Aromatization of Cyclohexanecarboxylic Acid

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SUMMARY
An enzyme which catalyzes the aromatization of cyclohexanecarboxylic acid has been isolated from whole liver as well as from liver mitochondria. Incubation of the soluble, partially purified enzyme with cyclohexanecarboxyl-CoA produces cyclohexene-1-carboxyl-CoA, benzoyl-CoA, and a polar compound which has not been further characterized.

All three isomers of cyclohexanecarboxyl-CoA are converted to benzoyl-CoA by the liver enzyme, but evidence is presented indicating that only cyclohexene-1-carboxyl-CoA lies on the path from cyclohexanecarboxyl-CoA to benzoyl-CoA.

Aromatization takes place either in air or anaerobically in the presence of artificial electron acceptors. The enzyme system is inactivated by low pH and activity is restored by the addition of flavin adenine dinucleotide.

In 1911, Friedmann showed that the injection of cyclohexanecarboxylic acid increases the urinary excretion of hippuric acid in dogs (1). Later, Bernhard (2, 3) provided evidence for the aromatization of cyclohexane derivatives containing either a ring-bound carboxyl group or substituents which are convertible to a ring-bound carboxyl group. Bernhard also established that the administration of deuterated cyclohexanecarboxylic acid leads to the excretion of labeled hippuric acid (4).

Dickens (5) first showed the conversion of cyclohexanecarboxylic acid to hippuric acid in a system in vitro. In his experiments with rabbit liver slices, the aromatization was shown to require oxygen and to be inhibited strongly by hydrogen cyanide (6, 7). Dickens further found that the three isomeric cyclohexanecarboxylic acids were also converted to hippuric or benzoic acid, but that none of the seven isomeric optically inactive hexenecarboxylic acids were also converted to hippuric or benzoic acid (7).

Aromatization takes place in air or anaerobically in the presence of artificial electron acceptors. The enzyme system is inactivated by low pH and activity is restored by the addition of flavin adenine dinucleotide.

EXPERIMENTAL PROCEDURE
Guinea pig liver acetone powder was purchased from Pentex Corporation. CoA (Boehringer, 79.9% by assay), α-ketoglutaric acid, glycine, FAD, FMN, phenazine methosulfate, and catalase (Boehringer) were obtained from Calbiochem. ATP and horse heart cytochrome c were purchased from Sigma. Diazal (N-methyl-N-nitroso-p-toluenesulfonamide) was obtained from Aldrich. Radioactive chemicals were purchased from New England Nuclear.

Materials for Chromatography—Aluminum Cyt gel was the generous gift of Dr. Ephraim Levin. Hydroxyapatite gel (Hypatite C) and silicic acid (Unil, 100 to 300 mesh) were purchased from Clarkson.

Techniques—Infrared spectra of the isomeric cyclohexanecarboxylic acids and of cyclohexene-1-carbonitrile were taken by placing the compounds between NaCl plates. For the spectral analysis of other compounds, KBr pellets were used. The instrument was a Perkin-Elmer Infracord. Gas-liquid chromatography was performed on a Research Specialties Series 600 instrument equipped with a model B600 ionization detector. Argon was the carrier gas. Radioactivity was determined with a Packard model 314 CX liquid scintillation counter by the usual techniques.

CoA Esters—These were synthesized by the method of Goldman and Vagelos (9). For the synthesis of cyclohexene-1-carboxyl-CoA, a 5-fold molar excess of mixed anhydride was added to the CoA solution in one portion rather than dropwise in order.

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† Holder of a Postdoctoral Fellowship from the Division of General Medical Sciences, National Institutes of Health.
to minimize sulfhydryl addition of CoA across the double bond of the olefinic acid. Cyclohexene-2-carboxyl-CoA was isolated in an atmosphere of helium to prevent autoxidation of this labile compound to benzoyl-CoA. The thioester was stored under helium at -20°.

