specific concentration or orientation previously proposed.

4. The inhibitory effects were much reduced by the presence of phosphate or by preincubation with 5 mM-*isocitrate*.

5. In the presence of sodium chloride the activity of the aconitase increased. With potassium chloride there was an increase followed by a fall. An increase in the concentration of both salts much reduced the inhibition of fluorocitrate.

6. The percentage inhibition with fluorocitrate was the same for $L_1(+)$ -*isocitrate* (D), though the rate for a given amount of *isocitrate* was doubled. There was no indication that the inactive isomer was an inhibitor.

7. Variation of temperature within $\pm 4^\circ$ was about 4% per degree. Variations in pH of ± 0.6 caused little change in fluorocitrate inhibition, though a fall in activity was found on making more alkaline.

Thanks are due to Miss P. Ivall for efficient technical assistance, and to Miss R. Shawdon for some of the preliminary work; to Dr P. F. V. Ward for the final purification of the fluorocitrate used, to others in the Depart-

ment for advice and to Dr D. H. Treble for reading the MS. Thanks are also due to Professor F. G. Young, F.R.S., for providing facilities for the finish of this work.

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The Metabolites of Piperic Acid and Some Related Compounds in the Rat

By R. M. ACHESON AND G. L. ATKINS
Department of Biochemistry, University of Oxford

(Received 18 October 1960)

Human urine contains many compounds which give coloured derivatives with Altman reagent on paper chromatograms (Acheson & Dearnaley, 1960; Smith, Paul, McGeer & McGeer, 1959; Armstrong, Shaw & Wall, 1956). This reagent (4-dimethylaminobenzaldehyde in acetic anhydride) reacts with aroyl-glycines and similar compounds to give coloured oxazolones, a number of which have been synthesized and their absorption spectra measured (Acheson, Booth, Brettle & Harris, 1960). 5-Phenylpenta-2:4-dienoylglycine gives, when heated with the reagent on paper chromatograms, a red-purple colour similar to those given by compounds 54 and 55, which were found in human urine by Acheson & Dearnaley (1960), but the three compounds had different R_f values. It was possible that compounds 54 and 55 were derived from a substituted 5-phenylpenta-2:4-dienoic acid which is a dietary constituent and

excreted as the glycine conjugate. In order to test this in connexion with piperic acid, which occurs as derivatives in pepper, piperoylglycine was prepared and the metabolism of piperic acid has been investigated in the rat.

MATERIALS AND METHODS

All melting points are uncorrected, and the absorption spectra were determined in methanol solution unless otherwise specified.

Piperonylic acid, m.p. 226-227°, λ_{max} 293 and 256 m μ (ϵ 6400 and 7500 respectively), was prepared by the oxidation of piperonal with neutral potassium permanganate (*Organic Syntheses*, 1943). 3:4-Methylenedioxyhippuric acid, m.p. 178°, was prepared as described previously by Acheson *et al.* (1960), and 3:4-methylenedioxy-cinnamic acid, m.p. 238°, was obtained from malonic acid and piperonal (*Organic Reactions*, 1942).

3:4-Methylenedioxy-cinnamoylglycine. The cinnamic acid (1.0 g.) was covered with ethyl aminoacetate (0.8 g.) in dry

tetrahydrofuran (6 ml.) and a solution of dicyclohexylcarbodi-imide (1.5 g.) in tetrahydrofuran (3 ml.) was added (cf. Sheehan & Hess, 1955). The mixture was well shaken and kept for $3\frac{1}{2}$ hr., and the precipitate collected. The filtrate was evaporated to dryness and the residue extracted with ethyl acetate (2×10 ml.). The combined extracts were washed with dilute aqueous hydrochloric acid and sodium carbonate and dried. Ethyl 3:4-methylenedioxy-cinnamoylaminoacetate (0.42 g.), m.p. 135° (cf. Acheson *et al.* 1960), was obtained as colourless crystals when the residue, obtained on evaporating the ethyl acetate, was triturated with light petroleum (b.p. 80 – 100°) and left overnight. Hydrolysis of the ester (Acheson *et al.* 1960) gave the glycine which separated from dioxan as colourless crystals, m.p. 210° .

