Metabolism of Xanthurenic Acid in the Rat

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The chromatographic pattern of metabolites of tryptophan and xanthurenic acid in urine of the normal rat has been described by Baglioni, Fasella, Turano & Siliprandi (1960). Administration of xanthurenic acid caused the excretion of three major components, one of which is xanthurenic acid. When heated at 100° in strongly acidic solutions, the other two compounds yielded xanthurenic acid. Dalglish (1952) reported the presence, in the urines of tryptophan-fed pyridoxine-deficient rats, of a compound in which xanthurenic acid is conjugated with sulphate and some other unknown residue. In the urine of xanthurenic acid-treated albino rats, Kotake & Nogami (1954) and Kotake (1957) found a double ethereal sulphate and a glucuronide derivative, and Rothstein & Greenberg (1957) found a diglucuronide-monoserine derivative and a monoglucuronide-monoserine derivative. In the present paper we describe the separation and identification of two metabolites of xanthurenic acid from rat urine.

EXPERIMENTAL

Separation and purification of metabolites. Xanthurenic acid (a kind gift of Professor Musajo, University of Padova) was administered to white albino rats of about 200 g. body wt. in amounts of 300 mg./kg. The urine passed in the 24 hr. after the administration was collected and adsorbed on and eluted from deactivated charcoal according to Dalglish (1955). The dry residue of the urinary extract was dissolved in 1 ml. of water; 0.05 ml. was analysed by electrochromatography (Baglioni *et al.* 1960) and 0.3 ml. was applied to the top of a Dowex-50 ion-exchange column (50 × 0.9 cm., 150–200 mesh) in the hydrogen form and eluted with water. The effluent was collected in fractions of 0.5 ml. at a rate of 1.5 ml./hr. The fractions were examined for fluorescence in ultraviolet light (366 mμ) and, after dilution to 3 ml. with water, for their extinction at 280 and 340 mμ. The fractions corresponding to the absorption and fluorescence peaks were collected and analysed by paper chromatography (Dalglish, 1956) and electrochromatography (Baglioni *et al.* 1960). Each of the components so separated was further purified by submitting it to electrophoresis on a cellulose column (51 cm. × 4 cm.; sodium acetate buffer, pH 5.4, 1.0 M; 600 v, 8 ma for 9 hr.). The bottom of the column was connected to the anode. On completion of the electrophoretic run the column was eluted with the same buffer at the rate of 12 ml./hr. and the effluent col-

lected in fractions of 3 ml. each. The effluent was examined for fluorescence and extinction as described above. To remove the buffer used in electrophoresis, the major component was concentrated under high vacuum and re-chromatographed on a Dowex-50 column as described above. Fractions containing the purified substance were collected and lyophilized.

Analytical tests. Sulphate ion was detected by precipitation with barium chloride and glucuronic acid by the naphtharesorcinol reaction. Amino acids were detected by paper chromatography in phenol (Consden, Gordon & Martin, 1944), lutidine–collidine–water (Dent, 1948) and 80% pyridine (Block & Bolling, 1951) and by paper electrophoresis at pH 6.0 in 0.02 M-potassium phosphate buffer. Pauly's reaction for phenolic groups was according to Feigl (1956).

RESULTS

The pattern of the electrochromatographic separation of the urinary extracts after administration of xanthurenic acid is shown in Fig. 1. The separation

Fig. 1. Electrochromatographic separation of metabolites of xanthurenic acid in rat urine. One-twentieth of the urine eliminated by one rat in the 24 hr. after the administration was applied at the corner of a square paper. The paper was first developed by descending chromatography (upward direction in the figure) and subsequently submitted to electrophoresis (horizontal direction in the figure). Spots 1, 2, 3 and 4 are derivatives of xanthurenic acid, 5 is xanthurenic acid and 6 is urinary indican. Spots 1 and 3 are substances I and II respectively. Spots 1–5 are fluorescent; spot 6 was detected with *p*-dimethylaminobenzaldehyde.

