The Metabolism of Cyclohexanecarboxylate in the Rat

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In the rat, cyclohexanecarboxylate was metabolized and excreted (mostly in the urine) as hippurate, hexahydrohippurate, 3,4,5,6-tetrahydrohippurate and benzoyl and cyclohexylcarbonyl β -glucuronides. The pattern of metabolism is dose-dependent. With decreasing dose a progressive increase in the conversion into hippurate occurred. This was largely at the expense of glucuronide formation, although the proportions of hexahydro- and tetrahydro-hippurate were also decreased. The observed formation of hexahydrohippurate and 3,4,5,6-tetrahydrohippurate substantiates the proposed mechanism of aromatization of cyclohexanecarboxylate. It appears that these compounds arise via glycine conjugation of active intermediates in the aromatization process. Hexahydrohippurate and 3,4,5,6-tetrahydrohippurate may occur in the urine of rats as new metabolites of shikimate, dependent for their formation on microbial metabolism.

Shikimic acid, a naturally occurring acid widespread in plants and certain fruits (Bohm, 1965), has attracted new attention (Stavric & Stoltz, 1976; Brewster *et al.*, 1976) since the report that the acid may possess carcinogenic properties (Evans & Osman, 1974). Introductory investigations into the metabolism of this compound have shown that gastrointestinal micro-organisms present in a variety of animal species both hydrogenate and dehydroxylate shikimate to cyclohexanecarboxylate (Brewster *et al.*, 1976, 1977). It has been proposed (Brewster *et al.*, 1976) that the urinary excretion of hippurate which follows oral administration of shikimate (Asatoor, 1965; Adamson *et al.*, 1970; Brewster *et al.*, 1976) originates from an aromatization of cyclohexanecarboxylate by the mammalian tissues. A complete knowledge of the metabolism of the latter is desirable, therefore, if the ultimate metabolic fate of shikimate is to be understood.

As early as 1911 it was shown that injection of cyclohexanecarboxylate into dogs increased the urinary excretion of hippurate (Friedmann, 1911). Subsequent investigations have supported this aromatization and conjugation with glycine (Baltes *et al.*, 1952) and have shown that the process occurs in both liver and kidney (Beer *et al.*, 1951). Experiments *in vitro* have shown that the liver is the most active tissue for aromatization (Beer *et al.*, 1951) and that the overall enzyme system for this oxidative process is localized in the mitochondria (Mitoma *et al.*, 1958; Babor & Bloch, 1966). No other metabolites of cyclohexanecarboxylate have as yet been reported.

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Materials and Methods

Chemicals

Cyclohexanecarboxylic acid was obtained from Aldrich Chemical Co., Gillingham, Dorset, U.K., and was distilled twice before use. Benzoic acid and Adam's catalyst (platinum dioxide) were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K., and [*ring*-U-¹⁴C]benzoic acid (sp. radioactivity 339 mCi/mmol) from The Radiochemical Centre, Amersham, Bucks., U.K.

Materials

Cannulae tubing (PP 120, PP 25, PP 10) was obtained from Portex Ltd., Hythe, Kent, U.K. Polystyrene collection tubes (LP 3) were obtained from Luckham Ltd., Burgess Hill, Sussex, U.K., and polypropylene analysis tubes (0.4 ml capacity) for urine collection from Sarstedt Ltd., Leicester, U.K.

