The Aromatization of Hydrogenated Derivatives of Benzoic Acid in Animal Tissues

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Aromatization within the animal body is a phenomenon of special interest in view of the occurrence in many highly active biological substances of one or more aromatic rings. The origin of such compounds as the natural oestrogenic hormones, in which ring A is always aromatic and sometimes ring B also, is at present unknown. Evidence of aromatization is therefore of potential value in considering the origin of these substances within the animal body. Further, it has been suggested as a possibility that carcinogens of the type of the well known carcinogenic polycyclic aromatic hydrocarbons might arise within the animal organism as a result of a pathological metabolism of naturally occurring polycyclic hydroaromatic ring systems, such as steroids, or perhaps from the natural sex hormones by a process involving the further aromatization of these compounds.

A detailed account of these suggestions and of some related investigations, with references to the literature, has recently been given (Dickens, 1950) so that it is unnecessary to repeat it here. Some preliminary investigations were reported by Dickens (1947). In the present paper an investigation is reported into the simpler aromatization reaction as it occurs in the course of metabolism of *cyclohexanecarboxylic acid* and its derivatives within the animal body.

The present knowledge of aromatization of the *cyclohexane* ring, to yield benzoic acid or its derivatives, begins with the studies on the metabolism of *laevoquinic acid* (1:3:4:5-tetrahydroxycyclohexanecarboxylic acid). In an attempt, not yet fully realized, to account for the normal excretion of benzoic acid (or hippuric acid in those species which normally effect the necessary conjugation with glycine), it was found by Lautemann (1863) that in man orally administered calcium quinate was converted to hippuric acid which was isolated from the urine. Quick (1931) showed that the excretion rate was slower than that for a corresponding dose of benzoic acid, and gave similar data for the increased hippuric acid excretion following a meal of fruits (prunes) believed to contain quinic acid. Vasiliu, Timoşencu, Zaimov & Coteleu (1940) report a 54% conversion of quinic acid to benzoic acid in the sheep, but less than 6% conversion in the dog. In man, however, they obtained only 10% of the theoretical

amount of benzoic acid, and this only when the diet was such that it produced an alkaline urine. In view of the discrepancy between their results and those of Lautemann and Quick, both of whom obtained considerable aromatization, we have re-investigated the fate of this acid, and have confirmed the work of Lautemann and Quick.

Apart from quinic acid, the aromatization of only a very limited number of hydroaromatic substances has hitherto been studied. Friedmann (1911) obtained some evidence of increased urinary excretion of hippuric acid in the dog after injection with *cyclohexanecarboxylic acid* or hexahydroanthranilic acid. The latter observation has not been confirmed (Bernhard, 1938*a*), but as a result of extensive investigations by Bernhard and his co-workers it is now clear that the dog, rabbit and man can aromatize *cyclohexanecarboxylic acid* and a limited number of closely related derivatives, e.g. hexahydro-*N*-methylbenzamide, hexahydrohippuric acid, *cyclohexylvaleric acid* (Bernhard, 1937, 1938*a, b*; Bernhard & Cafilisch-Weill, 1945). The related literature has recently been fully reviewed (Dickens, 1950). It is only necessary, therefore, to mention here that by the use of deuterium-labelled *cyclohexanecarboxylic acid*, Bernhard & Cafilisch-Weill (1945) were able to show that the source of the urinary benzoic acid was, in fact, the administered *cyclohexane* compound.

The object of the present investigation was twofold: (a) to obtain information on the chemical route and enzymic nature of this transformation of the *cyclohexane* to the benzenoid ring, (b) to obtain aromatization in isolated tissues, if possible; since this is the only practicable method of excluding the participation of intestinal flora in the reaction and of ascertaining the probable site at which it occurs in the animal body.

EXPERIMENTAL

Materials and methods

cycloHexane- and cyclohexene-carboxylic acids. *cycloHexanecarboxylic acid* and *cyclohexylacetic acid* were purchased (British Drug Houses Ltd.). The following compounds were synthesized as in the references cited: Δ^1 - and Δ^2 -*cyclohexenecarboxylic acids* and *cis*-3-hydroxycyclohexanecarboxylic acid (Boorman & Linstead, 1935); 1-hydroxycyclohexanecarboxylic acid (Bucherer, 1894); *trans*-

2-hydroxycyclohexanecarboxylic acid (Mousseron, Jullien & Winternitz, 1948); *trans*-3-hydroxycyclohexanecarboxylic acid (Perkin & Tattersall, 1907); *cis*-4-hydroxycyclohexanecarboxylic acid (Hardegger, Plattner & Blank, 1944).

The preparation and nature of *trans*-4-hydroxycyclohexanecarboxylic acid calls for special comment. The earlier experiments of Beer, Dickens & Pearson (1949, see also Dickens, 1950), were made with a specimen of the so-called *trans* isomer of this acid prepared according to Perkin (1904), m.p. 120–121°. When this work was nearly completed Campbell & Hunt (1950) showed that this was in fact a *cis-trans* mixture, the true *trans* isomer having m.p. 148°. We are greatly indebted to these authors for specimens of the *cis* and *trans* isomers of 4-hydroxycyclohexanecarboxylic acid with which we have repeated our earlier experiments. Only the results on the pure *trans* isomer are reported here.

cis-2-Hydroxycyclohexanecarboxylic acid was prepared by the method of Pascual, Sistaré & Regás (1949), except that the use of Raney nickel (prepared according to Delépine & Horeau, 1937) instead of platinum oxide shortened the time required for the catalytic reduction of the intermediate ethyl cyclohexan-2-one-1-carboxylate to approximately 90 min. at atmospheric temperature and pressure.

Δ^3 -cyclohexenecarboxylic acid: Butadiene (5 g.), methyl acrylate (12 g.) and hydroquinone (0.05 g.) were heated in a sealed tube at 130° for 10 hr. When cold the contents of the tube were diluted with ether (100 ml.), washed with 2*N*-NaOH and water, and dried (Na_2SO_4). Distillation gave methyl Δ^3 -cyclohexenecarboxylate (12.5 g.) as a clear colourless oil b.p. 69–71°/15 mm. The latter (12 g.) was refluxed for 2 hr. with a mixture of KOH (15 g.), ethanol (60 ml.) and water (10 ml.). After removing the ethanol in a current of steam, the alkaline solution was washed with ether, acidified with 5*N*-HCl and continuously extracted (12 hr.) with ether in an atmosphere of N_2 . The ethereal extract was dried (Na_2SO_4) and distilled, giving Δ^3 -cyclohexenecarboxylic acid as a colourless oil (9.75 g.) b.p. 120–122°/10 mm., which rapidly crystallized on cooling, m.p. 18–18.5°. (Sobecki (1910) gives m.p. 13–15°.) (Found: C, 66.55; H, 8.35. Calc. for $\text{C}_7\text{H}_{10}\text{O}_2$: C, 66.6; H, 8.0%). The acid, in cold CHCl_3 solution, rapidly absorbed the theoretical quantity of Br_2 required to saturate one double bond, no HBr being eliminated. After removal of the solvent the dibromide, crystallized from formic acid, had m.p. 85–86° (Perkin & Tattersall, 1907, m.p. 86°).

Where optical isomerides could exist all the above compounds were used as the racemic forms.

Estimation. Benzoic (or hippuric) acid was estimated by the colorimetric method of Dickens & Pearson (1950). All results are expressed as benzoic acid regardless of whether benzoic or hippuric acid was actually present in the samples. Details of any special preliminary treatment required to prepare urine or tissue extracts for analysis are mentioned in the appropriate sections below.

Diet and dosage. Rabbits of 2–3 kg. were maintained in metabolism cages on a diet of cabbage *ad lib*. The compounds were usually injected subcutaneously as single doses of about 10 ml. of the neutral aqueous solutions of their Na salts; in a few instances the Na salt was given by mouth. No toxic effects were observed at any of the dose levels employed, although occasionally there was a marked decrease in the volume of urine excreted during the 24 hr. following injection. The urine was, however, of usual appearance and pH.

Collection and estimation. (a) *Urine.* A control urine sample was collected during the 24 hr. period preceding in-

jection and usually for two subsequent 24 hr. periods. No preservative was used since no aromatization was observed when selected compounds were dissolved in normal urine and kept 24 hr. Similarly, benzoic acid added to urine was recovered undiminished after standing for 24 hr. Estimations were made on 5 ml. portions of the urine adjusted to pH 6.8 with HCl and filtered through gauze. The filtrate was treated with 5 ml. saturated Br_2 water and allowed to stand for 30 min. to precipitate phenolic compounds and saturate the double bonds of cyclohexene derivatives. After filtration, excess Br_2 was removed from the solution by the passage of a stream of N_2 . Portions of 1 ml. each were diluted to 10 ml. with 0.1*N*-HCl, extracted with isomyl alcohol, and the estimation completed as described by Dickens & Pearson (1950).