Piperoylglycine. Piperic acid, m.p. 213 – 215° , $\lambda_{\max}$. 226 $m\mu$ (ϵ 18 000), inflexions at 329, 304 and 248 $m\mu$ (ϵ 24 000, 20 000 and 15 000 respectively), was prepared from piperonal according to Lohaus (1929) and was condensed with ethyl aminoacetate as above. The crude ethyl piperoylaminoacetate (21% yield) had m.p. 123 – 125° , and four recrystallizations from aqueous 50% ethanol gave pale-yellow needles, m.p. 137° (Found: C, 63.9; H, 5.8; N, 4.1. $C_{18}H_{17}O_5N$ requires C, 63.4; H, 5.6; N, 4.6%). This ester was also obtained in 17% yield from piperoyl chloride, m.p. 203 – 205° [Lohaus (1929) gives m.p. 180°], prepared from the acid and thionyl chloride, and excess of ethyl aminoacetate. Hydrolysis of the ester (0.22 g.) in warm dioxan (4 ml.) was effected by *N*-sodium hydroxide (1.46 ml.) in 2 hr. at room temperature (cf. Harington & Mead, 1935). The pH was then adjusted to 7 with 2*N*-hydrochloric acid and the solvents were removed *in vacuo*. The residue was shaken with ethyl acetate (3×8 ml.), and the combined solutions were extracted with saturated aqueous sodium bicarbonate (2×5 ml.). The bicarbonate solution was extracted with ether and subsequently acidified, when crude piperoylglycine (0.06 g.), m.p. 192 – 197° , was precipitated. Four recrystallizations from aqueous 50% methanol gave yellow plates, m.p. 200 – 204° (Found: C, 61.1; H, 4.7; N, 5.0. $C_{18}H_{15}O_5N$ requires C, 61.0; H, 4.7; N, 5.1%). It had $\lambda_{\max}$. 336 and 243 $m\mu$ (ϵ 20 000 and 14 000 respectively) and inflexions at 310 and 255 $m\mu$ (ϵ 15 000 and 13 000 respectively).

Treatment of this acid with 4-dimethylaminobenzaldehyde and acetic anhydride (Acheson *et al.* 1960) gave a deep-red crystalline material which after six recrystalliza-

tions from benzene reached a constant m.p. 205 – 206° , and the ultraviolet-absorption spectrum in ethyl acetate was not changed by the last crystallization. It showed $\lambda_{\max}$. 488 and 363 $m\mu$ (ϵ 66 000 and 22 000) and after acidification with concentrated sulphuric acid $\lambda_{\max}$. 426, 345 and 308 $m\mu$ (ϵ 45 000, 15 000 and 13 000 respectively). Although sufficient material for analysis was not obtained, the method of preparation and the absorption spectrum suggest that the material is largely the expected oxazolone.

Animals, diet and dosage. Rats (70–100 g.) were kept in pairs in metabolism cages with an unrestricted water supply. They were placed in feeding cages twice daily for 30 min. periods during which time they did not urinate. Their normal diet was that supplied by Herbert C. Styles (Bewdley) Ltd. (diet 41). Each rat received piperic acid (50 mg.), 3:4-methylenedioxybenzoic acid (42 mg.), 3:4-methylenedioxy-cinnamic acid (32 mg.) or 3:4-methylenedioxy-cinnamoylglycine (32 mg.) mixed with rat-cake (20 g.), which was consumed during the experimental period of 48 hr. Piperoylglycine (10 mg.) in aqueous sodium bicarbonate (0.75 ml.) was fed by stomach tube once daily. The urine was collected for 48 hr.

Extraction of the urine and chromatographic procedures. The urine samples and cage-washings were saturated with ammonium sulphate, acidified to pH 1–2 with hydrochloric acid, and extracted ($3 \times$ half the sample volume) with ethyl acetate close to 0° (cf. Armstrong *et al.* 1956). The combined extracts were dried (Na_2SO_4), filtered and evaporated to about 1% of their original volumes, and 25 μ l. was chromatographed on Whatman no. 1 paper in solvent *A* (Table 1) in the first dimension and solvent *B* in the second. The chromatograms were examined under a Hanovia ultraviolet lamp emitting 90% of its radiation at 2537 Å, and were subsequently treated with Altman reagent. The identities of the methylenedioxy compounds on the urine chromatograms were shown by comparing their R_F values with those of the authentic materials and also by co-chromatographing the authentic materials with the urine extracts and observing the increase in intensity of the spots. Rough estimates of some of the compounds were made by comparing visually the spots on the urine chromatograms with those produced by known amounts of the authentic materials chromatographed under identical conditions. All chromatograms were run in duplicate and were repeated if good agreement was not obtained. The stability of the methylenedioxy compounds in the urine

Table 1. *Chromatographic behaviour of the authentic methylenedioxy compounds*

Solvents and times of run: *A*, propan-2-ol-aq. ammonia soln. (sp.gr. 0.88)–water (6:3:1, by vol.) (8 hr.); *B*, aqueous 20% potassium chloride ($1\frac{1}{2}$ hr.); *C*, butan-1-ol–acetic acid–water (4:1:1) (5–6 hr.); *D*, benzene–propionic acid–water (2:2:1), organic phase ($2\frac{1}{2}$ hr.); *E*, propan-2-ol–ammonia–water (8:1:1) (8 hr.). The ascending method on Whatman no. 1 paper was used.

