POLYHYDROXY (CATECHOLIC) PHENOLIC ACIDS—STUDIES OF THEIR METABOLISM IN MAN

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The urinary excretion of glycine and glutamine conjugates has been studied after the oral ingestion of some phenolic acids. The stability of such conjugates and also of the parent substances to various forms of acid hydrolysis has been made. Further observations on the 4-hydroxy-3-methoxy- and *m*-hydroxyphenolic acids present in human urine have been made.

The metabolism of aromatic substances in man is increasingly studied since it is believed that deviations from the normal may be associated with certain diseases. The metabolism of catecholic phenolic acids in man has been previously discussed (Tompsett, 1958a, 1959, 1960) and the present paper is an extension of these studies.

The following problems have been investigated. The effect of hot acid hydrolysis upon the stability of a number of phenolic acids. The evidence for conjugation with glycine or glutamine, or both, after the oral administration of a number of phenolic acids. The fractionation of methoxy phenolic acids present in urine. The presence of *m*-hydroxybenzoic and vanillic acids in urine and the metabolism of caffeic acid.

Experimental

Hydrolysis of urinary conjugates. (*a*) 10 ml. of urine and 1 ml. of 10 N hydrochloric acid were placed in boiling water bath for 1 hr., or (*b*) 10 ml. of urine and 10 ml. of 10 N hydrochloric acid were heated under a reflux condenser for 1½ hr.

Extraction of phenolic acids. Urine which had been treated as (*a*) or (*b*) above was extracted three times with 4 vol. of ether, and the extracts were evaporated to dryness.

The Determination of Conjugated Glycine and Glutamine

Glycine and aspartic and glutamic acids were measured in urine (1) untreated and (2) after hot acid hydrolysis. It was assumed that the difference between (2) and (1) would represent conjugation with glycine and glutamine respectively. To effect hydrolysis of the conjugates, 10 ml. of urine and 10 ml. of 10 N hydrochloric acid were boiled under a reflux condenser for 1½ hr. The mixture was then evaporated to dryness *in vacuo* in an all-glass still. The residue was dissolved in water, and the volume made up to 10 ml.

(i) *The determination of glycine.* The method of Smith (1953) was used. 1 ml. of urine [(1) before or (2) after hydrolysis with 5 N hydrochloric acid] was distilled in the presence of buffer pH 6·5, and ninhydrin. The formaldehyde content of the distillate, which is representative of the original amount of glycine present, was measured colorimetrically with

chromotropic acid. It has been shown that hippuric acid does not react until after hydrolysis in the presence of hot 5 N hydrochloric acid.

(ii) *The determination of aspartic and glutamic acids.* Aspartic and glutamic acids may be conveniently separated from the neutral and basic amino-acids by electrophoresis.

Apparatus: EEL electrophoretic apparatus. Whatman filter paper No. 1—34 × 5 cm. Reagent: phosphate buffer, pH 7·0/0·05 M.

Method. Examinations were duplicated. 20 μl. of urine [(1) before or (2) after hydrolysis with 5 N hydrochloric acid] were applied to the centre of each paper located in the electrophoresis apparatus. A potential difference of 2 mA per paper strip was applied for 6 hr. After drying, the positions of the three amino-acid fractions were located by means of the ninhydrin reaction on one strip. The dicarboxylic amino-acid fraction was then determined in the duplicate strip by the method of Smith and Tompsett (1954).

Paper Chromatography of Phenolic Acids

The Kawerau Unit was used and the developing solvent was benzene: acetic acid: water (Tompsett, 1958b).

Ether extracts of urine hydrolysed in the presence of 5 N hydrochloric acid (*b*) were prepared and alcoholic solutions of the residues applied to the paper. Extract equivalent to 2·5 ml. of urine was applied to the paper.

(i) *The fractionation of methoxy phenolic acids.* Developed paper chromatograms were dried and divided into 10 equal strips within the limits R_F 0·0 to 1·0. The strips were extracted with ethanol which was removed by evaporation and the methoxyl content of the residue determined (Tompsett, 1959). Owing to the low sensitivity of this reaction, the extracts from six separate chromatograms were combined.

(ii) m-*Hydroxybenzoic and vanillic acids.* The chlorimide reaction (Tompsett, 1958b; 1959; 1960) was applied to extracts obtained from strips corresponding to an R_F 0·1 to 0·5 for *m*-hydroxybenzoic acid, and an R_F 0·5 to 0·7 for vanillic acid.