Cyclohexene-1-carboxylic Acid and Cyclohexene-1-carboxylic Acid-7-14C—Cyclohexanonecarboxyhydrin prepared either with KCN or K14CN by the method of Fieser (10) was converted to cyclohexene-1-carboxylic acid by treatment with POCl₃ according to the method of Chakravarti (11). Hydrolysis of the nitrile by refluxing for 3 hours with approximately 10 volumes of a solution composed of equal volumes of water, 36% H₂SO₄, and glacial acetic acid yielded cyclohexene-1-carboxylic acid. The free acid was purified either by crystallization from n-pentane at -70° (nonradioactive acid) or by chromatography on silicic acid (radioactive acid). The nonradioactive acid melted at 34-38° (recorded (12), 36-37°), and was 99% pure as judged by gas-liquid chromatography.

The methyl ester of the radioactive acid had the same retention time relative to methyl cyclohexanecarboxylate as nonradioactive methyl cyclohexene-1-carboxylate and was 93% radiochemically pure as determined by coincidence of mass and radioactivity on chromatography. The specific activity of the radioactive acid was 0.16 mC per mmole.

Cyclohexene-2-carboxylic Acid—This was prepared by the method of Boorman and Linstead (13) and purified by recrystallization of the 2-benzyl-2-thiouronium salt (14). The melting point of the free acid could not be raised above -10° (reported, 6°) because the last traces of solvent were difficult to remove. The acid is very volatile and, therefore, cannot be dried thoroughly without large losses by evaporation. The following evidence established with reasonable certainty that the product is cyclohexene-2-carboxylic acid. It was generated by debromination of 2,3-dibromocyclohexanecarboxylic acid (m.p. 164-167°; recorded (13), 167°). On gas-liquid chromatography, the methyl ester of the product had the same retention time as methyl cyclohexene-3-carboxylate, and was separable from both methyl benzoate and methyl cyclohexanecarboxylate. The infrared spectrum was nearly identical with that of cyclohexene-3-carboxylic acid. However, in enzymatic experiments, the behavior of the putative β,γ-unsaturated acid was distinctly different from that of authentic cyclohexene-3-carboxylic acid.

Cyclohexene-3-carboxylic Acid (15)—Silver nitrate, 1.2 g (7.1 mmoles) in 6 ml of water, was mixed with 3.0 g (75 mmoles) of NaOH in 6 ml of water. The suspension of Ag₂O was cooled in an ice bath; 1 ml of cyclohexene-3-carboxaldehyde (Eastman) was added in three equal portions, and the mixture was shaken vigorously for 1 hour. After filtration, the supernatant solution was injected into a stainless steel column (6 feet X 4 inch) packed with 10% diethylene glycol succinate on Chromosorb W. The column temperature was 120° and the pressure was 20 pounds. Enzyme Assays—All components of the incubation mixture except enzyme were placed in a stoppered test tube for 2 to 3 min at 37°. Enzyme that had been warmed to the same temperature was then added to start the reaction. Anaerobic experiments were carried out in Thunberg tubes. The tubes were evacuated and refilled with helium six times before incubation. The enzymatic reaction was stopped by addition of 2 ml of 1 n NaOH and the mixture was heated to 40-50° for ½ hour to hydrolyze thioesters. The solution was then cooled and extracted twice with petroleum ether, which was discarded. After acidification of the aqueous phase with 4 N HCl to a methyl orange end point, the solution was extracted twice with 5-ml aliquots of ether. When necessary, emulsions were broken with methanol. The pooled ether extracts were washed once with 5 ml of 4 N HCl. The products were then extracted from the ether layer with a minimum volume of 1 n NaOH. The aqueous layer was transferred to a 3-ml centrifuge tube. All subsequent operations were designed to minimize losses of the highly volatile reaction products. The contents of the centrifuge tubes were acidified with the minimum quantity of 1 N HF. Six to eight drops of ether were then added, and the stoppered tubes were shaken vigorously. The mixture was allowed to stand in an ice bath for several minutes, after which the aqueous layer was drawn off and discarded. Two drops of freshly prepared saturated diazomethane solution were added to the ether phase and immediately afterward 1 drop of 1 n HF was added to destroy excess CH₂N₂. For gas chromatography, an aliquot of the ether solution was injected into a stainless steel column (6 feet X ½ inch) packed with 10% diethylene glycol succinate on Chromosorb W. The column temperature was 120° and the pressure was 20 pounds. A chromatogram of the various esters is shown in Fig. 1.