Synthesis of [*ring*-U-¹⁴C]cyclohexanecarboxylic acid

Cyclohexanecarboxylic acid was prepared by the catalytic hydrogenation of [*ring*-U-¹⁴C]benzoic acid. Resublimed benzoic acid (200 mg) and [*ring*-U-¹⁴C]benzoic acid (200 μ Ci) in ethanol (25 ml) were hydrogenated in a Baskerville and Lindsey hydrogenator at 8–10 MPa (80–100 atm) in the presence of Adam's catalyst (40 mg). After 12 h the solution was filtered, evaporated at 20°C and the product separated from any traces of unchanged benzoic acid by preparative-layer chromatography (see below) by using acetone/light petroleum (b.p. 40–60°C) (1:2, v/v) as developing solvent. After recovery, the [*ring*-U-¹⁴C]cyclohexanecarboxylic acid (120–180 mg; sp. radioactivity 0.95 μ Ci/mg) was

stored as an aqueous solution of the sodium salt at -40°C . The purity ($>98\%$) and benzoate content ($<0.8\%$) of the product were established by isotope dilution.

Animals

Male Wistar albino rats (220–250 g) were used in all experiments.

Animal surgery

Anaesthesia was induced and maintained by intraperitoneal administration of sodium pentobarbital (Nembutal), and the abdominal viscera exposed for surgery through a midline incision. Cannulation of the bile duct was achieved by using polypropylene tubing (Portex PP 25). In turn, each ureter was located, cleared of mesentery and ligated. Both ureters were cannulated (PP 10 tubing) when sufficiently dilated with urine to enable this operation to be carried out, usually within 10 min of ligation. To permit intraduodenal dosing of animals, a short length of cannula tubing (PP 25) was introduced into the duodenum via a small incision and secured to the wall by a single ligature. All cannulae were exteriorized before suturing. Bile-duct cannulae were 25 cm in length and ureter cannulae 8–10 cm.

Collection of urine and bile

Bile was collected in weighed polystyrene LP3 tubes and urine in weighed 0.4 ml polypropylene analysis tubes at 30 min intervals for up to 7 h, after which the animals were killed by an overdose of Nembutal. Samples were stored at -40°C until required for analysis.

Administration of dose

[^{14}C]Cyclohexanecarboxylic acid (5–6 μCi , 0.5–200 mg/kg) was administered as the sodium salt in 0.5 ml of water via the duodenal cannula and flushed through with a further 0.25 ml of 0.9% NaCl. Samples of the dosing solution were taken to determine the precise amount of ^{14}C administered.

Measurement of radioactivity

Urine (5 μl) and bile (50 μl) from all 30 min samples were dissolved in 0.4 ml of water and 4 ml of a Synperonic NXP (I.C.I., Billingham, Teesside, U.K.)/toluene/2,5-diphenyloxazole scintillant by the method of Wood *et al.* (1975). Radioactivity was measured in an LKB 1210 liquid-scintillation counter, and the excretion of radioactivity in the urine and bile was calculated as a percentage of the administered dose. Counting efficiencies were obtained by internal standardization by using either [^{14}C]toluene or [^{14}C]hexadecane.

Solvent extraction of urine and bile

Radioactive samples of urine and bile were

separately combined, diluted with an equal volume of water and acidified to pH 1 with 5 M-HCl. The solutions (2–3 ml) were extracted with five 6 ml portions of ethyl acetate in small (10 ml) screw-capped glass tubes and the layers separated by centrifugation (2000 g for 30 s). Extracts were combined, evaporated, and dissolved in methanol (100–200 μl) for examination by t.l.c.

Chromatography

T.l.c. was carried out on 0.25 mm-thick plates of silica gel GF₂₅₄ (Anderman and Co., East Molesey, Surrey, U.K.) and preparative-layer chromatography on silica-gel 60 F₂₅₄ plates of 2 mm thickness (E. Merck, Darmstadt, Germany). The following solvent systems were used: (a) acetone/light petroleum (b.p. 40 – 60°C)/acetic acid (20:40:1, by vol.); (b) propan-1-ol/ NH_3 solution (sp.gr. 0.88) (7:3, v/v); (c) butan-1-ol/acetic acid/water (4:1:1, by vol.). All solvents used in the purification of metabolites for either mass spectrometry or p.m.r. (proton-magnetic resonance) spectrometry were of AnalaR quality. System (a) separated all metabolites observed; systems (b) and (c) were used as means of further purification of the metabolites from endogenous impurities. Carboxylic acids were detected by spraying the dried plates with 0.04% Bromocresol Green in ethanol (Lugg & Overall, 1948) and glucuronic acid conjugates with 1% (w/v) naphtharesorcinol in acetone/10% (v/v) phosphoric acid (4:1, v/v) followed by heating at 100°C for 10 min (Bridges *et al.*, 1965).

