyrosine O-sulphate has now been detected and determined in the urine of the rabbit and other mammals. A preliminary report of this work has already been made (John, Rose, Wusteman & Dodgson, 1965).

MATERIALS AND METHODS

Reagents. All the chemicals used for the preparation of buffers and reagents were British Drug Houses Ltd. (Poole, Dorset) AnalaR grade. Potassium L-tyrosine O-sulphate was prepared as described by Dodgson *et al.* (1961).

Urine samples. Specimens (24 hr.) of the urine of all animals were collected in such a way as to avoid contamination of the urine with faeces. Samples (5 ml.) of the filtered urines were adjusted with conc. HCl to pH 2.5 and were then diluted with an equal volume of 0.05 N-HCl containing norleucine (0.15 mM) as internal standard.

Arylsulphatase. Electrophoretically purified arylsulphatase (aryl sulphate sulphohydrolase, EC 3.1.6.1) of *Alcaligenes metalcaligenes* was prepared according to the method described by Dodgson, Spencer & Williams (1955). The final preparation possessed no hydrolase activity towards phenolphthalein glucuronide, *p*-nitrophenyl phosphate or phenyl acetate but was strongly active towards L-tyrosine O-sulphate, 1 ml. of the enzyme solution liberating 0.6 μ mole of L-tyrosine/ml. from 0.01 M-L-tyrosine O-sulphate in 0.2 M-tris-acetic acid buffer, pH 9.0, at 37° over 1 hr.

Autoanalytical procedure. Analysis of the ninhydrin-positive constituents of urine was carried out by chromatography on Chromobead type A cation-exchange resin (Technicon Chemical Co. Inc., New York, N.Y., U.S.A.) with the aid of the Technicon Amino Acid Autoanalyzer. A procedure similar to that used by King (1964) for the analysis of human urine but with some modifications was employed. The column was equilibrated with sodium citrate-NaCl buffer (0.2 M with respect to Na⁺), pH 2.5. This was prepared by titrating stock M-sodium citrate buffer to pH 2.5 with 6 N-HCl (see the manual supplied by Technicon Instruments Co. Ltd., Chertsey, Surrey).

The use of a starting pH (2.5) lower than that recommended in the Technicon manual (pH 2.875) for the analysis of protein hydrolysates was necessary to achieve a satisfactory resolution of the strongly acidic components present in urine. The column was then loaded (hypodermic syringe) with approx. 1 ml. of the diluted acidified sample and eluted by either of the following procedures according to the information required with the sample being tested. For the determination of both L-tyrosine O-sulphate and L-tyrosine in the same sample, elution was achieved with a continuous gradient between 0.2 M-sodium citrate, pH 2.5, and 0.8 M-sodium citrate, pH 5.0. The Autograd was filled as for the gradient elution of amino acids from protein hydrolysates (Technicon manual), except that 0.2 M-sodium citrate

buffer, pH 2.5 (instead of at pH 2.875), was placed in the first two chambers. The elution required 20 hr. for completion. The pump rate varied slightly from one run to another, causing the position of L-tyrosine on the time scale to vary, but the latter could be easily identified by reference to the position of the norleucine standards or the adjacent very large ammonia peak.

When only L-tyrosine O-sulphate was being detected or determined in a sample, as in routine measurements, there was no necessity to run a full 20 hr. analysis by full gradient elution between pH 2.5 and 5.0. Instead, the elution was started with pH 2.5 buffer and allowed to continue for approx. 20 min., under which conditions the L-tyrosine O-sulphate emerged approx. 35 min. after frontal elution. The buffer was then changed abruptly (manually) to 0.8 M-sodium citrate, pH 5.0, and the elution continued until the norleucine standard appeared (approx. 90 min.). Estimations of ninhydrin-positive components were carried out by using the procedure described in the Technicon manual.