The values of urinary benzoic acid excretion given in this paper are corrected for the basal figures obtained during the 24 hr. preceding the administration of the dose. In rabbits fed solely on cabbage this basal value is favourably low, the mean on sixteen rabbits being 24 mg./24 hr. (cf. Table 1, column '– 24 to 0 hr.').

(b) *Blood.* The benzoic or hippuric acid in whole blood was determined colorimetrically on a 3.5 or 5 ml. sample collected from the ear vein of the rabbit (Dickens & Pearson, 1950).

Isolation of hippuric and benzoic acids from urine. Two methods were used. (i) The neutralized urine was evaporated to a syrup and the extraction and crystallization of hippuric acid followed as described by Friedmann (1911). (ii) The urine was acidified to congo red with HCl and continuously extracted with ether for 14 hr. Benzoic acid was readily isolated in this way, but hippuric acid, if present in the urine, is liable to become hydrolysed to benzoic acid and glycine during the extraction.

Tissue slices. Tissue slices (usually 300 mg. wet wt.) were incubated in O_2 at 37° in Warburg manometer vessels containing 2 ml. Krebs's Ringer solution, buffered with phosphate. In some experiments the gas was O_2 containing 5% CO_2 and the buffer used was then bicarbonate. Substrates were added as neutral solutions of their Na salts. At the end of the incubation period (usually 4 hr.), the pH was checked (6.8–7.2), and the whole contents of the vessels (after removal of the KOH used for absorption of CO_2) were then diluted to 15 ml. with 70% (v/v) ethanol. Benzoic acid was estimated colorimetrically in measured portions of the filtrate (Dickens & Pearson, 1950). Vessels from which the tissue was omitted were simultaneously incubated to provide a reagent blank and to reveal any tendency of the compounds to autoxidize to substances estimable as benzoic acid: this correction was always very small under the conditions employed.

Tissue homogenates. (a) *Preparation.* No success followed attempts to prepare active fatty acid oxidizing homogenates from rat or rabbit liver with a Waring type blender after the general procedure of Green, Loomis & Auerbach (1948). These authors, however, worked with much larger quantities than were used in the present work, and the enzyme system was evidently unstable under the conditions used by us.

By using a glass homogenizer (Potter & Elvehjem, 1936) and following a standardized procedure, no difficulty was experienced in preparing homogenates able actively to oxidize *n*-octanoic acid in a reproducible manner. The conditions finally adopted were as follows. The animal (rabbit or rat) was killed by a blow on the head, quickly bled and the liver immediately removed into crushed ice. Portions of 1.5 g. liver were weighed into 5 ml. ice-cold 0.15*M*-KCl, and

homogenized with a loosely fitting glass pestle for 20 sec. at 0°. A further 7.5 ml. ice-cold 0.15 M-KCl and 1 ml. 0.1 N-KOH were then added and homogenizing was resumed for a further 100 sec. The 10% homogenate so obtained had pH 7.0-7.4 and was readily pipetted. It was always prepared immediately before use, being the last component (1 ml.) to be added to the vessels before filling them with O₂.

(b) *Measurement of respiration.* The final concentrations in 3 ml. diluted homogenate contained in each Warburg vessel were: phosphate buffer pH 7.4, 0.015 M; MgCl₂, 0.013 M; cytochrome c, 10⁻⁵ M; adenosinetriphosphate, 0.001 M. The substrates were added as neutral solutions of their Na salts; final pH 7.0-7.2. Each vessel had homogenate equivalent to 100 mg. wet tissue. The flasks were gassed with O₂ at room temperature for 2 min., immediately placed in the bath at 37° and readings begun after 5 min. The oxidative activity of the homogenate was compared with that towards 0.001 M-n-octanoate in each experiment. Colorimetric benzoic acid determinations were made on the vessel contents by the method already described for tissue slices.

RESULTS

Aromatization in the intact animal

Urinary excretion in the rabbit. Table 1 summarizes the observations on the urinary excretion of benzoic or hippuric acids after the subcutaneous injection into rabbits of the compounds listed. Control experiments using benzoic acid showed that some 70% of the compound was recoverable in the urine as hippuric acid, elimination being virtually complete within 24 hr. (Table 1). This agrees well with the findings of Bray, Neale & Thorpe (1946).

Where an appreciable amount of benzoic acid was indicated by the colorimetric method of estimation, the aromatic acid responsible was isolated with the sole exception of Δ^1 -cyclohexenecarboxylic acid where isolation was not carried out. In all cases the product was benzoic or hippuric acid, and the

Table 1. *Benzoic acid excretion in the urine of rabbits receiving subcutaneous injections of cyclohexane-, cyclohexene- and monohydroxycyclohexane-carboxylic acid (as sodium salts)*

Compound	Rabbit wt. (kg.)	Dose* (mg.)	Benzoic acid* excretion (mg.) during hr.			Method†	Extra benzoic* acid (mg.)	Extra benzoic as percentage of dose
			-24 to 0	0 to 24	24 to 48			
Benzoic acid	2.42	1750	16.5	1346	28.2	c	1341.2	76.7
Benzoic acid	2.47	1640	37.7	1150	N.D.	c	1112.3	67.8
cycloHexanecarboxylic acid	1.91	1888	28.4	947	145.6	c	1035.8	55.0
cycloHexanecarboxylic acid	2.51	1077	18.3	550	35.7	c	549.1	51.0
cycloHexanecarboxylic acid	2.70	493	39.2	248.2	16.5	c	186.3	37.8
cycloHexanecarboxylic acid	N.D.	1300	N.D.		650	i	—	50 (approx.)
Δ^1 -cycloHexanecarboxylic acid	2.75	1642	28.0	1018	176	c	1138	69.3
Δ^2 -cycloHexenecarboxylic acid	2.67	1840	90.0	755	84.1	c	659.1	35.8
			—		669	i	—	32 (approx.)
Δ^3 -cycloHexenecarboxylic acid	2.87	1936	32.7	1020	111	c	1065.6	55.1
			—		1155	i	—	60 (approx.)
Δ^3 -cycloHexenecarboxylic acid	1.84	1788	4.8	512.5	56.5	c	559.4	31.3
Δ^3 -cycloHexenecarboxylic acid	3.08	2015	—		675	i	—	33 (approx.)
1-Hydroxycyclohexane-carboxylic acid	1.92	846	19.0	43.4	N.D.	c	24.4	2.9
cis-2-Hydroxycyclohexane-carboxylic acid	3.12	1580	4.7	47.3	N.D.	c	42.6	2.7
trans-2-Hydroxycyclohexanecarboxylic acid	2.22	1690	10.4	52.9	N.D.	c	42.5	2.5
cis-3-Hydroxycyclohexane-carboxylic acid	2.50	1780	25.4	49.7	0.0	c	24.3	1.4
trans-3-Hydroxycyclohexanecarboxylic acid	2.50	1830	13.6	67.7	N.D.	c	54.1	3.0
cis-4-Hydroxycyclohexane-carboxylic acid	2.73	410	5.3	9.4	N.D.	c	4.1	1.0
trans-4-Hydroxycyclohexanecarboxylic acid	2.50	770	20.4	62.7	68.2	c	90.1	11.7

N.D. = not determined.

* Doses and amounts of excreted hippuric acid are both expressed as the equivalent amounts of benzoic acid.

† Method: c = Colorimetric; i = isolation as hippuric acid. (Blanks are not subtracted in isolation experiments, which covered the period 0-48 hr.)

amount isolated corresponded with that found colorimetrically. Analysis of urine samples passed during the first and second 24 hr. periods showed that the excretion of the benzoic acid was usually virtually complete within 24 hr., in agreement with the analyses of blood benzoic acid described below which show that the aromatization is a comparatively rapid process. Fig. 1 shows the relationship of the amounts of benzoic acid excreted to the doses of *cyclohexane* compound injected.

Fig. 1. Excretion of benzoic acid by rabbits following the subcutaneous injection of hydroaromatic acids. Total height of columns = weight of compound injected/kg. rabbit (expressed as the theoretically corresponding amount of benzoic acid); solid portion of column = weight of extra benzoic acid found in urine.

Fig. 2. Level of benzoic acid in rabbit blood after subcutaneous injection of *cyclohexanecarboxylic acid*. Dose equivalent to 0.97 g. benzoic acid.