Radioautographs of thin-layer chromatograms were produced on medical X-ray films (BB 54; 18 cm $\times$ 24 cm; Kodak, Hemel Hempstead, Herts., U.K.) by contact exposure.

Mass spectrometry

Electron-impact mass spectra were recorded on an AEI MS12 mass spectrometer equipped with temperature-programming facility. Spectra were run at 70 eV by using direct sample insertion. Urine or bile extracts were applied to t.l.c. plates as 1–2 cm bands and developed in solvent system (a). For the identification of metabolites 1–3 (see Plate 1) the plates were then sprayed with aq. 7 M- NH_3 ; the metabolites were located by radioautography, then eluted with methanol and rechromatographed separately in solvent system (b). Radioactive zones were eluted with methanol (AnalaR grade) by the technique described by Rix *et al.* (1969), and the final eluates transferred to small, washed melting-point tubes. The methanol was evaporated by a stream of N_2 from a finely drawn capillary and the tubes were used as sample probes for direct-insertion mass spectrometry. For the identification of metabolites 4 and 5, urine or bile extracts were applied as 10 cm bands to t.l.c. plates and developed in solvent system (a). The compounds were located, then eluted as described above and

chromatographed as short (2cm) bands in solvent system (c). Radioactive zones were removed and eluted with methanol into small conical glass vials (conveniently prepared from the conical section of a Pasteur pipette). The methanolic solutions were first treated with excess of ethereal diazomethane (Vogel, 1956), evaporated by a stream of N_2 and left overnight with acetic anhydride (AnalaR grade, 15 μ l) and redistilled pyridine (15 μ l). Excess of reagents was removed under high vacuum and the resulting triacetyl-methyl glucuronates were prepared for direct-insertion mass spectrometry as described above.

P.m.r. spectrometry

P.m.r. spectra were recorded on a 90 MHz Bruker WH-90 Fourier-transform n.m.r. spectrometer operating at ambient temperature. $[^2H_6]$ Dimethyl sulphoxide was used as solvent and tetramethylsilane as internal standard. Chemical shifts (δ) are expressed in p.p.m. downfield from tetramethylsilane. For the purification of metabolites 1 and 2, the urine of three animals which had received $[^{14}C]$ cyclohexanecarboxylate (200mg/kg) was combined and extracted as described above. The extracts were chromatographed on preparative-layer (2mm thick) silica plates in solvent system (a), the metabolites located and eluted with methanol, then further purified in sequential fashion on t.l.c. plates by using solvent system (a), then system (b). The metabolites were recovered as free acids by elution with methanol, evaporation, acidification with 0.1M-HCl (1ml) and finally extraction into ethyl acetate (5 $\times$ 5ml).

Quantitative determination of metabolites in urine

Metabolites in urine were separated by t.l.c. of urine extracts by using solvent system (a) and were located by radioautography. Radioactive zones (see Plate 1) were removed and the powdered silica was suspended in 4ml of the scintillant described above to which 4% (w/v) thixotropic gel (Packard Instruments, Caversham, Berks., U.K.) had been added. Radioactive metabolites were determined as a percentage of the total radioactivity recovered from the plates and converted into percentage of administered dose by reference to the degree of biliary excretion at that dosage. All determinations were carried out in triplicate (which were to within 5% of each other) and the means obtained.