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Fig. 2. Separation of metabolites of xanthurenic acid on a Dowex-50 column. About one-third of the urine eliminated by one rat in the 24 hr. after the administration was washed down the column with water at the rate of 1.5 ml./hr. The effluent was collected in fractions of 0.5 ml. each. Extinction was determined after diluting each fraction to 3 ml. with water. —, at 340 $m\mu$; ---, at 280 $m\mu$. Fluorescence: , green-yellow; , blue. A, Urinary indican; B, substance I; C, unknown metabolite, present also before administration of xanthurenic acid; D, derivative of xanthurenic acid (spot 2 in Fig. 1); E, unknown metabolite, present also before administration of xanthurenic acid; F, substance II; G, derivative of xanthurenic acid (spot 4 in Fig. 1).

Fig. 3. Absorption spectra of substance I. Solvents: ---, 0.1 N-HCl; —, 33 mM-potassium phosphate buffer, pH 7.0; — · —, 0.1 N-NaOH. Solutions used for the determination of the spectrum contained in 3 ml. one-fiftieth of the amount of substance I eliminated by one rat in the 24 hr. after administration of xanthurenic acid.

Fig. 4. Absorption spectra of substance II. Solvents: ---, 0.1 N-HCl; —, 33 mM-potassium phosphate buffer, pH 7.0; — · —, 0.1 N-NaOH. Solutions used for the determination of the spectrum contained in 3 ml. one-fiftieth of the amount of substance II eliminated by one rat in the 24 hr. after administration of xanthurenic acid.

Table 1. *Properties of substances I and II*

	Substance I	Substance II
Before hydrolysis		
R_F in butanol-acetic acid-water (4:1:5, by vol.)	0.14	0.09
R_F in 20% (w/v) KCl	0.47	0.53
Anodic displacement* (cm.)	25.00	14.50
Pauly's reaction	Delayed	Negative
Ninhydrin test	Negative	Negative
After hydrolysis		
Period of hydrolysis in 5N-HCl at 100° (hr.) ...	3	6
Test for sulphate	Positive	Negative
Naphtharesorcinol test	Negative	Positive
Ninhydrin test	Positive	Positive
Chromatographic test (Dalglish, 1956) for xanthurenic acid	Positive	Positive

* In potassium phosphate buffer, pH 5.9, I 0.05; 10 v/cm. length of paper; 8 hr.

obtained by the first chromatography on Dowex-50 ion-exchange column is shown in Fig. 2. Fluorescence peaks *D* and *G*, which are hardly detectable in the extinction curve because the amounts are small, correspond respectively to spots 2 and 4 of the electrochromatogram. Substances I (peak *B*) and II (peak *F*) were separately submitted to electrophoresis on a cellulose column. Substance I was thus separated from urinary indican and from an unidentified substance present in very small amounts and characterized by a green fluorescence; substance II was separated from an unidentified impurity absorbing at $280\text{ m}\mu$. About 6 mg. of substance I and 4 mg. of substance II were obtained from the urine eliminated by one rat in the 24 hr. after the intraperitoneal administration of 60 mg. of xanthurenic acid.

The absorption spectra of purified substances I and II at different pH values are shown in Figs. 3 and 4. The properties of substances I and II are given in Table 1. The ninhydrin-positive substance obtained upon hydrolysis of both compounds had the same R_F values as glycine when chromatographed in phenol and collidine-lutidine. Other known ninhydrin-positive substances with a similar chromatographic behaviour and which could be confused with glycine are glutamic acid and serine. Glutamic acid, however, moves rapidly towards the anode when submitted to paper electrophoresis at pH 6.0, whereas the substance in question does not move at this pH. The R_F value of serine in 80% pyridine is 0.39, whereas that of our substance and of glycine is 0.27. Moreover, in all solvents used our ninhydrin-positive substance could not be separated from authentic samples of glycine, but was easily resolved from authentic samples of serine and glutamic acid; it seems highly probable therefore that the substance in question is glycine.