![Fig. 1. Gas-liquid chromatography of the methyl esters of cyclohexanecarboxylic acid, cyclohexene-1-carboxylic acid, and benzoic acid on a column (6 feet X ½ inch) of 10% diethylene glycol succinate on Chromosorb W. Temperature, 110°; pressure of carrier argon, 10 pounds.](http://www.jbc.org/)
The peak areas were determined by triangulation. When radioactive substrate was used, the peak fractions were collected in U-tubes cooled in a Dry Ice-acetone mixture, rinsed into liquid scintillation vials with toluene scintillator fluid, and counted.

Validity of Assay—Enzymatic experiments with cyclohexanecarboxyl-CoA-7-14C yielded three peaks identified as the methyl esters of cyclohexanecarboxylic acid, cyclohexene-1-carboxylic acid, and benzoic acid. The recovery of counts from the column was low, ranging from 20 to 30%. In experiments with radioactive substrate, yields of product were calculated by determining the fraction of the total collected radioactivity which was associated with the peak in question. With nonradioactive substrate, the corresponding calculations were based on peak areas. For all compounds to be analyzed, the peak areas were shown to be linearly related to the mass of injected material.

Since recovery of the methyl esters of substrate and of the reaction products was not quantitative, the following experiment was carried out to show that the percentage of recovery was the same for all components of the enzymatic reaction. Cyclohexanecarboxyl-CoA-1-14C, 0.18 μmole containing 98,300 dpm, was incubated for 4 min at 37° with 0.05 ml of mitochondrial enzyme (see below). An ether extract of the free acids was obtained as described previously. At this stage, 90,900 dpm, or 92.4% of the original radioactivity, were recovered. To a 40% aliquot (36,400 dpm) of this ether solution was added 102.5 mg of carrier benzoic acid, and, after crystallization to constant activity, this yielded benzoic acid with a specific activity of 112 dpm per mg, corresponding to a total of 11,500 dpm or 32% conversion of substrate to benzoate. The remainder of the above ether solution was used for gas chromatographic assay. Measurement of the relative peak areas gave a value of 36%, and measurement of the collected radioactive activity gave a value of 34% for the conversion of cyclohexanecarboxylate to benzoate. The satisfactory agreement between the results of two independent assays indicates that in spite of large losses the proportional recoveries of substrate and reaction product are the same in the analytical procedure used.

Protein Determination—Depending on concentration, protein determinations were carried out either by the biuret method, by ultraviolet absorption, or with the Folin-Cöocalteu reagent (17).

Thioesters—Thioesters were determined by the method of Lipmann and Tuttle (18).

Enzyme Isolation—Aromatizing activity could be extracted from acetone powders either of whole guinea pig liver or of guinea pig liver mitochondria. Whole guinea pig liver acetone powder was used initially because it was commercially available. However, it was found that enzyme preparations from this source contained an activity which converted cyclohexene-1-carboxyl-CoA (and, therefore, also cyclohexanecarboxylic acid; see below) to a compound which cochromatographed with benzoic acid on gas-liquid chromatography, but was not identical with benzoate, thereby invalidating the gas-liquid chromatography assay for measuring the aromatization of cyclohexanecarboxyl-CoA. (The gas-liquid chromatography assay remains valid for measuring the conversion of cyclohexanecarboxyl-CoA to cyclohexene-1-carboxyl-CoA.) Extracts of mitochondrial acetone powder contained much less of this activity, relative to the aromatizing activity. For this reason, the mitochondrial enzyme was used for most of the experiments to be described.

Enzyme from Whole Guinea Pig Liver Acetone Powder (Whole Liver Enzyme)—Commercially prepared acetone powder (25 g) of guinea pig liver was extracted by stirring for 1 h at room temperature with 250 ml of buffer (pH 4.1), prepared by mixing 122.8 ml of 0.1 M citric acid solution with 77.2 ml of 0.2 M Na2HPO4·7H2O. The suspension was centrifuged for 20 min at 30,000 × g, the sediment was discarded, and the supernatant fraction was dialyzed overnight against three changes of 0.01 M potassium phosphate buffer (pH 7.1) and then centrifuged to remove precipitated protein. The volume of enzyme solution was 227 ml, and the protein concentration was 10.0 mg per ml. The enzyme preparation is referred to as the whole liver enzyme.