A slight fall was observed in the percentage conversion to benzoic acid excreted as the dose level (mg./kg.) of *cyclohexanecarboxylic acid* was reduced (cf. Fig. 1). In considering the remaining compounds a striking difference is at once apparent between the hydroxycyclohexanecarboxylic acids,

none of which was much aromatized, and the three *cyclohexanecarboxylic acids*, all of which yielded large quantities of benzoic acid. The proportion of the latter excreted following the injection of the Δ^1 compound (69%) approaches very nearly that obtained by the direct administration of benzoic acid itself (67–76%). Δ^2 - And Δ^3 -*cyclohexanecarboxylic acids* appear to be rather less readily converted to benzoic acid than the Δ^1 compound in the whole rabbit.

Blood benzoic acid in the rabbit. Fig. 2 shows the increase of benzoic acid content of the blood following the subcutaneous injection into a 3.05 kg. rabbit of an aqueous solution containing *cyclohexanecarboxylic acid* (as sodium salt) equivalent to 0.97 g. benzoic acid. During the first 4 hr. after injection the blood benzoic acid rose rapidly, but 18–24 hr. after injection it had returned to a low level.

Urinary excretion in the rat. Twelve rats in metabolism cages were injected at 0 hr., after collection of urine for the preceding 24 hr., with a total amount of 24 ml. solution of sodium *cyclohexanecarboxylate*, equivalent to 2.42 g. benzoic acid. The urine was collected over the periods shown in Table 2 in which the colorimetrically estimated amounts of benzoic acid are shown. The residue from evaporation of the ether extracts was crystallized from hot water, but only minimal amounts of benzoic acid were found (Table 2).

Table 2. Urinary excretion of benzoic acid by twelve rats after subcutaneous injection of sodium *cyclohexanecarboxylate*

(Total dose equivalent to 2.42 g. benzoic acid.)

Period (hr.)	Vol. urine (ml.)	Benzoic acid excretion	
		(mg.)	Percentage of dose
–24 to 0	157	15.5	—
0 to 24	235	109.4*	3.9
24 to 48	144	30.9	0.6

* By ether extraction, hydrolysis with NaOH and recrystallization, benzoic acid, m.p. 121°, was identified.

Aromatization in isolated tissues

Tissue slices with cyclohexanecarboxylic acid as substrate

Ability of various tissues to aromatize cyclohexanecarboxylic acid. Both liver and kidney slices of rabbits and rats were found to produce benzoic acid when incubated with *cyclohexanecarboxylic acid* (Table 3). In both species liver slices were more efficient than kidney slices and the rabbit tissues were more active than the corresponding rat tissues in effecting the aromatization. None of the other rabbit tissues tested (Table 3) was found to yield other than small quantities of benzoic acid. (The

Table 3. *Formation of benzoic acid from cyclohexanecarboxylic acid by slices of various tissues*(Incubation at 37° in O₂; slices suspended in phosphate-Ringer solution.)

Animal	Tissue	Tissue wt. (mg.)	Incubation time (hr.)	Benzoic acid found (mg.)		
				Tissue only	Tissue + cyclohexanecarboxylate (concn.)	Formed
Rat 1	Liver	300	3	0.00	0.07 (0.005 M)	0.07
Rat 2	Kidney	200	2	0.026	0.040 (0.025 M)	0.014
Rabbit 1	Liver*	300	3	0.034	0.120 (0.004 M)	0.086
	Testis	300	3	0.034	0.034 (0.004 M)	0.00
	Spleen	300	3	0.081	0.082 (0.004 M)	0.001
	Skeletal muscle (mince)	400	3	0.050	0.055 (0.01 M)	0.005
	Blood (defibrinated)	(10 ml.)	3	0.203	0.224 (0.004 M)	0.021
Rabbit 2	Kidney	200	3	0.061	0.099 (0.05 M)	0.038
		200	1.5	0.061	0.083 (0.05 M)	0.022

* For further experiments on cyclohexanecarboxylic acid with rabbit liver see Tables 4-6.

Table 4. *Effect of incubation conditions on benzoic acid formation from cyclohexanecarboxylic acid by rabbit-liver slices*(Concn. of HCN, 10⁻³ M. Incubation period, 3 hr. P = phosphate, B = bicarbonate medium.)

Rabbit no.	Medium	cycloHexanecarboxylate concn. (M)	Gas phase	Inhibitor	Benzoic acid at end of experiment	
					Total (mg.)	Extra due to substrate (mg.)
1	P	0.00	O ₂	None	0.03	—
		0.02	O ₂	None	0.15	0.12
		0.004	O ₂	None	0.19	0.16
		0.001	O ₂	None	0.21	0.18
		0.00	N ₂	None	0.05	—
		0.002	N ₂	None	0.06	0.01
		0.001	N ₂	None	0.05	0.00
2	P	0.00	O ₂	None	0.034	—
		0.04	O ₂	None	0.120	0.086
		0.04	O ₂	HCN	0.064	0.030
3	P	0.00	O ₂	None	0.030	—
		0.01	O ₂	None	0.163	0.133
3	B	0.00	O ₂ + CO ₂	None	0.028	—
		0.01	O ₂ + CO ₂	None	0.228	0.200
		0.00	O ₂ + CO ₂	HCN	0.022	—
		0.01	O ₂ + CO ₂	HCN	0.070	0.048
4	P	0.00	O ₂	None	0.025	—
		0.002	O ₂	None	0.159	0.134
4	B	0.00	O ₂ + CO ₂	None	0.023	—
		0.002	O ₂ + CO ₂	None	0.229	0.206

value recorded in Table 3 for blood is doubtful as the final colour was atypical.)

This preliminary survey suggested that rabbit liver was the most suitable tissue with which to study the aromatization reaction, and before extending the investigation to the unsaturated and hydroxy acids an examination was made of the factors which might influence the formation of benzoic acid by rabbit-liver slices from cyclohexanecarboxylic acid.

Effect of incubation conditions. Cyanide powerfully inhibited the formation of benzoic acid by rabbit-liver slices from cyclohexanecarboxylic acid.

The replacement of oxygen by nitrogen in the incubation vessel also prevented aromatization (Table 4). Under aerobic conditions the yield of benzoic acid was slightly higher in a bicarbonate than in a phosphate-buffered Ringer solution (Table 4).

Extent of benzoic acid formation from cyclohexanecarboxylic acid by liver slices from different rabbits. It became apparent early in this work that the livers from different healthy rabbits varied considerably in their ability to produce benzoic acid from cyclohexanecarboxylic acid. As seen in Table 5, the benzoic acid produced in 4 hr. by 300 mg. of rabbit

Table 5. *Production of benzoic acid from cyclohexanecarboxylic acid by rabbit-liver slices*

(Warburg vessels contained: 300 mg. liver slices; 0.1M-phosphate Ringer solution and cyclohexanecarboxylate of stated molarity. Final volume of liquid 2 ml.; incubated at 37° in O₂. *a* And *b* here and in Tables 6 and 7 are estimations on different portions of slices from the same liver.)

Liver no.	Substrate concn. (M)	Incubation time (hr.)	Benzoic acid formed (mg.)	
			(a)	(b)
1	0.01	4	0.096	0.10
2	0.002	4	0.077	0.085
3	0.002	4	0.130	0.164
4	0.01	4	0.137	0.127
5	0.01	4	0.08	0.123
	0.01	8		
6	0.01	4	0.151	0.202
	0.005	4	0.154	0.225
7	0.01	4	0.135	0.135

liver slices varied from 0.077 to 0.225 mg. in different livers; there is some variation between different portions of slices from the same liver.

Effect of substrate concentration on the production of benzoic acid by liver slices. The results in Table 6 show that the production of benzoic acid from cyclohexanecarboxylic acid is nearly independent of substrate concentration between 0.025 and 0.005M. At 0.05M and above, some decline in degree of aromatization was found. The respiration rate of the slices was not noticeably affected by the addition of substrate up to a concentration of 0.1M. There was no autoxidation of cyclohexanecarboxylic acid incubated without tissue, nor was any benzoic acid found (Table 6).

Table 6. *Effect of substrate concentration on the aromatization of cyclohexanecarboxylic acid by rabbit-liver slices*

(Conditions as in Table 5; incubation time 4 hr.)