DISCUSSION

The above results show that substance I is a conjugate of xanthurenic acid with sulphate and glycine and substance II is a conjugate of xanthurenic acid with glucuronic acid and glycine. Some information on the structure of both substances is provided by their absorption spectra and reactivity with ninhydrin and Pauly's reagent. Neither substance I nor substance II reacts with ninhydrin before hydrolysis. The amino group of glycine is therefore involved in the bond, probably with the carboxyl group of xanthurenic acid. Substance II does not react with Pauly's reagent for phenols so that it seems unlikely that the —OH in position 8 of the xanthurenic acid residue is free (the —OH in position 4 is *para* to the nitrogen atom and has feeble phenolic properties, so that direct coupling with Pauly's reagent is unlikely). With substance I Pauly's reaction is positive but greatly delayed; this is compatible with substance I, which is readily hydrolysed, having a labile sulphate group at position 8.

Further evidence for the fact that in both compounds the hydroxyl group in position 8 is bound is provided by the absorption spectra. Neither shows a bathochromic shift at alkaline pH values. According to Ewing & Steck (1946), at alkaline pH 8-hydroxyquinolones show a shift of the absorption peak from $310\text{ m}\mu$ to higher wavelengths and this shift does not occur when the hydroxyl group in position 8 is bound. Conjugation is not likely at position 4 since in mammals 4-quinolones do not conjugate directly in position 4 but are first hydroxylated elsewhere, and are excreted as conjugates of hydroxyquinolones (Smith, 1953). This suggests that there is insufficient of the hydroxylated or lactim form present to permit conjugation and that, biologically, quinolones behave as if they were in the keto or lactam form.

The spectra of our substances are in agreement with the above general considerations. In both compounds the shift of the peak from 340 $m\mu$ to higher wavelengths at acidic pH values is very slight and is accompanied by a decrease in extinction. There are similar features in the spectrum of 8-methylxanthurenic acid ethyl ester (see Fig. 5), which has structural characteristics similar to those suggested for our compounds (the hydroxyl group in position 8 and the carboxyl group in position 2 are bound, whereas the hydroxyl group in position 4 is free). Compounds in which the hydroxyl group in position 4 is conjugated, e.g. 4-quinolylmethyl ether (see fig. 19 of Ewing & Steck, 1946) show a greater shift of the peak and no decrease of its intensity at acidic pH values. The above considerations suggest that the hydroxyl group in position 4 of the xanthurenic acid residue is free both in substance I and substance II.

It is concluded that substance I is a compound formed by xanthurenic acid, glycine and sulphate. Since the hydroxyl group in position 4 is free, sulphate probably esterifies the hydroxyl group in position 8, whereas glycine is bound by a peptide link to the carboxyl group (see formulae I and II).

Substance II is a compound formed by xanthurenic acid, glucuronic acid and glycine. Glycine is bound to the carboxyl group and glucuronic acid is linked to the hydroxyl group in position 8. Apart from any other consideration it seems probable that the latter bond is of glucuronide rather than of glucuronate nature since substance II is much more electronegative than xanthurenic acid (see behaviour in electrophoresis and on Dowex-50), which suggests that it has some free acidic group besides that of xanthurenic acid or glycine.

(I: R = SO_3H)

(II: R = $\text{C}_6\text{H}_4\text{O}_6$)

Fig. 5. Absorption spectra of 8-methylxanthurenic acid ethyl ester (46.5 μg . in 3 ml.) in: ---, 0.1N-HCl; —, 33 mM-potassium phosphate buffer, pH 7.0. In 0.1N-NaOH the substance was too insoluble to yield accurate extinction values, but showed a maximum at 240 $m\mu$ and a broad maximum in the region from 320 to 340 $m\mu$.