Preparation of Purified Liver Enzyme—Alumina gel Cy (910 mg) was washed with 0.01 M potassium phosphate buffer (pH 7.1) and then added to a solution of whole liver enzyme containing 22.7 g of protein in 227 ml. The suspension was left to stand at 0° for 10 min, and then centrifuged. The gel was eluted by suspending it successively in 100 ml of potassium phosphate buffers (pH 7.1) of the following strengths: 0.01 M, 0.05 M, 0.1 M, 0.2 M, 0.5 M, and 1.0 M. The last three fractions were pooled, the protein was precipitated by adding 168 g of (NH4)2SO4, and the precipitate was collected by centrifugation at 30,000 × g for 20 min. The protein was suspended in 40 ml of 0.01 M potassium phosphate buffer (pH 7.1) and dialyzed overnight against three changes of the same buffer. During the course of dialysis, the protein dissolved and was then partially precipitated again. This precipitate was removed by centrifugation. The dialyzed enzyme solution contained 238 mg of protein in 70 ml. The protein was next fractionated on a hydroxyapatite column (Hypatite C, Lot 6287). Sixty milliliters of the commercial gel were suspended in 100 ml of 0.01 M potassium phosphate buffer (pH 7.1), the gel was allowed to settle for 5 min, and the supernatant liquid was decanted. This procedure was repeated four times. The remaining gel was then packed in a column of 3 cm in diameter. The protein from the previous fractionation was placed on the column and eluted by successive 25-ml aliquots of potassium phosphate buffer (pH 7.1) of the following strengths: 0.15 M, 0.2 M, 0.35 M, 0.5 M, and 1.0 M. Enzyme activity was concentrated in the 0.35 M and 0.5 M buffer eluates. These were pooled after assay, and comprised the purified liver enzyme. The purification scheme is summarized in Table I.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Recovery</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extract</td>
<td>100%</td>
<td>11</td>
</tr>
<tr>
<td>Alumina Cy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total eluate</td>
<td>163%</td>
<td>18</td>
</tr>
<tr>
<td>Last three fractions</td>
<td>115%</td>
<td>80</td>
</tr>
<tr>
<td>Hydroxyapatite</td>
<td>36%</td>
<td>250</td>
</tr>
</tbody>
</table>

Mitochondrial Enzyme—Four guinea pigs were anesthetized with ether and decapitated, and their livers were immediately placed on ice. After washing with ice-cold distilled water, the livers (40 g) were minced in approximately 400 ml of 0.25 M sucrose containing 10−4 M EDTA. They were then homogenized in

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The specific activity of mitochondrial enzyme was several times higher than that of whole liver enzyme. The incubation mixtures contained 50 μmoles of potassium phosphate buffer (pH 7.4), 0.06 μmole of FAD, 0.5 μmole of phenazine methosulfate, 0.1 μmole of substrate, and enzyme (10 mg of whole liver enzyme or 1.2 mg of mitochondrial enzyme), in a total volume of 0.75 ml. Incubations were carried out at 37° for the times indicated in the table.

### TABLE II

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Time (min)</th>
<th>Disappearance of substrate (μmole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole liver enzyme</td>
<td>20</td>
<td>15</td>
</tr>
<tr>
<td>Cyclohexanecarboxyl-CoA</td>
<td>20</td>
<td>25</td>
</tr>
<tr>
<td>Cyclohexene-1-carboxyl-CoA</td>
<td>3</td>
<td>40</td>
</tr>
<tr>
<td>Mitochondrial enzyme</td>
<td>3</td>
<td>42</td>
</tr>
</tbody>
</table>

