the dose of benzoic acid is eliminated in 24 hr. after dosing. It is known that glycine is necessary for optimum growth in chickens and turkeys, but the glycine requirement of other birds is not known. It has been suggested that this requirement of glycine may be related to the biosynthesis of uric acid, which is a major nitrogenous waste product in birds, including the pigeons (see Arnstein, 1954). It is possible that the formation of hippuric acid is peculiar to the columbiformes, but many more species of birds have to be examined before it could be claimed that hippuric acid formation is characteristic of columbiformes.

SUMMARY

1. Benzoic acid has been administered to nine species of birds, namely the domestic fowl, turkey, goose, Muscovy duck, domestic pigeon, wood pigeon, Barbary dove, carrion crow and grey African parrot.

2. All these species of birds excrete benzoyl glucuronide.

3. The domestic fowl, turkey, goose and Muscovy duck also excreted L(+)-ornithuric acid but no hippuric acid.

4. The domestic pigeon, wood pigeon and Barbary dove also excreted hippuric acid but no ornithuric acid.

5. The carrion crow and grey African parrot did not appear to excrete hippuric acid or ornithuric acid.

6. [*carboxy*-¹⁴C]Benzoic acid was administered to hens and pigeons. The hens excreted about 25–30 % of the dose (240 mg./kg.) as glucuronide and 40–50 % as ornithuric acid in 24 hr. The pigeons excreted 25–30 % of the dose (200 mg./kg.) as glucuronide and 60 % as hippuric acid in 18.5 hr.

7. Chicken faeces contain an enzyme which decomposes L(+)-ornithuric acid via *N*⁵-benzoyl-ornithine. Monobenzoylornithines were not found as metabolites of benzoic acid in hens.

The work was supported entirely by a grant from Dr Salsbury's Laboratories, Charles City, Iowa. We are grateful to the Zoological Society of London for the loan of birds and to Mr T. Barton Mann and W. A. Motley Ltd., Andover, for the gift of turkeys.

REFERENCES

- Arnstein, H. R. V. (1954). *Advanc. Protein Chem.* **9**, 16.
 Baldwin, B. C., Robinson, D. & Williams, R. T. (1959). *Biochem. J.* **71**, 638.
 Barrass, B. C. & Elmore, D. T. (1957). *J. chem. Soc.* p. 3134.
 Jaffe, M. (1877). *Ber. dtsch. chem. Ges.* **10**, 1925.
 Karrer, P. & Ehrenstein, M. (1926). *Helv. chim. acta*, **9**, 323.
 McGilvery, R. W. & Cohen, P. P. (1950). *J. biol. Chem.* **183**, 179.
 Parke, D. V. & Williams, R. T. (1960). *Biochem. J.* **74**, 5.
 Robinson, D. & Williams, R. T. (1958). *Biochem. J.* **68**, 23P.
 Schachter, D., Manis, J. G. & Taggart, J. V. (1955). *Amer. J. Physiol.* **182**, 537.
 Williams, R. T. (1959). *Detoxication Mechanisms*, 2nd ed. p. 348. London: Chapman and Hall Ltd.

Biochem. J. (1960) **76**, 600

Studies in Detoxication

83. THE AROMATIZATION OF CYCLOHEXANECARBOXYLIC ACID IN HENS

By B. C. BALDWIN, D. ROBINSON AND R. T. WILLIAMS

Department of Biochemistry, St Mary's Hospital Medical School, London, W. 2

(Received 17 February 1960)

*cyclo*Hexanecarboxylic acid (hexahydrobenzoic acid) is aromatized to benzoic acid to a considerable extent in man, the rabbit and the dog (Bernhard & Caffisch-Weill, 1945) and to a minor extent in the rat (Beer, Dickens & Pearson, 1951). In man and the rabbit the main excretory product of *cyclo*-hexanecarboxylic acid is hippuric acid. This aromatization reaction has also been studied in tissue slices and homogenates (Beer *et al.* 1951; Mitoma, Posner & Leonard, 1958) and the last-named authors found that aromatization took place in guinea-pig- and in rabbit-liver mito-

chondria in the presence of adenosine triphosphate and glycine. A very weak aromatizing activity was found in rat-liver mitochondria, but no activity was found with liver mitochondria from man, cat, mouse, dog, monkey, toad, pigeon and chicken. This lack of activity was attributed to the lability of the enzyme. With chicken mitochondria replacement of glycine by ornithine did not produce aromatization. Furthermore, there is no evidence in the literature that the intact chicken can aromatize *cyclo*hexanecarboxylic acid. In the present paper it will be shown that chickens can

aromatize *cyclohexanecarboxylic acid* and that the main excretory products are benzoyl glucuronide and ornithuric acid.

