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THE METABOLISM OF INDOLE-3-CARBOXYLIC ACID BY THE RAT

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SUMMARY

Extracts of the urine of rats, fed or injected intraperitoneally with indole-3-carboxylic acid, contained about 20% of the original acid, the corresponding glucuronide and 6-hydroxyindole-3-carboxylic acid. *N*-(Indole-3-carbonyl)glycine was synthesised and shown absent from the urine extracts. Oxidation of indole-3-carboxylic acid by Udenfriend's method gave the 5- and 6-hydroxy derivatives, indole, anthranilic acid and *N*-formylanthranilic acid.

INTRODUCTION

Indole-3-carboxylic acid is present in peas[1], cabbage[2], brussel sprouts[3], cauliflower and savoy cabbage[4] and so its presence in human urine[5] is understandable without recourse to the suggestion[6] that it is formed from tryptophan. Rabbits excrete injected indole-3-carboxylic acid as a conjugate[7]. An unidentified compound appeared in the urine after feeding the acid to man[6], while earlier workers[8] recovered about 80% of the fed acid in the first 24 h; the procedure used, however, could easily have decomposed a labile conjugate. The fate of indole-3-carboxylic acid in the rat has now been investigated.

METHODS AND RESULTS

Animal experiments, chromatography and electrophoresis

Male albino rats (approx. 200 g) from the Departmental stock fed on diet No. 41, manufactured by Herbert C. Styles (Bewdley) Ltd., supplemented twice weekly by brown bread (13.4 g), milk (4 ml), Marmite (0.14 g), cod liver oil (0.14 ml), beans (9 g) and Bemax (0.42 g) per rat, were used.

After feeding, the rats were kept in pairs in metabolism cages with an unrestricted supply of water and the overnight urine collected. In experiments lasting more than 24 h the rats were removed once a day for 30 min for feeding.

Rats were either injected intraperitoneally with indole-3-carboxylic acid (30 mg per rat) in aqueous sodium bicarbonate (1 ml) or fed with the same quantity of the acid mixed with food. The acid was prepared[9] from indole and had m.p. 222–224° (with decomposition). The following 24-h urine and cage washings were combined, acidified to pH 3 with acetic acid and shaken for 3 min with activated charcoal (1 g, British Drug Houses) deactivated[10] with *n*-octadecylamine (6%). The mixture was allowed to settle and was then filtered under partial vacuum (approx. 55 mm) through

a porosity-3 sintered glass funnel; blockage of the filter was minimised under these conditions. The charcoal was washed with water (3 × 20 ml) and absorbed materials were eluted three times by stirring with water (10 ml) containing phenol (0.7 g). The charcoal was then washed with water (20 ml), the combined washing and eluates were diluted to 100 ml and evaporated to 5 ml *in vacuo* at 37°. The dilution and evaporation were repeated until the distillate gave no ferric chloride colour, whereupon the extract was concentrated to 0.5–1.0 ml.

Urine extracts (0.2–0.3 ml) were spotted on to Whatman No. 1 paper (23 cm^2) and chromatographed using ascending isopropanol–ammonia (d = 0.88)–water (8:1:1, v/v) (solvent A) in the first dimension (overnight run) and in ascending *n*-butanol–acetic acid–water (4:1:1) (solvent B) in the second dimension (about 7 h). Paper electrophoresis of the extracts was carried out on Whatman No. 1 paper in a Locarte apparatus at 7 kV using either acetic acid (23 ml), pyridine (8 ml) and water (800 ml) at pH 4.1, or 0.05 M $NaHCO_3$ (pH 8.1) as electrolytes. The chromatograms and electrophoresis papers were examined under ultraviolet light (2537 Å) and were subsequently sprayed with solutions mentioned later or with Ehrlich's reagent, 4-dimethylaminobenzaldehyde (2 %) in 2 N HCl. In this case after maximum colour development had taken place at room temperature the papers were heated with a hot air drier.

Efficiency of the extraction procedure

Indole-3-carboxylic acid (68 mg), in dilute $NaHCO_3$ (250 ml), was acidified, extracted and the concentrate chromatographed as for a urine sample. At the same time standard amounts of the acid were chromatographed in one dimension in solvent A. The chromatograms were developed simultaneously with Ehrlich's reagent and intensity comparisons of several runs showed that 37 % ± 10 % of the acid was recovered. The appearance of indole on the chromatograms of the recovered acid indicated that partial decarboxylation had occurred. A similar (35 % ± 10 %) recovery was obtained when the acid (0.4 mg) was added to rat urine (100 ml) which previously contained virtually none of the material, and the solution left at room temperature for 24 h, and subsequently at 0° for 48 h after acidification to pH 3 with acetic acid.