#### Identification of cyclohexene-1-carboxylic acid

The incubation mixture contained 1.5 μmoles of 2,6-dichlorophenolindophenol, 0.18 μmole of FAD, 100 μmoles of potassium phosphate buffer (pH 7.4), 0.5 μmole of cyclohexanecarboxyl-CoA-1-14C, and 36 mg of whole liver enzyme, in a total volume of 1.63 ml. It was incubated for 40 min at 37°. A Potter-Elvehjem homogenizer with a Teflon pestle. The homogenate was centrifuged twice at 600 X g for 10 min, and the precipitate was discarded each time. The 600 X g supernatant was then centrifuged at 26,000 X g for 20 min. The sediment was suspended in 400 ml of the above sucrose solution and recentrifuged at 26,000 X g for 20 min. The supernatant was then centrifuged at 26,000 X g for 20 min. The sediment was suspended in 20 ml of 0.01 M potassium phosphate buffer (pH 7.0). All steps were carried out at 3°. From this mitochondrial suspension, an acetone powder was prepared in the usual fashion. The enzyme was obtained by extracting the acetone powder with 0.5 M potassium phosphate buffer (pH 7.4) (10 ml of buffer per g of acetone powder), followed by centrifugation for 20 min at 30,000 X g. The protein concentration in the supernatant was 25 mg per ml. This extract is referred to as the mitochondrial enzyme.

### RESULTS

Typical data showing the conversion of cyclohexanecarboxylic acid to benzoic acid by the whole liver and mitochondrial enzymes are given in Table II. The specific activity of mitochondrial enzyme was several times higher than that of whole liver enzyme.

Identification of Reaction Products

Early in this investigation, it was observed that incubation of cyclohexanecarboxyl-CoA with whole liver enzyme yielded two peaks on gas-liquid chromatographic analysis with the retention times of the methyl esters of cyclohexene-1-carboxylic acid and benzoic acid, respectively. In order to verify their identity, the radioactive products collected from the gas-liquid chromatography were co-crystallized after addition of the authentic unlabeled acids.

Cyclohexene-1-carboxylic Acid—An incubation was carried out with 0.5 μmole (339,000 dpm) of cyclohexanecarboxyl-CoA-1-14C and whole liver enzyme. The reaction products were isolated as described and the radioactive peaks were collected from three separate runs by trapping in methanol. The methyl esters were saponified and unlabeled carrier (148.3 mg) was added to the column fraction containing the putative cyclohexene-1-carboxylic acid. The p-bromophenacyl ester was prepared (14), and recrystallized from ethanol and n-propyl alcohol to constant specific activity.

For further identification, the p-bromophenacyl ester was oxidized according to Lemieux and Von Rudloff (19), and the resulting adipic acid was crystallized to constant specific activity. The adipic acid was characterized by melting point (151-152.5°; reported (20), 151-153°), by gas-liquid chromatography of the dimethyl ester on a diethylene glycol succinate column, and by its infrared spectrum.

The results are shown in Table III. Comparison of the specific activity of the recrystallized p-bromophenacyl ester with the specific activity calculated on the basis of the activity of the original peak and the amount of carrier added showed that essentially all of the radioactivity co-crystallized with the derivative. The specific activity of the adipic acid was 77% of that expected if all the counts which cocrystallized with the p-bromophenacyl ester represented cyclohexene-1-carboxylic acid.

Benzoic Acid—A similar experiment was performed with 0.064 μmole (434,000 dpm) of cyclohexanecarboxyl-CoA-1-14C, but with mitochondrial enzyme instead of whole liver enzyme. The radioactive material from the benzoic acid peak was recrystallized from water with 98.9 mg of authentic benzoic acid. The specific activity of the recrystallized benzoic acid was over 95% of that expected if the peak in question represented pure benzoic acid (Table IV, Experiment 2).