Substrate concentration (M)	Tissue absent	Benzoic acid found (mg.)	
		Tissue present	
0.0	—	(a)	(b)
0.1	0.01	0.00	0.02
0.05	—	0.14	0.14
0.025	—	0.22	0.18
0.0125	0.00	0.24	0.13
0.01	—	0.21	0.16
0.005	0.01	0.24	0.17

Rate of formation of benzoic acid by tissue slices. Although the formation of benzoic acid from cyclohexanecarboxylic acid by liver slices has been shown to require active respiration, absence of increased respiration in the presence of substrate does not suggest a close connexion between aromatization and the extent of oxygen consumption by the tissue. The oxygen consumption and benzoic acid formation

in presence of cyclohexanecarboxylic acid were therefore determined at intervals during incubation (Fig. 3). It was found that the rate of benzoic acid formation fell off rapidly after 2 hr., whereas the oxygen consumption of the tissue fell only slightly compared with the control.

Fig. 3. Rate of formation of benzoic acid in relation to rabbit-liver slice respiration. 300 mg. slices in 2 ml. 0.1M-phosphate buffer (37° in O₂). ×—×, O₂ absorption, tissue only; ○—○, O₂ absorption, tissue + 0.005M-cyclohexanecarboxylate; extra benzoic acid formed in presence of cyclohexanecarboxylate (□ and ■, duplicate experiments).

Isolation of hippuric acid formed from cyclohexanecarboxylic acid by rabbit-liver slices. The liver of a freshly killed rabbit (wt. 2.8 kg.) was rapidly sliced, connective tissue (5 g.) being discarded. The material was equally divided between two flasks (42 g. in each) and rinsed in 100 ml. bicarbonate-Ringer solution while aerated with oxygen containing 5% carbon dioxide. The rinsing medium in both flasks was replaced by 430 ml. of fresh buffer, and to one of the flasks was added 0.5 ml. 1M-

sodium *cyclohexanecarboxylate*. Oxygen-carbon dioxide mixture was passed through the flasks whilst the latter were gently rocked at 37° for 3 hr. After cooling, the contents of the flasks were treated with 98 % ethanol (645 ml.), stood 1 hr., and filtered.

Colorimetric benzoic acid determinations made on samples (10 ml.) from each of the 1075 ml. filtrates thus obtained, gave the figures: *cyclohexanecarboxylic acid* absent, 1.1 mg.; *cyclohexanecarboxylic acid* present, 33.4 mg.; extra benzoic acid found, 32.2 mg. in the total filtrate.

The tissue retained on the filter was washed with 60 % aqueous ethanol (100 ml.), and the latter combined with the portion of the appropriate filtrate remaining after the foregoing colorimetric estimations. After evaporation *in vacuo* to about 230 ml. each of the concentrates was treated with 8 ml. 2*N*-hydrochloric acid and 1.5 g. sodium tungstate in 3 ml. water. After 1 hr. the precipitated material was removed by filtration and washed with 20 ml. water. The combined filtrates were adjusted to pH 8.5 with approximately 5 ml. 2*N*-sodium hydroxide and concentrated *in vacuo* to about 50 ml. After acidification with 2.5 ml. concentrated hydrochloric acid, the solutions were extracted continuously with ether for 24 hr. The residue remaining after evaporating the ether was recrystallized from 2.5 ml. hot water, yielding 21 mg. of hippuric acid, m.p. 184–185°. The mixed melting point with an authentic specimen (m.p. 187°) was 186–187°. The *p*-nitrobenzyl ester was prepared by refluxing the isolated hippuric acid (20 mg.) for 1 hr. with *p*-nitrobenzyl bromide (35 mg.), *N*-sodium hydroxide (0.12 ml.) and ethanol (0.35 ml.). On cooling *p*-nitrobenzyl hippurate (21 mg.) crystallized, m.p. 131°, raised after one recrystallization from ethanol to 135°, alone or mixed with authentic material. (Found: C, 61.6, H, 4.4; N, 8.2. Calc. for $C_{16}H_{14}O_5N_2$: C, 61.1; H, 4.5; N, 8.9 %.)

Tissue slices with cyclohexene- and hydroxycyclohexene-carboxylic acids as substrates

The marked difference in the capacity of various rabbit livers to aromatize *cyclohexanecarboxylic acid* has already been mentioned. In comparing, therefore, the ability of other substrates to act as precursors of benzoic acid the several compounds were either used as substrates for slices from the same liver or, when working with livers from different animals, *cyclohexanecarboxylic acid* was used as a common reference substance. The amounts of benzoic acid obtained with the three *cyclohexene-carboxylic acids* as substrates (0.01*M*) are shown in Fig. 4. Whilst Δ^1 -*cyclohexenecarboxylic acid* was aromatized to barely half the extent of *cyclohexanecarboxylic acid*, the Δ^3 compound produced almost double the amount of benzoic acid given by that substance. The Δ^2 acid yielded about the same

amount of benzoic acid as the *cyclohexanecarboxylic acid* control.

A number of the hydroxycyclohexanecarboxylic acids was also studied as substrates for aromatization by liver slices. Some reserve is necessary in interpreting the results of the colorimetric benzoic acid estimations in media containing compounds of this class, since they alone gave quite appreciable blank values for benzoic acid. This is probably due to the formation of a *cyclohexenecarboxylic acid* as a result of the dehydrating action of the sulphuric acid during the nitration stage. The unsaturated acids

Fig. 4. Aromatization of *cyclohexane-* and *cyclohexene-carboxylic acids* by rabbit-liver slices. 300 mg. slices incubated 4 hr. in 2 ml. 0.1*M*-phosphate buffer (at 37° in O_2). Substrate concn. 0.01*M*. Open columns, rabbit 1; solid columns, rabbit 2.

have been shown (Dickens & Pearson, 1950) to return appreciable values for benzoic acid in this method unless they are saturated by bromination, and they would therefore be expected to yield some substance which is estimated as benzoic acid under the above conditions. In fact, during the incubation with liver slices the amount of some of the added hydroxy compounds appeared to diminish, presumably by oxidative breakdown (see below). The result of this would be to cause a fall in the 'blank' value due to the hydroxy compound; and this is presumably the reason for the negative values of 'benzoic acid' reported in Table 7. This table, summarizing the results obtained on incubating the hydroxy compounds with rabbit-liver slices, shows, however, that even if the compound blank is ignored entirely, the benzoic acid values are not much

Table 7. *Hydroxycyclohexanecarboxylic acid as substrates for respiring rabbit-liver slices*

(Conditions as in Table 5. Incubation time, 4 hr.; period of measurement of respiration also 4 hr. except where otherwise stated.)

Substrate		O ₂ absorbed (μl.)		Benzoic acid found (mg.)		
Position of substituent	Concn. (M)	Compound absent	Compound present	Compound absent	Compound present	
1-OH	0.008	224	208	0.076	0.068	0.018
	0.005	—	—	0.076	0.090	0.060
<i>cis</i> -2-OH	0.010	576	550	0.016	0.019	0.019
	0.005	576	602	0.016	0.006	0.006
<i>cis</i> -3-OH	0.010	288†	255†	0.006	0.057	0.007
	0.008	288†	254†	0.006	0.061	0.000
<i>cis</i> -4-OH	(a) 0.030	772	787	0.000	0.135	-0.226
		772	730	0.000	0.048	-0.049
		772	756	0.000	0.025	-0.029
	(b) 0.008	248	342	0.008	0.057	-0.024
		248	293	0.008	0.035	-0.013
		772	1000	0.000	0.206	0.016
<i>trans</i> -4-OH	0.010	772	850	0.000	0.074	0.009
	0.005	772	830	0.000	0.050	0.016

* Corrected for amount of 'benzoic acid' as estimated on the quantity of substrate originally present in the vessels (see text).

† O₂ uptake for first 155 min. only.

greater than those in the control without substrate, and after subtraction of the analytical correction for the compound blank they are generally lower than the control and may even be negative as explained above.

Also included in Table 7 are some values for the oxygen consumption of the liver slices with and without the hydroxy compounds as substrates. Further estimations (not included in Table 7) showed that whilst 1-hydroxy-, *cis*-2-hydroxy- and *cis*-3-hydroxy-*cyclohexanecarboxylic* acids had no significant effect, an appreciable increase in respiration was sometimes observed when the substrate was *cis*- or *trans*-4-hydroxy-*cyclohexanecarboxylic* acid (see Table 7).