MATERIALS AND METHODS

Materials. *cycloHexanecarboxylic acid* (L. Light and Co. Ltd.) had b.p. 230° after distillation. The amide, m.p. 188°, was prepared via the acid chloride (cf. Lumsden, 1905).

cyclo[carboxy-¹⁴C]Hexanecarboxylic acid was prepared from [*carboxy-¹⁴C]benzoic acid according to the method of Mitsubara & Perkin (1905). The benzoic acid (4 g., 7 μ C/g.) in boiling isoamyl alcohol (200 ml.) was reduced with sodium (20 g.) and the procedure of Mitsubara & Perkin (1905) followed exactly to the stage where the *cyclohexanecarboxylic acid* is purified by distillation. Instead of distillation, the acid in 20 ml. of ether was treated with 3.6 g. of benzylamine. The benzylamine salt which separated was then recrystallized to constant activity (yield 1.2 g. containing 3.56 μ C/g.). For feeding experiments the whole of this salt was dissolved in water (60 ml.) with warming, and the solution treated with conc. HCl (5 drops) and extracted with ether (3 $\times$ 100 ml.). The extract was dried over anhydrous CaCl₂ and then evaporated in a current of warm air. This gave 0.47 g. of *cyclohexanecarboxylic acid* (b.p. 229°), which solidified on cooling.*

Other compounds used are described in the preceding paper (Baldwin, Robinson & Williams, 1960).

Hens. The hens used were Brown Leghorns. The *cyclohexanecarboxylic acid* was administered by crop tube in water containing just sufficient NaHCO₃ to dissolve the acid. The hens were deprived of food the evening before administration of the compound, and the urine was collected in flasks containing 2 ml. of saturated aqueous mercuric chloride (see Baldwin *et al.* 1960).

Isotope methods. Benzoyl glucuronide and ornithuric acid were determined as described by Baldwin *et al.* (1960).

cycloHexanecarboxylic acid. The urine (20–40 ml.) was heated under reflux for 3 hr. with an equal volume of 40% (w/v) NaOH. To the cooled solution *cyclohexanecarboxylic acid* (0.6–0.9 g.) in a little *n*-NaOH was added, and the solution was then neutralized with 30% (w/v) H₂SO₄ and extracted with ether. The extract was dried and the *cyclohexanecarboxylic acid* was precipitated as its benzylamine salt, which was recrystallized from ethyl acetate to constant activity. The salt was reconverted into the free acid, which was methylated with ethereal diazomethane. The ethereal solution of the methyl ester was then saturated with dry ammonia, and the *cyclohexanecarboxylic acid* amide was recrystallized from water (m.p. 188°) to constant activity.

Chromatography. The methods used are described by Baldwin *et al.* (1960).

RESULTS

Detection of benzoyl glucuronide and ornithuric acid

A portion of the urine from the hens which had received the *cyclo[¹⁴C]hexanecarboxylic acid* was acidified with strong HCl and extracted with ethyl acetate. The extract was concentrated to a small volume and suitable amounts were chromato-

graphed on Whatman no. 4 paper in solvent systems A, C and D of Baldwin *et al.* (1960). These chromatograms were left in contact with X-ray film for 14 days. The resulting radioautographs showed spots corresponding in *R_F* values to benzoyl glucuronide and ornithuric acid in the three solvent systems. Hippuric acid was not found. Chromatograms were also prepared, in solvent systems A, B, C, D and F of Baldwin *et al.* (1960), from the urine of hens dosed with non-radioactive *cyclohexanecarboxylic acid*. Only benzoyl glucuronide and ornithuric acid were detected in these urines by *R_F* values and colour reactions.

Isolation of metabolites

Benzoic acid. A hen (wt. 1.65 kg.) was given orally 1 g. of *cyclohexanecarboxylic acid* as the sodium salt in water. The liquid excreta (130 ml.) were collected for 24 hr. and were boiled (reflux) for 3 hr. with 40% NaOH (65 ml.). After cooling, the mixture was acidified with 50% (v/v) H₂SO₄ and steam-distilled until 1.5 l. of distillate had been collected. The distillate was extracted with 2 $\times$ 250 ml. of ether, and the extract dried over anhydrous CaCl₂ and then evaporated in a current of cold air to yield 800 mg. of benzoic acid, m.p. 116°. This corresponded to 88% of the dose of *cyclohexanecarboxylic acid*. On recrystallization from water and then from light petroleum, pure benzoic acid, m.p. and mixed m.p. 121°, was obtained. This was confirmed by preparing the benzylamine salt, m.p. and mixed m.p. 127°.

Benzoyl glucuronide. A total of 6 g. of *cyclohexanecarboxylic acid* was fed to six hens. The 24 hr. urine was acidified to pH 2–3 with conc. HCl and then continuously extracted with ether for 16 hr. After drying over anhydrous Na₂SO₄ the extract was evaporated. The residue was taken up in ethanol (10 ml.) and light petroleum (b.p. 60–80°) added. Benzoyl glucuronide (120 mg.) separated and after three recrystallizations from ethanol–light petroleum mixtures had m.p. and mixed m.p. 186° and $[\alpha]_D^{25} = -23.6^\circ$ (c, 1 in water) [light absorption, λ_{max} 230 and 274 μ with corresponding ϵ_{max} 12 400 and 1120 in ethanol; see Baldwin, Robinson & Williams (1959)].