By contrast, the benzoic acid peak from the previous experiment with whole liver enzyme evidently contained another substance (Table IV, Experiment 1). In order to verify its identity, the benzoic acid peak obtained from the previous experiment was isolated, and recrystallized from ethanol and 98.9 mg of authentic benzoic acid. The specific activity of the recrystallized benzoic acid was over 95% of that expected if all the counts which cocrystallized with the benzoic acid peak in question represented pure benzoic acid.
carboxylic acid, and benzoic acid are completely eluted from silicic acid by 20% ether in pentane. However, the products of the enzymatic reaction contain an additional component which requires 70% ether in pentane for complete elution. Thus, in one of the experiments, there was a 15% conversion of substrate to benzoate according to gas-liquid chromatography, whereas the conversion was only 7% as determined by crystallization of the benzoate fraction to constant specific activity. Therefore, 8% of the radioactivity was not accounted for. Analysis of another aliquot from the same reaction mixture by silicic acid chromatography showed that 9% of the product mixture remained on the column when the solvent was 20% ether in pentane. This component was eluted only with the more polar solvent mixture. Thus the quantity of polar product reaction is approximately the same as the quantity of the material which accompanies benzoic acid on gas-liquid chromatography but separates from benzoic acid on crystallization. While the polarity of the unknown as judged from its behavior on silicic acid is that of a hydroxy fatty acid, the relative retention time of the methyl ester of the product (3.7 with respect to methyl cyclohexanecarboxylate) is considerably smaller than the recorded values for methyl esters of hydroxy acids (7.8 with respect to the unsubstituted ester) (21). However, abnormally low retention times of 3.7 to 4.0 have been observed for the methyl esters of the β-hydroxy derivatives of capric, lauric, and myristic acids relative to the unsubstituted esters and, therefore, the assignment of a β-hydroxy structure is compatible with the behavior of the unknown on gas chromatography. Hydrogen bonding between the hydroxy group and the carboxyl oxygen of the ester function is likely to occur in esters of β-hydroxy acids and may account for the reduced polarity which is manifest on gas chromatography. This reasoning leads to the suggestion that the enzymatic product under consideration may be 2-hydroxy-cyclohexanecarboxylic acid. Other observations already mentioned indicate that the compound is formed from cyclohexene-1-carboxyl-CoA, i.e. by hydration of an α,β-enoyl-CoA, or a reaction of the enoyl hydrase type (22). Enoyl hydrase is present in the soluble fraction of liver cells and this agrees with the observation that the postulated hydration is much more extensive in extracts of whole liver than in extracts of mitochondrial acetone powder. In any event, the formation of the polar product is presumably a side reaction and unrelated to the aromatization of cyclohexanecarboxyl-CoA.

Role of Oxygen

It had previously been reported that the conversion of cyclohexanecarboxylic acid to benzoic acid requires oxygen (8), but it had not been established whether the aromatization involved an oxygen-dependent hydration of the cyclohexane ring followed by dehydroxylation, or whether oxygen served merely as a terminal electron acceptor in a direct dehydrogenation of the hydroaromatic ring. As the results of Table V show, certain artificial electron acceptors can replace oxygen in the dehydrogenation of cyclohexanecarboxyl-CoA. It should be noted that, although FAD is a prosthetic group of the aromatizing enzymes (see below), free FAD will not serve as substitute electron acceptor for oxygen. Whole liver enzyme was used in these experiments. Since some of the artificial electron acceptors undergo photoreduction with the production of H₂O₂, all incubations were carried out in the presence of catalase, and in the dark. The exclusion of light also prevented the photodecomposition of FAD and FMN. Similar results were obtained with mitochondrial enzyme, with the use of cyclohexene-1-carboxyl-CoA as the substrate (Table VI). Hence hydroxylation (or reactions of the oxygenase type) can be ruled out as intermediary steps in aromatization of cyclohexanecarboxyl-CoA.

Neither free FAD nor FMN is among the electron acceptors which can replace oxygen in the aromatization of the cyclohexane ring. However, the data in Table VII show that the aromatizing enzymes are flavoproteins. Enzyme preparations (purified liver enzyme) which have been exposed to low pH and have been dialyzed are inactive. For restoration of activity,
Acid was substantially lower than that of benzoic acid. The specific activity of cyclohexene-3-carboxylic acid was equal to or higher than that of benzoic acid in every case. The specific activity of cyclohexene-1-carboxylic acid was normalized to an assigned specific activity of 1.0 for cyclohexanecarboxyl-CoA. The role of the isomers in benzoic acid formation was examined by trapping experiments with mitochondrial enzyme. Incubations were carried out for 10 min at 37°C. On the other hand, cyclohexene-2- and -3-carboxyl-CoA do not appear to participate in the aromatization reaction, in either as precursors or as products of cyclohexene-1-carboxylate.