Results and Discussions

The Hydrolysis of Conjugates

Although phenolic substances can be detected in urine in the free state, they are for the most part excreted as conjugates with sulphate, glucuronic acid, glycine and sometimes glutamine. The mode of conjugation can be of interest, yet confusion can result in the identification and determination of individual phenolic substances unless these are examined in the free state. Adequate methods of hydrolysis are however necessary.

Hydrolysis in the presence of N hydrochloric acid generally liberates phenolic substances from conjugation with sulphate, acetic acid and glucuronic acid. Conjugates containing glycine are quite resistant to such treatment, hydrolysis in the presence of 5 N hydrochloric acid being required. It is assumed that glutamine conjugates require the same

method of hydrolysis. The methoxyl group is resistant to both hydrolytic treatments, heating with concentrated sulphuric acid being essential.

It was considered of importance to examine the effects of acid hydrolysis upon the stability of a number of parent substances. The following substances were found to be unaffected by hot acid hydrolysis in the presence of 5 N hydrochloric acid:

o-, *m*- and *p*-Hydroxybenzoic acids, *o*-, *m*- and *p*-hydroxyphenylacetic acids, 3,4-dihydroxybenzoic acid, vanillic acid and *o*- and *p*-aminobenzoic acids. Substituted cinnamic acids, e.g., caffeic, ferulic and *o*-hydroxycinnamic acids, are completely destroyed by such treatment and cannot be

TABLE I

THE URINARY EXCRETION OF HIPPURATES AFTER THE ORAL INGESTION OF SOME PHENOLIC ACIDS AND RELATED COMPOUNDS

	Hippurates (mg. glycine/8 hr. urine)
Control	125
After 1 g. salicylic acid	210
Control	110
After 1 g. salicylic acid	225
Control	98
After 1 g. salicylic acid	235
Control	96
After 1 g. *m*-hydroxybenzoic acid	198
Control	88
After 1 g. *m*-hydroxybenzoic acid	215
Control	115
After 1 g. *m*-hydroxybenzoic acid	218
Control	92
After 1 g. *p*-hydroxybenzoic acid	238
Control	82
After 1 g. *p*-hydroxybenzoic acid	242
Control	78
After 1 g. 3,4-dihydroxybenzoic acid	198
Control	82
After 1 g. 3,4-dihydroxybenzoic acid	225
Control	110
After 1 g. caffeic acid	210
Control	82
After 1 g. caffeic acid	240
Control	115
After 1 g. tryptophan	238
Control	98
After 1 g. tryptophan	184

Combined glutamic acid (glutamic conjugates)—not detectable.

recognised by the usual reactions. These substances appear, however, to be stable to hot acid hydrolysis in the presence of N hydrochloric acid.

Armstrong and Shaw (1955) have reported the instability of *m*-hydroxyphenylhydracrylic acid when heated in the presence of strong mineral acid.

Glycine and Glutamine Conjugation

An assessment was made of glycine and glutamine conjugation as the result of the oral administration of some phenolic acids. Examinations were made on the night urine (11 p.m. to 7 a.m.) to minimise the effect of diet. Assessment was made on changes in the quantity of combined glycine or the dicarboxylic amino-acid (glutamic + aspartic) fraction. The results of this investigation are shown in Table I. In each experiment there was evidence of glycine conjugation but none for conjugation with glutamine.

The Fractionation of the Methoxy Phenolic Acids of Urine

The object of the investigation was to identify the principal methoxy phenolic acids of urine and in particular whether dimethoxyphenolic acids existed in any quantity. An assessment of the ferulic and 4-hydroxy-3-methoxymandelic acid contents were excluded by the drastic method of

TABLE II

THE DISTRIBUTION OF METHOXY PHENOLIC ACIDS FROM A URINE EXTRACT ON A PAPER CHROMATOGRAM (BENZENE : ACETIC ACID : WATER)

	R_F (average)	Per cent of the total
Homovanillic acid fraction	0·50	52
Vanillic acid fraction	0·75	38
Methoxyphenylacetic acid fraction	0·90	8
	Colorimetric reactions. Folin-Ciocalteu reaction	2,6-Dichloroquinone chlorimide reaction
Homovanillic acid	+	−
Vanillic and ferulic acids	+	+
p- and *m*-Methoxyphenylacetic and veratric acids	−	−

acid hydrolysis employed which results in the destruction of these substances.