Results

After intraduodenal administration of $[^{14}C]$ cyclohexanecarboxylate, radioactivity was rapidly eliminated in the urine and bile. Extraction with ethyl acetate at pH1 removed 97–98% of the radioactivity present in the urine and 91–92% of that in the bile. The values were taken as representing complete extraction, since the non-extractable radio-

activity represented no more than 2–3% of the administered dose.

Urinary and biliary excretion of radioactivity

Intraduodenal administration of $[^{14}C]$ cyclohexanecarboxylate at a dosage of 200mg/kg led to the ready elimination of radioactivity in both urine and the bile (Fig. 1). At lower dosages the rate of elimination of the dose was very rapid indeed (see Fig. 1). Not only does the rate of excretion increase with decreasing dose, but the extent of biliary excretion is lowered. At the lowest dose studied (0.5mg/kg) about 95% of the administered dose was excreted within the first 90min.

Metabolites of cyclohexanecarboxylate

Examination of the ethyl acetate extracts of urine and bile from animals which received $[^{14}C]$ cyclohexanecarboxylate (200mg/kg) showed the presence of five different metabolites (see Plate 1). The

Fig. 1. Effect of dose on the urinary and biliary excretion of radioactivity after intraduodenal administration of $[^{14}C]$ -cyclohexanecarboxylate to rats

Cyclohexanecarboxylate (5–6 μ Ci) was administered as the sodium salt in 0.5 ml of water: $\square$, 200mg/kg; $\bullet$, 100mg/kg; $\circ$, 35mg/kg; $\blacktriangledown$, 5mg/kg; $\triangle$, 0.5mg/kg. Broken lines represent urinary excretion, solid lines represent biliary excretion. Mean flow rates were respectively: urine, 280, 210, 250, 140, 170 μ l/h; bile, 1.22, 1.00, 0.80, 1.20, 0.82 ml/h.

Table 1. *Electron-impact mass spectra (70 eV) of cyclohexanecarboxylate and its metabolites in the rat*
Mass/charge ratios (m/e) of the six predominant ions are given and their relative abundances included in parentheses.

Compound	Mol. wt.	m/e						
Hexahydrohippurate	185	55 (100)	83 (71)	41 (59)	130 (22)	111 (15)	*185 (13)	
3,4,5,6-Tetrahydrohippurate	183	109 (100)	108 (95)	81 (79)	79 (41)	57 (27)	*183 (10)	
Hippurate	179	105 (100)	77 (87)	135 (33)	106 (25)	134 (16)	*179 (2)	
Cyclohexylcarbonyl glucuronide†	444	83 (100)	43 (93)	111 (90)	55 (23)	155 (18)	*444 (1)	
Benzoyl glucuronide†	438	105 (100)	43 (78)	60 (27)	77 (15)	106 (8)	*438 (3)	
Cyclohexanecarboxylate	128	73 (100)	55 (88)	41 (75)	83 (61)	*128 (60)	56 (60)	

* Ions of highest m/e in the spectra (M^+).

† Determined as their triacetylmethyl esters.

Table 2. *P.m.r. data of metabolites of cyclohexanecarboxylate in the rat*

Spectra were recorded in $[^2\text{H}_6]$ dimethyl sulphoxide with tetramethylsilane as internal standard. Chemical shifts (δ) are in p.p.m. downfield from tetramethylsilane. Abbreviations: (d) doublet; (t) triplet; (b) broadened.

	H ₈	H ₇	H ₁	H ₂	H ₃ , H ₆	H ₄ , H ₅
Hexahydrohippurate (I)	3.69 (d) $J_{7,8}$ 6 Hz	7.96 (t) $J_{7,8}$ 6 Hz	2.10 (b)	—	1.1–1.8 (b)	—
3,4,5,6-Tetrahydrohippurate (II)	3.74 (d) $J_{7,8}$ 6 Hz	7.96 (t) $J_{7,8}$ 6 Hz	—	6.57 (b)	2.14 (b)	1.56 (b)

metabolites, detected by radioautography of thin-layer chromatograms developed in solvent system (a), were identified by mass spectrometry and p.m.r. as: (1) hexahydrohippurate; (2) 3,4,5,6-tetrahydrohippurate; (3) hippurate; (4) cyclohexylcarbonyl β -D-glucuronide; (5) benzoyl β -D-glucuronide. The mass spectra of metabolites 1–5 and the p.m.r. spectra of metabolites 1 and 2 are listed in Tables 1 and 2 respectively.