As shown in Table VIII, mitochondrial enzyme catalyzes the conversion of all three isomeric cyclohexanecarboxylates to benzoic acid. The role of the isomers in benzoic acid formation was examined by trapping experiments with mitochondrial enzyme. Incubations were carried out with enzyme, 14C-labeled cyclohexanecarboxyl-CoA, and the nonradioactive CoA esters of one of the three isomers of cyclohexanecarboxylic acid. The reaction products were analyzed by gas-liquid chromatography, and the material from the peaks was collected and assayed by the methods described. There was no further addition of carrier substrate before assay so that the specific activities of the various components could be directly determined by dividing the collected radioactivity by the area of the corresponding peak. Each value was normalized to an assigned specific activity of 1.0 for cyclohexanecarboxylic acid. The results are shown in Table IX. When cyclohexanecarboxyl-CoA was aromatized in the presence of unlabeled trapping acids, the specific activity of cyclohexene-1-carboxylic acid was equal to or higher than that of benzoic acid in every case. The specific activity of cyclohexene-3-carboxylic acid was substantially lower than that of benzoic acid. The cyclohexene-2-carboxylic acid in Table IX, Experiment 3, had an apparent specific activity approximately equal to that of benzoic acid. However, the cyclohexene-2-carboxylic acid peak was small and not too well separated from the very large peak of saturated acid, and, therefore, the relatively high specific activity in this peak could have been due to tailing of radioactivity from cyclohexanecarboxylic acid.

Experiment 3 was therefore repeated. One portion of the reaction products was chromatographed as before, and the specific activity was determined by crystallizing the products with carrier benzoic acid to constant specific activity.

### Table VI

| Tube | Atmosphere | Phenazine methosulfate | Time | Benzoic acid
|------|------------|-----------------------|------|---------------
| 1    | Helium     | +                     | 3 min| 34 (33)*      |
| 2    | Helium     | +                     | 3    | 3             |
| 3    | Helium     | -                     | 3    | 1 (0)         |
| 4    | Helium     | +                     | 3    | 1             |
| 5a   | O2         | -                     | 3    | 1             |
| 6    |            | 60                   | 0.6  | 5.5           |

* Figures in parentheses represent benzoic acid formation as measured by crystallizing the products with carrier benzoic acid to constant specific activity.

### Table VII

**FAD requirement of acid-treated enzyme**

The incubation mixture for Experiment 1 contained 50 μmoles of potassium phosphate buffer (pH 7.4), 0.5 μmole of phenazine methosulfate, 0.22 mg of purified liver enzyme (extracted from acetone powder at pH 4.1 (see text)), 0.1 μmole of substrate, and, when present, 0.09 μmole of flavin nucleotide, in a total volume of 0.75 ml. The incubations were carried out for 1 hour at 37°C. Experiment 2 was carried out to check the formation of polar product. The incubation conditions were identical with those of Experiment 1, except that 0.17 μmole of substrate and 0.18 μmole of enzyme were used. Benzoic acid was assayed both by gas-liquid chromatography and by recrystallization, as previously described.

| Substrate | Addition | Disappearance of substrate
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>μmole</td>
</tr>
<tr>
<td></td>
<td>FAD</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>FMN</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>FAD</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>FMN</td>
<td>11</td>
</tr>
</tbody>
</table>

* Figures in parentheses indicate benzoic acid production as determined by recrystallization assay.

### Table VIII

**Incubation of cyclohexene-2- and -3-carboxyl-CoA; mitochondrial enzyme**

Incubation mixtures contained 50 μmoles of potassium phosphate buffer (pH 7.4), 0.09 μmole of FAD, and 0.3 μmole of phenazine methosulfate, in a total volume of 0.75 ml. In each tube, 0.1 μmole of substrate was used. Tubes 1 and 3 contained 6 mg and tubes 2 and 4 contained 1.2 mg of mitochondrial enzyme. Incubations were carried out for 10 min at 37°C.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Benzoic acid formed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μmole</td>
</tr>
<tr>
<td>1. Cyclohexanecarboxyl-CoA</td>
<td>86</td>
</tr>
<tr>
<td>2. Cyclohexene-1-carboxyl-CoA</td>
<td>79</td>
</tr>
<tr>
<td>3. Cyclohexene-2-carboxyl-CoA</td>
<td>96</td>
</tr>
<tr>
<td>4. Cyclohexene-3-carboxyl-CoA</td>
<td>70</td>
</tr>
</tbody>
</table>
activities of the components were determined. Another portion was dissolved in ether, and after a carbon tetrachloride solution of cyclohexene-2-carboxylic acid (obtained by debrominating 200 mg of 2,3-dibromocyclohexanecarboxylic acid with zinc (13)) had been added the mixture was immediately treated with 0.4 ml of a solution of bromine in CCl₄ (1:9, v/v). Excess bromine was destroyed with cyclohexene. The bromination product, 2,3-dibromocyclohexanecarboxylic acid, was isolated, converted to the p-bromophenacyl ester, and crystallized repeatedly from ethanol. Under the conditions employed, only the radioactive cyclohexene-2-carboxylic acid along with the carrier acid should be brominated, whereas cyclohexene-1-carboxylic acid, benzoic acid, and cyclohexanecarboxylic acid should fail to react (23–25).