Ehrlich-positive constituents of rats urine

Thirty samples of urine from the normal rats were examined separately and the Ehrlich-positive constituents are listed in Table I. The identifications are based on comparisons of the R_F values and colour reactions with those described in similar studies on rat[11] and human urine[6,12,13], the unidentified Ehrlich-positive compounds are probably indoles, pyrroles or phenols[14]. Differences between the present chromatographic picture (Table I) and that reported earlier[11] are ascribed to the different diets used.

The urine from two rats injected with indole-3-carboxylic acid was collected every 24 h for 7 days, and each collection was separately extracted and quantitatively chromatographed. Assuming a 35 % efficiency for the extraction procedure for indole-3-carboxylic acid, 20 % of the injected acid was excreted unchanged in the urine and 90 % of this was excreted in the first 24 h. The subsequent urine collection showed an almost normal excretion pattern.

In addition to the injected acid the chromatograms possessed a large area containing compound 30, the major metabolite, much smaller quantities of compound 28, and indole (compound 29) which may have been formed from the indole-3-carboxylic acid during the extraction, increased amounts of compounds 2, 5 and 8. An identical excretion pattern was shown by rats fed with indole-3-carboxylic acid.

TABLE I

EHRLICH-POSITIVE CONSTITUENTS OF RATS URINE

Compound number	Rf's × 100 in solvents		Colour observed with Ehrlich's reagent		Identification
	A	B	Initial	After 24 h	
Urine from normal rats					
1	2	61	Purple	Fades	—
2	2	24	Purple	Fades	—
3	4	6	Purple	Fades	—
4	7	10	Purple	Fades	—
5	9	25	Blue*	Blue-green	—
6	9	74	Blue**	Blue	—
7	12	35	Pink*, ***	Pink	—
8	14	37	Blue*	Blue	—
9	17	28	Purple	Purple	—
10	19	83	Purple**	Fades	—
11	20	72	Purple**	Purple	5-Hydroxyindole-3-carboxylic acid?
12	21	33	Purple	Fades	—
13	21	47	Purple	Purple	—
14	22	44	Purple	Fades	—
15	23	51	Purple	Blue	—
16	23	73	Blue	Blue	5-Hydroxyindole-3-acetic acid
17	30	30	Blue**	Blue	—
18	31	8	Pink**	Fades	—
19	31	39	Purple	Fades	Tryptophan
20	35	71	Blue-green**	Blue-green	—
21	35	87	Pink*, ***	Pink	Indole-3-carboxylic acid
22	39	85	Pink*	Pink	Indole-3-glycolic acid
23	40	79	Purple	Blue	*N*-(Indolyl-3-acetyl)glycine?
24	41	92	Purple	Blue-grey	Indole-3-acetic acid
25	52	44	Brown	Brown	Indican
26	58	48	Blue	Blue	6-Sulphatoxyskatole
27	78	76	Purple**	Fades	—
Additional materials in urine from treated rats					
28	15	70	Blue	Blue	6-Hydroxyindo' -3-carboxylic acid
29	96	92	Pink	Pink	Indole
30	24	46	Pink***	Pink	Indole-3-carboxylic acid glucuronide

* Observed on approx. 50 % of chromatograms.
** Observed on under 25 % of chromatograms.
*** Develops on warming.

Investigation of substance 28

This material gave a pink colour with diazotised sulphanilic acid[15], a blue-green colour with diazotised 4-nitroaniline[16], black with ammoniacal silver nitrate, appeared dark under ultraviolet light and gave no reaction with Altman's reagent[17] or ninhydrin. It moved 9.0 cm towards the anode in 20 min on paper electrophoresis in the bicarbonate. Its colour reactions, and chromatographic and electrophoretic

properties were identical with those of 6-hydroxyindole-3-carboxylic acid alone or mixed.