Tissue homogenates

Rat-liver homogenate. In the initial experiments rat-liver homogenates were used in order to find if a system able rapidly to oxidize fatty acids such as *n*-octanoic could also utilize the *cyclohexane*- and *cyclohexene*-carboxylic acids as substrates, effecting thereby their aromatization to benzoic acid. The advantage to be gained by working with a homogenate instead of liver slices is clear from Fig. 5 which shows the relatively small increase in the respiration rate of slices with added *n*-octanoate in comparison with the homogenate. In these experiments the primary intention was to find an enzyme system capable of aromatizing *cyclohexane* derivatives, and therefore no attempt was made to purify the crude homogenate as was done, for example, in the cyclophorase or mitochondrial fatty acid oxidizing preparations used by Graffin & Green (1948) and

Schneider (1948). Such purification might have involved the loss of a component of the system which oxidizes the *cyclohexane* derivatives.

Fig. 5. Time course of oxidation of *n*-octanoate by rat-liver slices and homogenate. Slices: 300 mg. in 2 ml. 0.1M-phosphate buffer (at 37° in O₂), □- - -□, tissue only; ■-■, tissue + 0.001 M-*n*-octanoate; O₂ uptake plotted for 100 mg. wet tissue. Homogenate: conditions as described in 'Methods', ○- - -○, homogenate only; ●-●, homogenate + 0.001 M-*n*-octanoate.

However, even the crude rat-liver homogenates, though their oxidizing activity towards normal

Table 8. *Oxygen uptake and benzoic acid formation in rat-liver homogenates in presence of various substrates*
(Experimental details as described under 'Methods'.)

A	B	Oxygen (μ l.) absorbed in 30 min.				G
		C	D	E	F	
Acid	Concn. (M)	Tissue only	+ <i>n</i> -octanoate (0.001 M)	+ compound as in columns A and B	+ compound + <i>n</i> -octanoate (0.001 M)	Benzoic acid formed (columns E-C, mg.)
None	—	73 (av.)	192 (av.)	—	—	—
Benzoic	0.001	95	229	62	197	—
<i>cyclo</i> Hexanecarboxylic	{ 0.001 0.003	77 77	190 190	48 57	160 117	0.00 N.D.
Hexahydrohippuric	0.001	95	229	69	203	0.00
Δ^1 - <i>cyclo</i> Hexenecarboxylic	0.001	69	205	54	202	0.00
Δ^2 - <i>cyclo</i> Hexenecarboxylic	{ 0.001 0.003	69 (80)	160 198	53 53	163 N.D.	N.D. 0.017
Δ^3 - <i>cyclo</i> Hexenecarboxylic	0.003	(83)	210	87	116	0.00
1-Hydroxycyclohexane-carboxylic	0.001	49	185	39	178	N.D.
<i>cis</i> -2-Hydroxycyclohexane-carboxylic	0.003	80	202	44	195	0.00
<i>trans</i> -2-Hydroxycyclohexane-carboxylic	0.003	80	202	69	204	0.00
<i>cis</i> -3-Hydroxycyclohexane-carboxylic	0.003	90	206	80	189	N.D.
<i>trans</i> -3-Hydroxycyclohexane-carboxylic	0.003	90	206	80	206	N.D.
<i>cis</i> -4-Hydroxycyclohexane-carboxylic	{ 0.003 0.010	78 78	203 203	103 124	N.D. 244	- 0.030 - 0.065
<i>trans</i> -4-Hydroxycyclohexane-carboxylic	{ 0.003 0.010	78 78	203 203	72 71	N.D. 195	N.D. - 0.045
<i>cyclo</i> Hexylacetic	{ 0.001 0.003	77 77	199 199	54 52	84 69	N.D. 0.0
Palmitic	0.001	60	156	78	N.D.	N.D.
<i>n</i> -Butyric	0.005	65	204	103	226	N.D.
<i>iso</i> Butyric	0.005	65	204	53	199	N.D.

N.D. = Not determined; values in parentheses are estimated by average.

fatty acids was comparable with that of the above authors' preparations, showed no comparable degree of activity in aromatizing the *cyclo*hexane derivatives. In fact benzoic acid, if found at all, was present in amounts too low to be estimated in the incubation mixture with all the compounds tested, which are included in Table 8. Again the disappearance of the 4-hydroxy compounds is indicated by the negative 'benzoic acid' values.

The effect of *cyclo*hexane-, hydroxycyclohexane- and *cyclo*hexene-carboxylic acids on homogenate respiration is also shown in Table 8. The fatty acid oxidizing capacity has been taken to be measured by the increased oxygen consumption in the presence of *n*-octanoic acid (columns D-C, Table 8).

Most of the compounds were found to depress endogenous respiration of the homogenate without markedly interfering with the extra oxygen uptake in the presence of *n*-octanoic acid. Octanoate oxidation, however, was strongly depressed in presence of

*cyclo*hexanecarboxylic acid and Δ^3 -*cyclo*hexene-carboxylic acid (0.003 M). *cyclo*Hexylacetic acid is an even stronger inhibitor and in 0.001 and 0.003 M concentration depressed oxygen uptake by 58 and 65 % respectively. Since it had much less effect on the liver respiration in absence of octanoate (depression 30-32 %), it is evidently a specific inhibitor of fatty acid oxidation (cf. inhibitory effect of 2-phenylbutyric acid mentioned by Grafflin & Green, 1948).

On the other hand *cis*- but not *trans*-4-hydroxycyclohexanecarboxylic acid, particularly in higher concentration, increased the respiration of rat-liver homogenate in absence of *n*-octanoate, and the *cis* isomer also increased the total oxygen uptake of the homogenate in presence of *n*-octanoate (Table 8).

Previous workers (Grafflin & Green, 1948) have found with fairly crude kidney homogenates that the branching of the alkyl chain of a fatty acid frequently hinders oxidation beyond the substituted

carbon atom, which they considered led to the accumulation of α - or β -alkyl fatty acids. Liver homogenates unless too far purified were, however, able to oxidize branched-chain acids (e.g. *isocaproic* and *isobutyric*) to carbon dioxide and water (Grafflin & Green, 1948, footnote p. 106).

In the present work the straight-chain fatty acids, *n*-butyric and palmitic, both increased the respiration of the rat-liver homogenate (Table 8). On the other hand, 0.005M-*isobutyric* acid (which may be considered to have a structural resemblance to *cyclohexanecarboxylic* acid) was apparently not oxidized by our preparation, being perhaps slightly inhibitory to oxidation with or without added *n*-octanoic acid.

Rabbit-liver homogenates. Although no benzoic acid formation was found using rat-liver homogenates the possibility still existed that a rabbit-liver preparation might be more active, since slices of this tissue have been shown above to be more effective in aromatizing *cyclohexanecarboxylic* acid.

The oxidation of *n*-octanoic acid by rabbit-liver homogenate was more variable, somewhat slower and more sustained than by the corresponding preparation from rat liver. *cycloHexanecarboxylic* acid (0.003M) only slightly decreased homogenate respiration when used as the sole substrate, but it almost completely inhibited the ability of the homogenate to oxidize *n*-octanoic acid (Fig. 6). Similarly, Δ^1 - and Δ^2 -*cyclohexanecarboxylic* acids and hexahydrohippuric acid all failed to act as oxidizable substrates—the respiration curves being almost identical with that for *cyclohexanecarboxylic* acid shown in Fig. 6. Only traces of benzoic acid were found after the incubation of any of these substances (Table 9);

these are too near the experimental limit of the estimation method to be significant. It is clear, however, that neither the rabbit-liver nor rat-liver

Fig. 6. Respiration of rabbit-liver homogenate in the presence of *cyclohexanecarboxylate*. Conditions as in 'Methods'. $\times-\times$, homogenate alone; $\bullet-\bullet-\bullet$, homogenate + 0.001M-*n*-octanoate; $\blacksquare-\blacksquare-\blacksquare$, homogenate + 0.003M-*cyclohexanecarboxylate*; $\circ-\circ-\circ$, homogenate + 0.001M-*n*-octanoate + 0.003M-*cyclohexanecarboxylate*.

fortified homogenates were able to increase the aromatization reaction as they do the oxidation of straight-chain fatty acids in comparison with tissue slices (cf. Fig. 5).