The assignment of structure to the spectra shown was quite straightforward, although the p.m.r. spectrum of metabolite 2 requires further explanation. The mass spectrum (M^+ , m/e 183) was consistent with that of an isomeric tetrahydrohippurate, and the presence of a broad olefinic multiplet at δ 6.57 in the p.m.r. spectrum supported this. Integration of the olefinic signal showed it to be equivalent to exactly one proton when compared with the two-proton doublet at δ 3.74 of the methylene protons of the glycine moiety. This can only be consistent with the 3,4,5,6-tetrahydrohippurate. The downfield position of the olefinic resonance supported the proposed $\alpha\beta$ -unsaturation in the metabolite.

All five metabolites were present in urine extracts and gave acidic reactions with ethanolic Bromocresol Green. Hippurate gave a strong quench and

tetrahydrohippurate a weak quench of the fluorescence of short wave (254 nm) u.v. light. Glucuronic acid conjugates of cyclohexanecarboxylate and benzoate were the major products observed in the bile (see Plate 1) and gave positive colour reactions with the naphtharesorcinol reagent.

Metabolism-cage experiments in which animals received $[^{14}\text{C}]$ cyclohexanecarboxylate (100 mg/kg) orally showed a similar metabolic pattern. Almost all (>98%) of the administered dose was recovered in the urine, and negligible amounts (<1%) were in the faeces and expired air. The metabolic pattern in the urine was qualitatively the same as in biliary-cannulation experiments, which suggests that the animal surgery described did not influence the metabolism of cyclohexanecarboxylate to any great extent.

Effect of dose on metabolic profile

Changes in the metabolic pattern occurred when cyclohexanecarboxylate was administered at different doses ranging from 0.5 to 200 mg/kg (see Table 3). What is most apparent with decreasing dosage is the decrease in glucuronide conjugates and the increase

EXPLANATION OF PLATE I

T.l.c. in system (a) of urine extracts (UE) and bile extracts (BE) from rats given [¹⁴C]cyclohexanecarboxylate
 X-ray film was exposed by direct contact with developed chromatograms and then photographed by contact printing.
 Key to metabolites: (1) hexahydrohippurate; (2) 3,4,5,6-tetrahydrohippurate; (3) hippurate; (4) cyclohexylcarbonyl
 β -D-glucuronide; (5) benzoyl β -D-glucuronide.

Table 3. *Effect of dose on the metabolism of [¹⁴C]cyclohexanecarboxylate in the rat*

Ethyl acetate extracts of urine were subjected to t.l.c. in solvent system (a) and the separated components were then subjected to quantitative radioassay.

Dose (mg/kg)	Biliary excretion (% of dose)†	Metabolites in urine				Total percentage of dose accounted for
		Benzoyl and cyclohexyl- carbonyl glucuronides (% of dose)	Hippurate (% of dose)	3,4,5,6-Tetra- hydro- hippurate (% of dose)	Hexahydro- hippurate (% of dose)	
200*	8.0 ± 0.3	18.0 ± 0.6	48.2 ± 2.0	12.4 ± 0.8	10.5 ± 0.3	97.1 ± 4.0
100	7.7	13.6	56.1	9.9	10.0	97.3
35	3.9	6.9	70.5	8.6	6.1	96.0
5	3.8	2.4	75.0	9.2	6.6	97.0
0.5	3.0	1.4	81.0	8.1	4.9	98.4

* Values at this dosage represent the means for three animals ± S.E.M.