It should be noted that the Amino Acid Autoanalyzer system is so designed that the absolute area under individual peaks in the elution pattern depends on several factors (volume loaded, fraction of effluent entering the analytical system etc.) that need not be reproducible from one analysis to the next. For this reason, all analyses require a suitable internal standard. In the present case norleucine was found to emerge in a position well separated from urine components. A known quantity of norleucine was therefore added to each urine sample before loading on to the column so that the areas of the relevant peaks appearing on the recording chart in different experiments could be related to each other in terms of the norleucine peaks.

The relationship in ninhydrin colour values between L-tyrosine O-sulphate, free L-tyrosine and norleucine was determined in separate experiments in which standard mixtures of the three compounds were chromatographed under elution conditions similar to those used in the urine experiments.

For simplicity and to enable direct comparison of different experiments to be made, the chromatograms obtained in the present work were redrawn (see Figs. 1, 2, 3 and 4) so that they are directly comparable with each other. This was achieved by converting the original records of optical extinction versus time into a record of norleucine equivalents/min. against time (1 norleucine equivalent is that amount of a ninhydrin-positive component that would give rise to the same extinction as 1 μ mole of norleucine when both are subjected to chromatography in the same sample).

EXPERIMENTAL AND RESULTS

Analysis of normal rabbit urine. The complete analysis of a sample (approx. 1 ml.) of normal rabbit urine by the procedure described above gave a pattern of acidic components (Fig. 1) that was very similar to that obtained by King (1964) for human urine. One of the components in rabbit urine, which had a mobility comparable with an unidentified component detected by King (1964), could be increased by the addition of authentic L-tyrosine O-sulphate to the urine before analysis (Fig. 2). More-

Fig. 1. Elution pattern of normal rabbit urine, showing the position of emergence of the component under investigation. Experimental details are given in the text. Peaks A and B are ascribed to L-tyrosine O-sulphate and L-tyrosine respectively.

over, in this experiment, the increase in the size of the chromatographic peak representing the component was equal to the amount of ester added. These observations indicated that the component might be L-tyrosine O-sulphate.

Acid hydrolysis. Support for this claim was obtained by subjecting the urine to mild acid hydrolysis before analysis. The urine (2.5 ml.) was acidified with concentrated hydrochloric acid to 0.4 N and then heated on a boiling-water bath for 30 min. The hydrolysate was clarified by centrifugation, 1 ml. of the supernatant was mixed with 1 ml. of norleucine standard and approx. 1 ml. of the mixture analysed as before. The component was no longer present in the urine sample after this treatment (Fig. 3). The free L-tyrosine content had, however, increased by a corresponding amount, assuming that the component was L-tyrosine O-sulphate and that it had undergone complete hydrolysis.

These results indicated that the component was an acid-labile conjugate of L-tyrosine but the possibility that the component was the sulphuric acid ester required further verification. This was

Fig. 2. Elution pattern of normal rabbit urine to which had been added authentic L-tyrosine *O*-sulphate ($0.2 \mu\text{mole/ml.}$). Experimental details are given in the text. Peak A is ascribed to L-tyrosine *O*-sulphate.

achieved by treating the urine before hydrolysis with a preparation of a purified arylsulphatase active towards L-tyrosine *O*-sulphate.

Treatment with arylsulphatase. The urine (2ml.) was incubated for 1 hr. at 37° with 0.2ml. of a solution of the purified enzyme, the mixture being previously adjusted to pH 9.0 with a trace of 0.1N-sodium hydroxide. The treated urine was then subjected to analysis as before. A comparison of the chromatograph of the treated urine (Fig. 4) with that of the untreated urine (Fig. 1) shows that the component was removed by treatment with the arylsulphatase. The amount of free L-tyrosine in the sample was simultaneously increased.

Chromatography on Dowex 1. Rabbit urine could not be chromatographed satisfactorily on Dowex 1 without some preliminary purification. In all the following experiments the theoretical position of emergence of L-tyrosine *O*-sulphate from the columns was determined in a separate experiment in which L-tyrosine $O[^{35}\text{S}]$ -sulphate had been added to the urine. A 24hr. specimen of rabbit urine (140ml.) was treated with saturated barium

Fig. 3. Elution pattern of normal rabbit urine after acid-hydrolysis treatment. Experimental details are given in the text. Peak B is ascribed to L-tyrosine.