Compound	Colour in ultraviolet light	Colour with Altman reagent	R_F in solvent				
			<i>A</i>	<i>B</i>	<i>C</i>	<i>D</i>	<i>E</i>
3:4-Methylenedioxybenzoic acid	Green fluorescence	None	0.81	0.63	0.92	0.96	0.49
3:4-Methylenedioxy-cinnamic acid	Blue	None	0.87	0.25	0.93	0.98	0.48
Piperic acid	Green–blue	None	0.90	0.05	0.87	0.99	0.46
3:4-Methylenedioxybenzoylglycine	Faint blue	Yellow–orange	0.84	0.73	0.84	0.43	0.43
3:4-Methylenedioxy-cinnamoylglycine	Bright blue	Orange–red	0.76	0.25	0.80	0.50	0.35
Piperoylglycine	Green–blue	Red–purple	0.87	0.08	0.88	0.56	0.43
54	Absorbs	Red–purple	—	—	0.30	0.00	0.02
55	Absorbs	Red–purple	—	—	0.21	0.00	0.02

Table 2. *Metabolites of methylenedioxy compounds in rat urine*

The compounds indicated were detected on paper chromatograms. + Indicates present, ++ present in greater amount and - not detected. Piperic acid was not detected in any of the urines.

Compound fed	Compound in urine				
	3:4-Methylene-dioxybenzoic acid	3:4-Methylene-dioxybenzoic acid	3:4-Methylene-dioxybenzoyl-glycine	3:4-Methylene-dioxybenzoyl-glycine	Piperoyl-glycine
3:4-Methylenedioxybenzoic acid	+	-	+	-	-
3:4-Methylenedioxybenzoic acid	-	-	++	+	-
Piperic acid	+	-	++	+	-
3:4-Methylenedioxybenzoyl-glycine	-	+	+	+	-
Piperoyl-glycine	-	-	+	+	+

was checked by adding the compounds to a sample of rat urine and working up one half of the urine immediately and the other half after a delay of 72 hr. Chromatograms of equivalent amounts of the extracts were identical. In a similar way the stability of the methylenedioxy compounds in the rat-cake for 72 hr. was ascertained, and the recovery of added 3:4-methylenedioxybenzoic acid and its glycine conjugate from rat urine shown to be about 80%.

RESULTS AND DISCUSSION

The results of the feeding experiments are given in Table 2. None of the methylenedioxy compounds could be detected in normal rat urine. No piperoyl-glycine could be detected as a metabolite of piperic acid, and the results (Table 2) suggest that four carbon atoms of the side chain can be eliminated in two stages through the corresponding cinnamic acid; a simultaneous direct removal of the four carbon atoms is not excluded. These results are in line with the conversion, by the rabbit, of 5-phenylpenta-2:4-dienoic acid into benzoic acid and cinnamic acid and the corresponding glycine conjugates (Friedman & Mai, 1931). About 40% of the administered piperic acid was eliminated as 3:4-methylenedioxyhippuric acid, and about 0.3% as 3:4-methylenedioxybenzoic acid, within 72 hr. of the beginning of the experiment.

As the side chains of piperic acid and 5-phenylpenta-2:4-dienoic acid are oxidized so readily in the rabbit it is perhaps unlikely that compounds analogous to piperoyl-glycine will be found in normal human urine. Although the colour developed by piperoyl-glycine with Altman reagent is similar to that produced by the compounds 54 and 55 present in human urine the chromatographic properties of the three compounds are widely different.

SUMMARY

1. 3:4-Methylenedioxy-benzoic acid and -cinnamic acid, and piperic acid, and their glycine conjugates have been synthesized.

2. None of these compounds was detected in the urine of rats fed with the standard diet and the three acids were partially metabolized to 3:4-methylenedioxyhippuric acid.

3. The chromatographic properties of piperoyl-glycine are not identical with two other compounds giving a similar Altman reaction and which are present in human urine.

This work was supported in part by grants from the Rockefeller Foundation and from the National Institutes of Health United States Public Health Service (Grant no. A3369) to the Department of Biochemistry, University of Oxford.

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