† Radioactivity in the bile was almost entirely glucuronide conjugates of cyclohexanecarboxylate and benzoate.

Scheme 1. *Metabolism of cyclohexanecarboxylate in the rat*

Key to metabolites: (1) hexahydrohippurate; (2) 3,4,5,6-tetrahydrohippurate; (3) hippurate; (4) cyclohexylcarbonyl β-D-glucuronide; (5) benzoyl β-D-glucuronide. Pathways indicated* are dose-dependent and are unimportant at low dosages (<1 mg/kg). References: (a) Mitoma *et al.* (1958); (b) Babior & Bloch (1966); (c) Schachter & Taggart (1953).

in hippurate. The fact that glucuronide formation is greatest at the higher dosages seems to account for the fact that biliary excretion, although a minor route of excretion of cyclohexanecarboxylate, is greatest at the higher dosages (see Fig. 1).

Discussion

In the rat, cyclohexanecarboxylate was metabolized to hippurate, hexahydrohippurate, 3,4,5,6-tetrahydrohippurate and benzoyl and cyclohexyl-

carbonyl glucuronides, of which only hippurate has previously been reported (Friedmann, 1911; Beer *et al.*, 1951; Baltes *et al.*, 1952; Mitoma *et al.*, 1958; Babor & Bloch, 1966). The metabolism of cyclohexanecarboxylate is dose-dependent, with considerable amounts of benzoyl and cyclohexylcarbonyl glucuronides being formed only at higher dosages. This dose-dependence of cyclohexanecarboxylate metabolism raises important considerations in the metabolism of shikimate, a potential carcinogen (Evans & Osman, 1974) which is metabolized by rat caecal micro-organisms to cyclohexanecarboxylate (Brewster *et al.*, 1976). Although shikimate itself is poorly absorbed from the gut, it is slowly converted by microbial action into cyclohexanecarboxylate, which is then absorbed and metabolized in the mammalian tissues. The microbial reduction of an oral dose of shikimate to rats yields cyclohexanecarboxylate at a rate of approx. 1–2 mg/h per kg body wt. (Brewster, 1977). Clearly the metabolism of cyclohexanecarboxylate at low doses is most relevant to the metabolic fate of shikimate, and hexahydro- and 3,4,5,6-tetrahydro-hippurates should occur therefore as metabolites of shikimate in the urine, in addition to hippurate, a known metabolite.

Since shikimate occurs widely in plants and fruits (Bohm, 1965) and is converted into cyclohexanecarboxylate by a variety of animal faecal microflora (Brewster *et al.*, 1977), then hexahydro- and 3,4,5,6-tetrahydro-hippurate should occur naturally in the urine of animals as does hippurate. Hexahydro-hippurate has been detected in the urine of cattle (Suemitsu *et al.*, 1971) and therefore the microbial conversion of dietary shikimate into cyclohexanecarboxylate followed by metabolism in the animal tissues appears to offer a viable explanation for this observation. This mechanism is an alternative to the pathway suggested by Rho & Evans (1975), namely that hexahydrohippurate may arise from a microbial reduction of benzoate or hippurate, and is strengthened by the fact that rat gastrointestinal micro-organisms do not appear to reduce benzoate either *in vitro* or *in vivo* (Brewster, 1977). The presence of 3,4,5,6-tetrahydrohippurate in animal urine has yet to be reported.

These new metabolites of cyclohexanecarboxylate, namely hexahydro- and 3,4,5,6-tetrahydro-hippurate, provide further information on the mechanism of its

aromatization by mammalian tissues to hippurate. The process probably involves dehydrogenation of intermediate CoA esters before conjugation of benzoyl-CoA with glycine (see Scheme 1). The urinary excretion of hexahydro- and 3,4,5,6-tetrahydro-hippurates substantiates these proposed intermediates.

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