1(+)-Ornithuric acid. A hen that had been starved for 17 hr. was given orally 2 g. of *cyclohexanecarboxylic acid*

Table 1. *Metabolites of cyclo[¹⁴C]hexanecarboxylic acid in the hen*

For details see text.

Expt. no.	...	...	1	2
Wt. of hen (kg.)			1.5	1.8
Dose of acid (mg./kg.)			260	205
Dose of ¹⁴ C (μ C/hen)			0.77	1.57
Metabolites excreted in 24 hr. (% of dose)*				
Benzoyl glucuronide			49	57
Ornithuric acid			17	11
<i>cycloHexanecarboxylic acid</i>			6	3
Sum of metabolites			72	71
¹⁴ C in 24 hr. urine			82	67
¹⁴ C in 48 hr. urine			82	69

* Determined by isotope dilution.

as the sodium salt in water. The 24 hr. urine was acidified and extracted with ether as above. The extract was dried and evaporated as before and the residue was treated with 100 ml. of CHCl_3 at 60° and the extract filtered. Evaporation of the CHCl_3 gave a clear gum, which was taken up in 10 ml. of ethanol and then 30 ml. of water was added. On keeping overnight L(+)-ornithuric acid (80 mg.) separated. It had m.p. 184° , not lowered by the authentic acid, and $[\alpha]_D^{25} + 5.5^\circ$ (c, 1.3 in 0.1N-NaOH) (light absorption in 0.1N-HCl, λ_{max} , 229 m μ , ϵ_{max} , 20 100 with a slight inflexion at 270 m μ).

The results of the isotope-dilution experiments are shown in Table 1.

DISCUSSION

The aromatization of *cyclohexanecarboxylic acid* has been shown to occur in guinea-pig-liver mitochondria and the end product of the process is hippuric acid. Apparently the process takes place in three steps, the first being the activation of *cyclohexanecarboxylic acid* by formation of *cyclohexanecarbonyl-coenzyme A*, the second the dehydrogenation of *cyclohexanecarbonyl-coenzyme A* to benzoyl-coenzyme A, and the third the formation of hippuric acid by the reaction of benzoyl-coenzyme A with glycine. In the absence of glycine, aromatization occurs only to a very minor extent (Mitoma *et al.* 1958), for removal of the aromatization product, benzoic acid, as hippuric acid greatly accelerates the aromatization step. This process was also studied in the liver mitochondria of two birds, the pigeon and the hen, but Mitoma *et al.* (1958) were unable to show aromatization in these species, even when glycine was replaced by ornithine in the system used. The present work shows that the intact hen can aromatize *cyclohexanecarboxylic acid* very efficiently and that the main product is benzoyl glucuronide. Table 1 shows that 70–80 % of the metabolites (50–60 % of the dose) of *cyclohexanecarboxylic acid* consist of benzoyl glucuronide and about 20 % (10–20 % of the dose) of ornithuric acid.

Mitoma *et al.* (1958) were also unable to detect

aromatization of *cyclohexanecarboxylic acid* in the liver mitochondria of the cat, mouse, dog, monkey and human, although aromatization is known to occur in man and the dog *in vivo*. It is possible that aromatization might have been detected in the mitochondria of some of these species if glycine had been replaced by a source of biologically active glucuronic acid such as uridine diphosphate glucuronic acid, which would have allowed the benzoic acid to be removed as glucuronide.

SUMMARY

1. *cyclo[carboxy- ^{14}C]Hexanecarboxylic acid* has been prepared by the reduction of [*carboxy- ^{14}C]-benzoic acid.*

2. *cycloHexanecarboxylic acid* is extensively aromatized when administered orally to hens. This was proved by the isolation of benzoyl glucuronide and L(+)-ornithuric acid from the urine. By isotope dilution it is shown that of the ^{14}C administered orally to hens as *cyclohexanecarboxylic acid* (dose 200–260 mg./kg.) about 80 % is excreted in 24 hr.; 50–60 % of the dose is excreted as benzoyl glucuronide and 10–20 % as ornithuric acid. About 5 % of the *cyclohexanecarboxylic acid* is excreted unchanged.

This work was supported entirely by a grant from Dr Salsbury's Laboratories, Charles City, Iowa.

REFERENCES

- Baldwin, B. C., Robinson, D. & Williams, R. T. (1959). *Biochem. J.* **71**, 638.
- Baldwin, B. C., Robinson, D. & Williams, R. T. (1960). *Biochem. J.* **76**, 595.
- Beer, C. T., Dickens, F. & Pearson, J. (1951). *Biochem. J.* **48**, 222.
- Bernhard, K. & Cafilisch-Weill, H. (1945). *Helv. chim. acta*, **28**, 1697.
- Lumsden, J. S. (1905). *J. chem. Soc.* **87**, 90.
- Mitoma, C., Posner, H. S. & Leonard, F. (1958). *Biochim. biophys. Acta*, **27**, 156.
- Mitsubara, K. & Perkin, W. H. (1905). *J. chem. Soc.* p. 661.