Results of this investigation are shown in Table II. It will be noted that the greatest part of the methoxy phenolic acids exist as vanillic and homovanillic acids. Dimethoxy phenolic acids, if present, constitute a minor fraction.

m-*Hydroxybenzoic and Vanillic Acids in Urine*

Determinations have been made on 10 urines and the results are shown in Table III. The ranges of excretion are very wide. This is to be expected since these substances have a dietary origin. Urinary vanillic

TABLE III

VANILLIC AND *m*-HYDROXYBENZOIC ACIDS IN HUMAN URINE. THE RESULTS ARE EXPRESSED IN MG./DAY

	Vanillic acid	*m*-Hydroxybenzoic acid
1	12·6	12·6
2	23·8	31·8
3	19·2	43·6
4	30·8	18·2
5	45·6	46·8
6	28·4	18·2
7	41·6	35·2
8	35·8	41·6
9	49·2	78·6
10	128	310

acid is undoubtedly derived from two distinct sources, from the metabolism of 3,4-dihydroxyphenolic substances, e.g., caffeic acid and from ingested 4-hydroxy-3-methoxyphenolic substances, e.g., vanillin.

Since a large number of phenolic acids have been identified in urine, some reference to substances estimated by the chlorimide method is merited. The drastic form of acid hydrolysis employed, eliminates those

phenolic acids possessing a cinnamic acid structure and prevents interference by hippurates in the paper chromatographic procedure. It is believed that vanillic acid measured after separation by paper chromatography does represent a specific determination.

An extract obtained after paper chromatography would contain, in addition to *m*-hydroxybenzoic acid, such substances as *o*- and *m*-hydroxyphenylacetic acids, both of which react to produce blue colours. *m*-Hydroxyphenylacetic acid behaves similar to *m*-hydroxybenzoic acid in that the blue colour is not extractable by butanol. Since both appear to have a similar metabolic origin, this should produce little confusion in the interpretation of investigations concerning the formation of *m*-hydroxyl compounds. Under normal conditions, the urinary excretion of *o*-hydroxyphenylacetic acid is about 1 mg./day (Armstrong and others, 1955). This substance reacts to produce a blue colour which is extractable by butanol, hence little interference should result from its presence.

The Metabolism of Caffeic Acid

The metabolism of caffeic acid in man is of interest since in the form of the conjugate, chlorogenic acid, it has a wide distribution in natural

TABLE IV

URINARY EXCRETION OF METABOLITES AFTER THE ORAL INGESTION OF 1 G. CAFFEIC ACID. URINE WAS COLLECTED FOR 8 HR. AFTER INGESTION

Catecholic phenolic acids*	117 mg.
m-Hydroxybenzoic acid†	184 mg.
Vanillic acid†	110 mg.
Other methoxy phenolic acids‡	210 mg.

* Identified by paper chromatography to consist almost entirely of 3,4-dihydroxybenzoic acid. Expressed in terms of 3,4-dihydroxybenzoic acid.

† Determined by means of the chlorimide reaction.

‡ Calculated by difference between total methoxy phenolic and vanillic acid content. Expressed in terms of vanillic acid.

products. Chlorogenic acid, a conjugate of caffeic acid with quinic or isoquinic acid, is readily hydrolysed by treatment for 1 hr. in a boiling water bath in the presence of N hydrochloric acid. In the Mitchell reaction, chlorogenic acid produces a yellow colour and caffeic acid the typical purple colour.

An examination has been made of the urinary excretions of some metabolites after the ingestion of 1 g. of caffeic acid. The night urine was used and a correction has been applied by the examination of a control. Three determinations were made, total methoxy phenolic acids (Tompsett, 1959), phenolic acids reacting in the Mitchell reaction (Tompsett, 1958a), and vanillic and *m*-hydroxybenzoic acids. Acid hydrolysis in the presence of 5 N hydrochloric acid was made as a preliminary to these examinations.

Typical results are shown in Table IV. Naturally only some of the varied products would be measured. The catecholic phenolic fraction would not include caffeic acid since this substance is destroyed in the initial hot acid hydrolysis. For similar reasons, ferulic acid cannot be identified individually. By means of paper chromatography, it was

found that the catecholic phenolic fraction consisted very largely of 3,4-dihydroxybenzoic acid.

Human urine has been shown to contain a variety of phenolic acids. The majority of these may be described as metabolites, the result of methylation, de-hydroxylation, oxidation, reduction, or conjugation. Against such a complex background, the detection and determination of individual phenolic acids may prove a difficult and laborious process. Many investigations are concerned mainly with the examination of a particular mode of metabolism and such, the patient's health permitting, are simplified by the study of the fate of an administered substance.

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