Table 9. *Rabbit-liver homogenate: effect of cyclohexane- and cyclohexene-carboxylic acids on respiration*

Substrate	O ₂ absorbed (μl.)	Benzoic acid formed during incubation (mg.)
Rabbit 1: incubation period 1 hr.		
None	75	0.00
0.001M- <i>n</i> -octanoate	114	N.D.
0.003M- <i>cyclohexanecarboxylate</i>	45	0.03
0.003M- Δ^1 - <i>cyclohexenecarboxylate</i>	67	0.01
0.003M- Δ^2 - <i>cyclohexenecarboxylate</i>	62	0.03
0.003M-hexahydrohippurate	60	0.01
Rabbit 2: incubation period 2 hr.		
None	115	0.00
0.001M- <i>n</i> -octanoate	267	N.D.
0.001M- <i>n</i> -octanoate + 0.003M- <i>cyclohexanecarboxylate</i>	115	0.03
0.003M- <i>cyclohexanecarboxylate</i>	N.D.	0.02
Rabbit 3: incubation period 2 hr.		
None	159	0.00
0.001M- <i>n</i> -octanoate	330; 306	N.D.
0.001M- <i>n</i> -octanoate + 0.003M- <i>cyclohexanecarboxylate</i>	158	0.03
0.003M- <i>cyclohexanecarboxylate</i>	152; 140	0.02

N.D. means not determined.

Table 10. *Urinary excretion of hippuric acid in man following oral administration of laevoquinic acid*

(Dose of free acid 6.0 g., given at '0 hr'.)

Subject	Period (hr.)	Vol. urine (ml.)	Hippuric acid isolated (g.)		Hippuric acid isolated, increase over blank, as percentage of theoretical	
			Nearly pure	Pure	Nearly pure	Pure
C.T.B.	-24 to 0	2550	1.532	0.910	74	68
	0 to 24	2785	5.612	4.723		
F.D.	0 to 6	445	1.482	Total pure 3.330	77*	43*
	6 to 12	670	2.000			
	12 to 24	690	2.310			
		Total 1805				

* Corrections for previous 24 hr. excretion based on the above experiment have been applied.

The metabolism of quinic acid in man and other mammals

Man. Two healthy males received by mouth in water 6 g. laevoquinic acid of purity checked by melting point (164°) and analysis. (Found: C, 43.9; H, 6.4. Calc. for $C_7H_{12}O_6$: C, 43.8; H, 6.3 %.)

Urine was collected at intervals before and after this dose and the hippuric acid extracted from the neutralized, concentrated urine as described under Methods. After recrystallization it had m.p. 186°, unchanged on admixture with a genuine specimen. The results (Table 10) show that the increase of nearly pure hippuric acid excretion in the 24 hr. following the dose may amount to about 70 % of the theoretical amount corresponding to the quinic acid given. This excretion is shown by the second experiment of Table 10 to be spread over the 24 hr. period rather evenly, in contrast to the excretion of hippuric acid following the oral administration of benzoic acid in similar dose which, as in the familiar test for hepatic efficiency, is very much more rapid.

Species other than man. In the rabbit (Table 11) negligible residues were obtained in attempts to isolate hippuric acid from the urine after an oral dose of 2 g. quinic acid. Colorimetric estimations similarly showed only a slight increase above normal. In rats a similar result on colorimetric analysis was observed. The guinea pig after subcutaneous injection of quinic acid also showed little increase in the excretion, and the cat even showed a diminished excretion (Table 11). Consequently none of these species can be considered to aromatize quinic acid appreciably.

Tissue slices. In view of the negative results reported above it was not thought necessary to investigate slices of organs from the above species. The liver and kidney of the sheep were studied since the whole sheep has been stated to aromatize quinic acid (Vasiliu *et al.* 1940).

Slices (300 mg. fresh wt.) of liver and kidney of a recently killed sheep were incubated aerobically as usual in the presence of quinic acid concentrations

from 0.04 to 0.005M. The amounts of benzoic acid, colorimetrically estimated after incubation, were negligible (0.0004 mg.) with both tissues.

Table 11. *Urinary excretion of hippuric (or benzoic) acid in animals following administration of laevoquinic acid**Rabbits*

i, By stomach tube 2 g. quinic acid to each of two rabbits. Urine passed in next 24 hr. evaporated and extracted as usual. Yield of non-crystalline residues, 22 mg., 87 mg.
ii, Another rabbit, as above, dose 1.9 g. quinic acid at 0 hr.:

Colorimetric estimations

Period (hr.)	Vol. urine (ml.)	Benzoic acid (mg.)
-24 to 0	252	15.8
0 to 24	195	45.7

Rats

(2 g. quinic acid by stomach tube distributed among 6 rats. Pooled urine collected and analysed colorimetrically.)

Period (hr.)	Vol. urine (ml.)	Benzoic acid (mg.)
-24 to 0	77	45.4
0 to 21	64	83.4
21 to 45	60	54.0

Guinea pigs

(1.874 g. quinic acid (as neutral Na salt) was injected subcutaneously, the total dose being distributed among six guinea pigs. Pooled urine analysed as above.)

Period (hr.)	Vol. urine (ml.)	Benzoic acid (mg.)
-24 to 0	316	74.4
0 to 24	415	112.0

Cats

(Two cats each received 2 g. quinic acid as a subcutaneous injection of neutral Na salt.)

Period (hr.)	Vol. urine (ml.)	Benzoic acid (mg.)
Cat A -24 to 0	355	31.9
0 to 24	360	9.0
Cat B -24 to 0	None passed	—
0 to 24	105	8.1

*Fatty acid dehydrogenase and cyclohexane
carboxylic acid*

A fatty acid dehydrogenase preparation was made as described by Annau (1940) and Annau, Eperjessy & Felszeghy (1942) from horse liver. From the red extract obtained after heating briefly to 60° (Annau, 1940), the fraction obtained by 0.4 saturation with ammonium sulphate was rejected and active fractions obtained by successive 0.5 saturation ('Lang enzyme', see Lang, 1939) and 0.6 saturation ('Annau enzyme'). The clear red supernatant fluid from the latter precipitate was brought to 0.7 saturation with ammonium sulphate when a further precipitate ('authors' enzyme') was obtained, of which the water-soluble part was used without dialysis in the experiment reported below.

All the above preparations, and also that of Burton (1948), were found to be somewhat unstable and generally far from satisfactory, especially as the amount of 'coenzyme' (adenylic acid, hypoxanthine, cozymase) used was in several cases found to be sufficient to act as a substrate for the reduction of a considerable part of the methylene blue employed as hydrogen acceptor. Many of these preparations contain active xanthine oxidase; a fact which does not seem to have been sufficiently emphasized in the publications cited.

(1937) and Bernhard & Caffisch-Weill (1945). In the dog 45–59 % of the dose was aromatized, in man 55–91 %, and in two rabbits studied very variable results were obtained; 93.5 % conversion in one animal, but only a non-crystallizable oil could be isolated from the urine of another rabbit.

The results given above (Table 1) for this reaction in the rabbit show a more consistent behaviour in our animals. The basal excretion of benzoic acid (mainly as hippuric acid) in the rabbit maintained on a diet of cabbage only is favourably low and reasonably constant. After subcutaneous injection of amounts of cyclohexanecarboxylic acid within the range of 0.5–2.0 g., extra hippuric acid equivalent to 40–55 % of the dose is regularly excreted in the urine (cf. Table 1). (Similar results were observed after oral administration in several unpublished experiments.) Consequently, the rabbit is in our experience a very suitable species for this investigation. The rat, on the contrary, excretes a very much lower percentage (4 %) of benzoic acid following the administration of cyclohexanecarboxylic acid (cf. Table 2). A marked species specificity has frequently been observed by us in these aromatization reactions, as, for example, in the behaviour of different species towards quinic acid.

In the rabbit the excretion of subcutaneously injected sodium benzoate as urinary hippuric acid is

Table 12. *Action of fatty acid dehydrogenase from horse liver on cyclohexanecarboxylate, palmitate and laurate*

(Each Thunberg tube contained in the main part 1.5 ml. 0.3M-potassium phosphate buffer, pH 7.45, or solution of the substrate (0.02M) in the same buffer adjusted to same pH; 1.0 ml. 'authors' enzyme' (see text); 0.15 ml. adenylic acid solution in above buffer or 0.15 ml. buffer only. The hollow stopper contained 0.2 ml. methylene blue 0.05 %; this was added after evacuation, filling with nitrogen and re-evacuation of the tubes, followed by 5 min. equilibration in the thermostat (temp. 37°). In some experiments 0.004M-cyclohexanecarboxylate was used without any alteration of the reduction time.)

Substrate	Palmitate		Laurate		cycloHexane- carboxylate		None added	
Adenosine-5-phosphoric acid (40 µg.)	-	+	-	+	-	+	-	+
Decolorization time (t, min.)	23	21	40	36	43	44.5	38	43.5
1/t (mean)	0.045		0.027		0.023		0.025	

The results of action of the 0.7 saturation fraction on palmitate, laurate and cyclohexanecarboxylate are shown in Table 12. With the preparation used the rate of reduction of methylene blue was increased in presence of palmitate, possibly very slightly so by laurate, and not significantly affected by cyclohexanecarboxylate. Since this work was completed another fatty acid dehydrogenase preparation has also been found to be inactive towards cyclohexanecarboxylate by Fantl & Lincoln (1949).