The chromatographic properties of substances I and II correspond to two compounds found by Dalglish (1956) in the urine of tryptophan-fed, pyridoxine-deficient rats and tentatively identified as derivatives of xanthurenic acid. Moreover, Dalglish (1952) described a strongly acidic compound very similar in spectrophotometric and chromatographic properties to our substance I. The hydrolysis products of this compound suggested that xanthurenic acid was conjugated with sulphate and some other unknown substance. It seems reasonable to assume that the compound of Dalglish is the same as substance I.

In the urines of rats given xanthurenic acid, Kotake & Nogami (1954) and Kotake (1957) reported the presence of a double ethereal sulphate and of a glucuronide derivative, but these authors did not note the presence of an amino acid among the hydrolysis products of either compound. Rothstein & Greenberg (1957) described a diglucuronide-monoserine derivative and a monoglucuronide-monoserine derivative of xanthurenic acid in the urine of rats. In view of the improbability of conjugation in position 4 (Smith, 1953) and our own results more direct evidence is needed for the existence of xanthurenic acid derivatives conjugated in position 4. The conclusions of the Japanese authors might have been affected by the presence of contaminants in their preparations of xanthurenic acid derivatives; the latter were isolated by paper chromatography, a technique which in our hands proved unsuitable for complete purification. The assumption of Rothstein & Greenberg rests on the observation of two glucuronic acid derivatives of xanthurenic acid, one of which can be transformed into the other by mild treatment

with acid; no direct proof, however, is given that this transformation is brought about by detachment of a glucuronic residue from the hydroxyl group in position 4 of xanthurenic acid. The fact that the R_f values of Rothstein & Greenberg's compounds B and D in butanol-acetic acid-water (4:1:5) differ from those of our compounds indicate that they are not the same substances. The difference might tentatively be ascribed to the fact that xanthurenic acid can be conjugated in the living organism in several ways and that differences exist not only between various species, e.g. the rabbit and the rat (Rothstein & Greenberg, 1957), but also between different races of the same species.

SUMMARY

1. Two major metabolites of xanthurenic acid were found in the urine of albino rats after intraperitoneal administration of xanthurenic acid.

2. These metabolites were isolated by chromatography on Dowex-50 and column electrophoresis on cellulose powder.

3. The spectral and chemical properties of the two compounds indicate that one is a derivative of xanthurenic acid in which the hydroxyl group in position 8 is bound to sulphate and the carboxyl group to glycine; the other compound differs in

that the hydroxyl group in position 8 is bound to glucuronic acid.

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The Breakdown of Pyruvate by Cell-Free Extracts of the Rumen Micro-organism LC

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The micro-organism LC, isolated from the sheep rumen by Elsden, Volcani, Gilchrist & Lewis (1956), ferments lactate and carbohydrates with the formation of volatile acids of chain length up to hexanoate. Washed cells break down pyruvate, giving carbon dioxide, acetate and butyrate as the main products (Elsden & Lewis, 1953). Supplementing the pyruvate with acetate, propionate or butyrate results in the formation of large amounts of butyrate, pentanoate or hexanoate respectively. Elsden & Lewis concluded that in LC the higher volatile fatty acids are produced by a mechanism similar to that established in *Clostridium kluyveri* by Barker and his colleagues (Barker, 1957a). Whereas the latter organism obtains acetyl-coenzyme A for fatty acid synthesis by the anaerobic oxidation of ethanol, it was presumed that

in LC this intermediate is derived from pyruvate. Preliminary experiments by S. R. Elsden & C. Warner (unpublished work) indicated that dried preparations catalyse the anaerobic oxidation of pyruvate to acetate and a more detailed investigation of this reaction was thus of importance in relation to fatty acid synthesis in LC. Previous studies on the oxidative decarboxylation of pyruvate have revealed certain well-defined differences between the systems from different micro-organisms. LC is difficult to place taxonomically (Elsden *et al.* 1956), although it has recently been suggested that it should be placed in the genus *Peptostreptococcus* (Gutierrez, Davis, Lindahl & Warwick, 1959). It bears some biochemical resemblance to clostridia but is otherwise quite distinct from all the organisms previously used in studies of pyruvate breakdown.