The isolation and reactions of substance 30

1. The urine concentrate from 8 rats injected with indole-3-carboxylic acid was diluted with water to 30 ml, saturated with $(NH_4)_2SO_4$ and extracted with ethyl acetate (3 × 30 ml). Chromatography showed that all the metabolite had been extracted and that no other Ehrlich-positive material was present which moved to the same position in solvent B. The extract was evaporated to 1.35 ml, applied equally to three Whatman No. 3 papers (23 cm^2) as 18-cm stripes, and the papers were run in ascending solvent B. The areas containing the metabolite, which appeared dark under ultraviolet light, were cut out, eluted for 12 h with water and the combined eluates concentrated to 2 ml. Chromatography showed that the metabolite was now free from other Ehrlich-positive material. Further evaporation gave a gum which over P_2O_5 *in vacuo* gave a glass (62.5 mg). It had $[\alpha]_{5461}^{20}$ −21° in water (*c*, 0.6 %); light-absorption maxima in methanol–water (3:2, v/v) at 232 and 285 mμ (ε 8400 and 8900 respectively), inflexion about 247 mμ (ε 6500). Comparison with the light-absorption of methyl indole-3-carboxylate in methanol, 281 and 286 mμ (ε 12100 and 11600 respectively), inflexions at about 225 and 244 mμ (ε 18500 and 8800 respectively) suggests that the glucuronide is about 75 % pure.

2. Substance 30 gave no visible reaction with Ehrlich's reagent on paper chromatograms until heat was applied when a very intense pink colour developed. It appeared as a large dumb-bell shaped spot on the urine chromatograms, but after isolation appeared as a single spot and it traveled as a single material on electrophoresis. It moved 10.4 cm in 20 min, and 25.4 cm in 105 min, towards the anode in the buffers of pH 8.1 and 4.1 respectively. It reduced Fehling's solution on warming, gave a white colour against a grey background with ammoniacal silver nitrate, an indecisive reaction with Altman's reagent, and no reaction with diazotised sulphanilic acid or 4-nitroaniline, or with Cl_2 followed by starch–KI, or with ninhydrin on paper chromatograms.

3. About 30 mg of substance 30 in water (3 ml) was heated at 85–90° for 2 h with 2 N NaOH (3 ml) under N_2. After acidification (HCl) the solution was chromatographed at 1 ml/min on an 8-cm column of Amberlite CG-120 (100–200 mesh, sulphuric acid 4.5 % cross linked), which had been previously treated with 2 N HCl (25 ml) followed by water until the eluate was neutral. Elution with water and chromatographic examination of this eluate showed only indole-3-carboxylic acid.

4. Substance 30 (about 30 mg) in water (3 ml) was heated at 85–90° with 2 N HCl for 2 h and the resulting dark solution was applied to a charcoal column prepared as described[18]. Elution with water (50 ml) removed all chloride ions but apparently no sugar derivatives. The subsequent 30 ml water–ethanol (20:1, v/v) eluate was concentrated to 1 ml and chromatographed on Whatman No. 4 paper using the pad technique and ascending (a) isopropanol–water (4:1, v/v) (b) isopropanol–*n*-butanol–water (7:1:2) and (c) *n*-butanol–acetic acid–water (12:3:5) alongside authentic D-glucuronic acid, D-glucuronolactone and D-glucose. The dried chromatograms, sprayed with ammoniacal silver nitrate, *N*,*N*-diphenylaniline, aniline hydrogen phthalate or naphthoresorcinol, showed two compounds with identical colour reactions to those of D-glucuronic acid and D-glucuronolactone and similar $R_{glucose}$

values. The chromatograms were run until the lactones had moved about 20 cm. The glucuronic acid streaked badly in the first two solvents. The $R_{glucose}$ values for the authentic glucuronic acid and the lactone respectively, and those of the hydrolysate constituents (in parentheses) for the respective solvents are: a, 0.18–0.57 (0.29–1.13), 1.49 (1.50); b, 0.19–0.64 (0.33–1.12), 158 (155); c, 1.00 (1.00), 170 (164).

5. Substance 30 (0.98 mg) in 0.004 M sodium acetate–acetic acid buffer (3 ml) at pH 3.95 was incubated with mollusc β-glucuronidase (0.80 mg, Light and Co. Ltd.) in buffer (3 ml) at 37° for 3 h alongside a control without the enzyme. Chromatography now showed that substance 30 was unchanged in the control, but that the enzyme-treated sample contained none of this material and that the sole Ehrlich-positive material present was indole-3-carboxylic acid.