DISCUSSION

The urinary excretion of benzoic acid following the administration of cyclohexanecarboxylic acid to men, dogs and rabbits was studied by Bernhard

also favourable (Table 1), about 70 % of a similar dose being excreted in this form in the subsequent 24 hr. This agrees well with the experience of Bray *et al.* (1946), who recovered on the average 77 % of orally administered sodium benzoate as hippuric acid and 11 % as glucuronide from rabbit urine. We have not distinguished between these two excretion products in our experiments, since the main object was to determine the degree of aromatization of the cyclohexane derivatives.

The rapid rate of aromatization of cyclohexanecarboxylic acid in the rabbit is shown not only by the fact that excretion of the benzoic acid derived from it is almost complete in 24 hr. (Table 1), but also by the rapid rise in the level of blood benzoic acid shown in Fig. 2. 2–4 hr. after the injection of an amount of

cyclohexanecarboxylic acid equivalent to about 1 g. benzoic acid, the basal level is raised by a factor of 3–4.5, from which a nearly normal value is regained after about 24 hr. Whether this circulating benzoic acid is free or combined has not been investigated.

Bernhard's extensive researches on aromatization left unsolved two major points: the nature of the intermediate products by way of which the benzenoid ring arises from the fully saturated *cyclohexane* derivatives, and the sites in the animal body at which this reaction occurs. The main object of the present paper was to investigate these two important aspects of biological aromatization.

that none of these is aromatized in the rabbit to anything approaching the extent of aromatization of *cyclohexanecarboxylic acid* itself. The slight degree of aromatization, e.g. with *trans*-4-hydroxy*cyclohexanecarboxylic acid* (11.7 % of dose), may perhaps be due to partial dehydration to the Δ^3 compound within the body. There is also the probability that some of these hydroxy compounds undergo oxidative degradation in the body: this is discussed in detail below under the behaviour of tissue slices and homogenates towards these compounds. In this way they may be removed from the pathways leading to aromatization.

Fig. 7. Hydroxycyclohexanecarboxylic acids and cyclohexenecarboxylic acids studied as possible intermediates in the aromatization of *cyclohexanecarboxylic acid*. Compounds able to exist as optically active isomers are marked with an asterisk *.

The overall result of the aromatization of *cyclohexanecarboxylic acid* is the formation of three unsaturated HC=CH bonds from three —CH₂—CH₂— linkages. In the present work consideration has been given to two of the possible mechanisms for the stepwise formation of these bonds; one a direct process, involving dehydrogenation, the other indirect, in which the double bonds arise by the elimination of a previously introduced substituent together with a hydrogen atom. The substituent considered here is the hydroxyl group whose elimination, together with an atom of hydrogen as water, would lead to the appearance of an unsaturated link. Even simple reactions of this type offer numerous pathways to the aromatization of the *cyclohexane* ring and Fig. 7 shows the possible compounds resulting from the introduction of either one double bond or one hydroxyl group. Subsequent steps in the aromatization might proceed by several alternative routes and a combination of two mechanisms would greatly increase the number of hypothetical intermediates. To mention only one possibility, the further dehydrogenation of the three *cyclohexenecarboxylic acids* could lead to five possible dihydrobenzoic acids.

All seven monohydroxycyclohexanecarboxylic acids shown in Fig. 7 were injected into rabbits with results as shown in Table 1 and Fig. 1. It is evident

On the other hand, each of the three *cyclohexenecarboxylic acids* yields much higher amounts of benzoic acid in the rabbit (Table 1 and Fig. 1), the percentage conversion of the Δ^1 and Δ^3 compounds being 60–70 %, i.e. equal to or greater than that of *cyclohexanecarboxylic acid* itself. (The fate of these compounds in isolated tissue is described below.) Thus on the evidence derived from the intact rabbit, while the Δ^2 -*cyclohexenecarboxylic acid* appears to be aromatized too slowly to be an intermediate (cf. Table 1), either the Δ^1 - or Δ^3 -*cyclohexenecarboxylic acid* could be a possible intermediate in this reaction.

It is to be noted that the hydroxy derivatives of *cyclohexanecarboxylic acid* used were all optically inactive preparations, being the synthetic mixtures of the two forms, but even if the body were selectively to aromatize only one isomer, the resulting benzoic acid should have been easily detectable since the doses given were fairly large.

The metabolism of *laevoquinic acid* presents some points of special interest. In our experience the formation of benzoic acid from this compound is confined to man among the species studied, and it was not appreciably converted in rabbit, rat, guinea pig or cat. However, the conversion in man is remarkably high showing that at least this particular polyhydroxy derivative of *cyclohexanecarboxylic acid* is

capable of undergoing biological reactions which must involve dehydrations and reduction to yield benzoic acid. The mechanism of this is at present quite unknown. It cannot be excluded that the intestinal flora are responsible.

Aromatization in tissue slices and homogenates

In order to obtain further evidence on the site and nature of the aromatization reaction we have investigated the behaviour of isolated tissues in the form of slices and homogenates. Bernhard & Caffish-Weill (1945, p. 1703) mention that with minced liver, kidney and muscle of dogs and horses they did not succeed in obtaining dehydrogenation of hexahydrobenzoic acid, and this is apparently the only instance of the investigation of aromatization hitherto made under these conditions. However, it was shown (Dickens, 1947) that sliced liver and kidney of the rabbit are able to effect this aromatization *in vitro*, and more detailed information is given in the present paper. Table 3 includes a number of rabbit tissues of which only the liver and to a less extent the kidney were highly active, the values for rabbit-liver slices given in Tables 4 and 5 being more typical than the unusually low figure cited in Table 3. Rabbit blood even in 10 ml. amounts gave only slight and rather dubious aromatization (see Experimental section), and here the result is perhaps confused by the considerable content of preformed colour-forming substance (*p*-aminobenzoic acid?) present in blood. Consequently the liver appears on the present evidence to be the main site of aromatization in the animal body.

The species difference noted in the whole animal is again evident in a comparison of rabbit- and rat-liver slices, the latter being less active in aromatization just as the whole rat is less efficient in this respect (cf. Tables 2, 3, 4 and 5), though the difference is less marked in the isolated tissue.

The aromatization reaction is shown by the experiments of Table 4 to be an aerobic process in rabbit-liver slices, being strongly inhibited or completely absent in non-respiring slices, whether tissue respiration is checked by anaerobic conditions or by the presence of cyanide. It is somewhat dependent on the suspension medium, being rather greater in presence of bicarbonate than in phosphate-buffered Ringer solution. As regards the effect of substrate concentration, the early results reported by Dickens (1947) have not been repeated in subsequent experiments (Table 6), although the higher concentrations of cyclohexanecarboxylic acid still appears to be occasionally slightly inhibitory. The reason for these variations is not clear.

The formation of hippuric acid from cyclohexanecarboxylic acid in slices of rabbit liver has been confirmed by isolation of the product, as has been described in detail in the Experimental section.

There can, therefore, be no doubt that both the aromatization and conjugation with glycine do actually occur in isolated liver tissue, but whether the conjugation is subsequent to or precedes the aromatization is not known, though the former assumption seems more probable. However, hexahydrohippuric acid and hexahydrobenzamide have also been found to yield corresponding aromatic compounds in rabbit-liver slices (Dickens, 1947).

The time course of the aromatization reaction shown in Fig. 3 indicates that the reaction rate falls with time of incubation much more rapidly than the tissue respiration. Whether this is due to the accumulation of hippuric acid in the medium has not been ascertained.

We may therefore conclude that aromatization is a progressive process associated with active respiration of the tissue and, in rabbit liver at least, it is accompanied by conjugation of the benzoic acid with tissue glycine to yield hippuric acid.

Further light on the mechanism of aromatization is provided by the study of the action of tissue slices on the hydroxycyclohexane derivatives and of the three unsaturated compounds shown in Fig. 7. As in the whole rabbit, none of the seven hydroxy derivatives shown in Table 7 yields more than traces of benzoic acid when incubated with rabbit-liver slices. On the other hand, the experiments represented in Fig. 4 show that while Δ^1 -cyclohexenecarboxylic acid is aromatized under these conditions at only about one-third to one-half of the rate of cyclohexanecarboxylic acid, the Δ^2 compound almost attains the same rate as the fully saturated compound. But by far the most vigorously aromatized of these compounds is the Δ^3 -cyclohexenecarboxylic acid, which in presence of respiring rabbit-liver slices yields benzoic acid at a rate much greater than cyclohexanecarboxylic acid in the same liver preparation.