Fig. 4. Elution pattern of normal rabbit urine after sulphatase treatment. Experimental details are given in the text. Peak B is ascribed to L-tyrosine.

hydroxide solution until no more precipitation occurred. The precipitate was separated by centrifuging for 10 min. at 2000g and the clear supernatant was then evaporated to dryness *in vacuo* at 40°. The residue was dissolved in 7 ml. of water, and 2 ml. of the resultant solution was then applied to a column (10 cm. × 1 cm.) of Dowex 50 ion-exchange resin (200–400 mesh; H⁺ form) and eluted with water. The first 4 ml. of the acidic eluate was rejected, and the next 10 ml. was collected, neutralized with 2N-potassium hydroxide and concentrated to dryness *in vacuo* at 40°. The residue, dissolved in 2 ml. of water, was subjected to chromatography on Dowex 1 ion-exchange resin (200 mesh; acetate form) by using the procedure described by Tallan *et al.* (1955) for the determination of L-tyrosine O-sulphate in human urine. The appropriate fractions of the eluate from four such columns were combined (120 ml.), passed through a column (30 cm. × 2 cm.) of Dowex 50 (20–50 mesh; H⁺ form) and freeze-dried at –15° to remove acetic acid. The residue was taken up in 2 ml. of ice-cold water and neutralized with N-potassium hydroxide.

Autoanalysis of this solution by the technique described above revealed the presence of a component identical with that already detected in rabbit urine directly. This component behaved like L-tyrosine O-sulphate on hydrolysis with acid and sulphatase. Further, when the solution was subjected to thin-layer chromatography by the procedure described by Wusteman, Dodgson, Lloyd, Rose & Tudball (1964), the presence of a considerable quantity of a component having the same chromatographic mobility as L-tyrosine O-sulphate could be detected.

Determination of the ester in the urine of rat, mouse, sheep and calf. Autoanalysis of the urine of these animals gave chromatographic patterns that were all similar to that obtained for rabbit urine (cf. Fig. 1). All possessed the component identifiable with L-tyrosine O-sulphate. The concentrations of the ester in all these animal urines (in mg./24 hr./kg. body wt.) are as follows: rat, 0.11; sheep, 0.30; human, 0.32; calf, 0.36; rabbit, 0.64; mouse, 0.82.

DISCUSSION

Contrary to the findings of Grimes (1959), which suggested that L-tyrosine O-sulphate was not a constituent of normal rabbit urine, a material having the properties of this ester has now been shown to be excreted by these animals. The excretion of this material by other animals also conflicts with these earlier findings and suggests that the ester may be a common constituent of mammalian urine. Moreover, the quantity of the ester

excreted daily by these animals is comparable, on a body-weight basis, with the amount excreted by the human (Tallan *et al.* 1955).

The observations support the findings of Jones *et al.* (1963), which suggested that L-tyrosine O-sulphate might be a constituent of rabbit urine. The presence of L-tyrosine O-sulphate in rat urine is particularly interesting for two reasons. First, the ester is known to undergo rapid deamination when injected into these animals. Secondly, the source of the ester is obscure since free tyrosine does not undergo sulphonation in the liver 3'-phosphoadenylyl sulphate-phenol-sulphotransferase system (EC 2.8.2.1) *in vitro* (Segal & Mologne, 1959; Grimes, 1959; Vestermark & Boström, 1959) and, further, rat tissues are not known to contain any sulphated tyrosine residues. However, Jones & Dodgson (1965) have found that L-tyrosine methyl and ethyl esters can be sulphated and then degraded to free L-tyrosine O-sulphate by rat-liver preparations.

The method described for the determination of urinary L-tyrosine O-sulphate is sensitive and can be used to determine the ester directly in any mammalian urine.

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