Oxidation of indole-3-carboxylic acid

The acid (0.3 g) in ethanol (10 ml) was shaken with ascorbic acid (3 g), ferrous sulphate (0.2 g), disodium hydrogen phosphate (0.95 g), potassium dihydrogen phosphate (1.36 g) and EDTA (7.6 g) in water (250 ml), for 12 h under oxygen[19] and after filtration the ether-soluble material was collected and examined by two dimensional chromatography in the usual way (Table II). Unchanged indole-3-carboxylic acid was by far the major constituent, and the anthranilic acid, *N*-formylanthranilic acid, indole and the 6-hydroxyindole-3-carboxylic acid had the same chromatographic properties and colour reactions as authentic specimens, alone or mixed (Table II). The compound labeled 5-hydroxyindole-3-carboxylic acid was tentatively identified by comparison with published data[5].

TABLE II

OXIDATION PRODUCTS FROM INDOLE-3-CARBOXYLIC ACID

Identification	*Rf's* × 100 *in solvents*		*Colours observed with*			
			Ultraviolet light	*Ehrlich's reagent*	*Diazotised*	
	A	*B*			*Sulphanilic acid*	*4-Nitroaniline*
—	11	75	Dark	Blue	Orange	Blue
6-Hydroxyindole-3-carboxylic acid	15	70	Dark	Blue	Pink	Blue-green
5-Hydroxyindole-3-carboxylic acid	22	71	Dark	Purple	Orange	Pink
Anthranilic acid	45	88	Blue	Yellow	Yellow	Yellow
N-Formylanthranilic acid	58	90	Blue	Yellow	None	None
Indole	96	92	Dark	Pink	None	None

N-(Indole-3-carbonyl)aminoacetic acid

Indole-3-carboxylic acid (1.6 g) was covered with ethyl aminoacetate (1.2 g) in sodium-dried tetrahydrofuran (10 ml). Dicyclohexylcarbodiimide (2.4 g) in dry tetrahydrofuran (5 ml) was added, the mixture was shaken, and after 3.5 h the precipitate of dicyclohexylurea was collected[20]. Evaporation of the filtrate *in vacuo* gave a solid which was washed with a little ethyl acetate, and subsequent crystallisation from ethanol containing some water gave ethyl-*N*-(indole-3-carbonyl)aminoacetate as colourless flakes (2.3 g, 94 %), m.p. 154°. (Found: C, 63.4; H, 5.9; N, 11.4. $C_{13}H_{14}N_2O_3$

requires C, 63.4; H, 5.7; N, 11.4%.) This ester (1.8 g) was shaken with 0.8 N NaOH for 1.75 h and the resulting solution acidified with hydrochloric acid. The precipitate crystallised from water giving *N*-(indole-3-carbonyl)aminoacetic acid as colourless plates (1.36 g, 85%), m.p. 191–192°. (Found: C, 60.1; H, 4.6; N, 12.9. $C_{11}H_{10}N_2O_3$ requires C, 60.6; H, 4.6; N, 12.8%.) It gave a very distinct yellow colour with Altman's reagent, no colour with Ehrlich's reagent under the usual conditions, and had R_F 0.35 and 0.75 in solvent A and solvent B respectively. On electrophoresis in the pyridine–acetic acid buffer it moved 5.1 cm in 40 min towards the cathode.

DISCUSSION

Extracts of the urine of rats, fed or injected with indole-3-carboxylic acid, had identical chromatographic patterns which suggests that gut bacteria are not materially involved in the metabolism of the acid. A small proportion of the acid was hydroxylated at position 6, as are other indoles by rat enzymes[12,21]. The major metabolite is the glucuronide, and has the interesting property of failing to react with Ehrlich's reagent on paper chromatograms until heated with a hair drier. Hydrolysis to indole-3-carboxylic acid will then occur; the pink colour produced by the acid and the metabolite are identical. This type of heating also caused a very marked intensification of most of the other coloured areas produced by Ehrlich's reagent, and may have other applications. Indolyl-3-carbonylglycine, which was synthesised but could not be detected in any of the urine extracts, also gave no Ehrlich reaction.

BALAKRISHNAN AND RODNIGHT[6] have isolated a compound from human urine which they considered was probably indole-3-carboxylic acid glucuronide. This conclusion is difficult to reconcile with (a) the rapid purple colour given by this compound with Ehrlich's reagent, as indole-3-carboxylic acid, its methyl ester and glycine conjugate fail to react or give pink colours slowly and (b) with the observation[6] that feeding indole-3-carboxylic acid did not increase the excretion of this compound. Instead a further compound was produced which gave a pink Ehrlich colour only after exposure to HCl vapour for a day. This suggests that hydrolysis was necessary before the Ehrlich reaction could occur, as with our metabolite; these two compounds may be identical.

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