If the criterion be adopted that an intermediate in metabolism should be capable of transformation to the end product at a rate at least equal to that of the original substrate, Δ^3 -cyclohexenecarboxylic acid, alone among the compounds studied, qualifies for the role of intermediate in the aromatization of cyclohexanecarboxylate. This is certainly true of the behaviour in liver slices; in the whole animal, however, either Δ^1 - or Δ^3 -cyclohexenecarboxylic acid could be considered eligible. Possibly elsewhere than in liver the animal body can attack the Δ^1 compound, though we have no direct evidence of this. Since, however, the Δ^3 compound is the only one which both the whole animal and the liver slice are able to aromatize at a sufficient rate, it appears on the present evidence to be the most probable primary intermediate in this aromatization reaction. Of the nature of this dehydrogenation of cyclohexanecarboxylic acid to the Δ^3 compound we have

as yet little information. The known fatty acid dehydrogenase preparations of animal origin do not appreciably dehydrogenate *cyclohexanecarboxylic acid*, as we have shown, and as Fantl & Lincoln (1949) have independently found also. These dehydrogenase preparations, however, are known to be somewhat restricted in their attack to certain long-chain fatty acids.

We therefore turned to the use of tissue homogenates which are known to be capable of oxidizing a wide range of fatty acids, to see if these preparations can effect the aromatization. Rat-liver homogenates, which have been more extensively investigated hitherto than rabbit-liver preparations, gave no detectable amount of benzoic acid from any of the substrates used (Table 8) which included *cyclohexanecarboxylic acid*, its seven monohydroxy derivatives, and all three mono-unsaturated derivatives. Hence even these preparations which are exceedingly active in oxidizing fatty acids such as octanoic acid do not have the ability to aromatize. This is not due to the use of rat- instead of rabbit-liver tissue, for the latter when homogenized similarly had almost negligible aromatizing activity (Table 9). This dependence of the aromatization reaction on the integrity of cell structure is a difficulty in the way of more detailed investigation of the enzyme mechanism concerned in aromatization.

SUMMARY

1. The conversion of *cyclohexanecarboxylic acid* to benzoic acid, with urinary excretion of hippuric acid, has been studied in the intact rabbit. The observations of Bernhard (1937, 1938*a, b*) have been confirmed, but the excretion in our animals was more consistent, being equivalent to between 38–55 % of the dose. In the rat it is lower.

2. The intact rabbit is unable to aromatize appreciably any of the seven possible monohydroxy derivatives of *cyclohexanecarboxylic acid*, with the exception that *trans*-4-hydroxycyclohexanecarboxylic acid is feebly (10 %) aromatized.

3. The intact rabbit aromatizes Δ^1 - and Δ^3 -*cyclohexenecarboxylic acids* to an extent similar to that of *cyclohexanecarboxylic acid*. The Δ^2 -acid is somewhat less aromatized.

4. The reaction has been studied in excised tissues by means of tissue slices, homogenates and extracts.

5. Of the rabbit tissues examined in the form of slices, the liver was most active in aromatization and the kidney next in activity. The effect of varying the conditions of incubation was studied, and the product formed by rabbit-liver slices from *cyclohexanecarboxylic acid* was proved to be hippuric acid by isolation.

6. In agreement with the behaviour of the intact rabbit, rabbit-liver slices do not form appreciable quantities of benzoic acid from any of the monohydroxycyclohexanecarboxylic acids.

7. Rabbit-liver slices are able to aromatize Δ^3 -*cyclohexenecarboxylic acid* to an extent greater than that observed with *cyclohexanecarboxylic acid*. The Δ^1 compound was much less rapidly converted to benzoic acid, while the Δ^2 compound occupied a middle position between the other two *cyclohexene* derivatives. Since the Δ^2 compound in the whole animal is less readily aromatized than either the Δ^1 - or Δ^3 -*cyclohexenecarboxylic acid*, the latter is the only one of the three unsaturated compounds which is aromatized at a sufficient rate both in the whole animal and in liver slices for it to be a possible intermediate.

8. Homogenates of rat liver which vigorously oxidize fatty acids do not aromatize *cyclohexanecarboxylic acid*, or any of its monohydroxy or mono-unsaturated derivatives to any considerable extent. Only the *cis*-4-hydroxy derivative was vigorously oxidized, whereas most of the other hydroxy compounds depressed the oxygen uptake of the homogenate. *cyclohexanecarboxylic acid* and its Δ^3 derivative (0.003*M*) depressed the oxidation of octanoate by liver homogenate. Rabbit-liver homogenates behaved similarly.

9. Fatty acid dehydrogenase preparations prepared from liver tissue were not able to dehydrogenate *cyclohexanecarboxylic acid*.

10. Quinic acid (1:3:4:5-tetrahydroxycyclohexanecarboxylic acid) is converted to hippuric acid in good yield by man, but not by the other species in which it was tested.

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A Note on the Racemization of Serine

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According to Daft & Coghill (1931), the successful preparation of DL-serine from sericin by the method of Cramer (1865) depended on the intermediate mixture of serine and other amino-acids being kept for 1 hr. at room temperature in the presence of baryta at pH 9 before the final crystallization stages were carried out. These authors believed that the baryta treatment caused racemization of the serine, but no experimental evidence was produced in support of this idea. In view of the resistance to racemization by alkali observed in the case of certain

amino-acids, it seemed remarkable that serine should be so labile. L-Tyrosine, for example, is completely racemized only after 30 hr. boiling with 5N-sodium hydroxide (Gunness, Dwyer & Stokes, 1946). L-Serine was required for certain investigations in this laboratory, and therefore it was a matter of practical importance as well as of theoretical interest to study the effect of alkali in more detail.

The results obtained under various conditions are shown in Table 1. It will be seen that even in the

Table 1. *Optical rotation and racemization studies of D-serine and D-alanine at various pH values*

Amino-acid	$[\alpha]_D^{20}$	Solvent	<i>c</i>	Exp. no.	Initial $[\alpha]_D$ in NaOH† solution*	pH	Duration of racemization exp. (hr.)	Temp. (°)	Final $[\alpha]_D$ of solution*
Serine	$+6.65 \pm 0.05^\circ$	Water	9.7	1	$+6.4^\circ \pm 0.3^\circ$ (18°; <i>c</i> , 1.0)	8.9	192	18	$+6.4^\circ \pm 0.3^\circ$ (18°; <i>c</i> , 1.0)
				2	$+4.6^\circ \pm 0.3^\circ$ (18°; <i>c</i> , 0.9)	9.9	18	18	$+4.6^\circ \pm 0.3^\circ$ (18°; <i>c</i> , 0.9)
							2.25	100	$+4.6^\circ \pm 0.3^\circ$ (18°; <i>c</i> , 0.9)
				3	$+3.0^\circ \pm 0.1^\circ$ (19°; <i>c</i> , 10.5)	11.4	2.5	18	$+3.0^\circ \pm 0.1^\circ$ (18°; <i>c</i> , 10.5)
							4.25	105	$+2.7^\circ \pm 0.1^\circ$ (18°; <i>c</i> , 10.5)
							17	105	$+1.9^\circ \pm 0.1^\circ$ (18°; <i>c</i> , 10.5)
Alanine	$-10.0^\circ \pm 0.2^\circ$	N-HCl	5.8	4	$+3.2^\circ \pm 0.1^\circ$ (19°; <i>c</i> , 10.0)	> 13	22	18	$+3.0^\circ \pm 0.1^\circ$ (18°; <i>c</i> , 10.0)
				5†	$+3.4^\circ \pm 0.1^\circ$ (20°; <i>c</i> , 4.3)	10	18	18	$+3.4^\circ \pm 0.1^\circ$ (20°; <i>c</i> , 4.3)
				6	$-3.52^\circ \pm 0.1^\circ$ (20°; <i>c</i> , 8.0)	12.4	16	105	$-3.52^\circ \pm 0.1^\circ$ (20°; <i>c</i> , 8.0)
	$-10.7^\circ \pm 0.2^\circ$	N-HCl	5.8	7	$-3.95^\circ \pm 0.1^\circ$ (20°; <i>c</i> , 7.3)	13.4	16	105	$-3.95^\circ \pm 0.1^\circ$ (20°; <i>c</i> , 7.3)

* *t* and *c* values in parentheses.

† Exp. no. 5, Ba(OH)₂ and not NaOH was used.