After three recrystallizations, the specific activity of the bromophenacyl ester dropped to an insignificant value. From these data, the true specific activity of cyclohexene-2-carboxylic acid was calculated to be no greater than 11% that of benzoic acid (Table X).

Any intermediate in the conversion of cyclohexanecarboxylic acid to benzoic acid must have a specific activity at least equal to that of benzoic acid. Of the three isomeric monounsaturated acids, only cyclohexene-1-carboxylic acid satisfies this requirement. It is therefore clear that only cyclohexene-1-carboxylic acid is on the direct path from cyclohexanecarboxylic acid to benzoic acid. The ready conversions of the β,γ- and γ, δ-unsaturated acids to benzoate may be the result of direct aromatization or they may occur after isomerization to cyclohexene-1-carboxylic acid. The relative importance of these two alternative pathways cannot be estimated from the present experiments.

CoA Esters as Products of Aromatization Reaction

Since the CoA ester of cyclohexanecarboxylic acid is the substrate in the aromatization reaction (8), and since hippuric acid is formed from benzoyl-CoA and glycine the ultimate product of the desaturation reactions is likely to be benzoyl-CoA rather than benzoic acid. Attempts to isolate the CoA esters of products in the aromatization reaction have failed, but it could be shown that addition of hydroxylamine after incubation led to the formation of hydroxamates of benzoic acid and probably also cyclohexene-1-carboxylic acid.

Two incubations were carried out: one with cyclohexanecarboxyl-CoA-1-14C and mitochondrial enzyme and one with the same substrate but boiled enzyme. After incubation for 4 min, 0.2 ml from each of the tubes was added to 0.5 ml of neutral hydroxylamine solution. Simultaneously, 1.5 ml of 1.0 N NaOH were added to the remainder of the incubation mixtures. After 10 min, 0.5 ml of 1.0 N NaOH was added to each of the hydroxylamine-treated portions of the incubation mixtures, and 10 min later the pH of all four solutions was adjusted to 6.6. After the addition of 0.5 ml of 0.005 M disodium EDTA and 0.2 ml each of carrier 0.01 M benzoylhydroxamic acid and cyclohexanecarboxylhydroxamic acid, the samples were passed through Dowex 50 columns and eluted with distilled water, and the eluates (10 ml, pH 7 to 8) were passed through Dowex 1 columns. After elution of the Dowex 1 eluates with distilled water (20 ml), the solutions containing the hydroxamates were brought to dryness on a rotary evaporator and the residue was extracted with 2 to 3 ml of absolute ethanol. These extracts were brought to dryness, the residue was extracted with ether, and the contents of the extract were streaked on strips of Whatman No. 3MM paper. Descending chromatography was carried out with n-butyl alcohol saturated with 2.7 N NH₄OH as the developing solvent. The dried strips were counted in a Nuclear-Chicago strip counter model. The strip was then sprayed with an ethanolic solution of FeCl₃ (26) to visualize the hydroxamic acids. The relative location of radioactivity and of the ferric chloride-positive areas is shown in Fig. 2.

On inspection of the records of strip counting, radioactivity is found to coincide with the benzoylhydroxamate spot and there is also a suggestion of radioactivity in the area corresponding to cyclohexene-1-carboxylhydroxamate, which migrates between cyclohexene-2-carboxylhydroxamate and benzoylhydroxamate. In control experiments with boiled enzyme, the only radioactive spot was in the area of cyclohexene-2-carboxylhydroxamate. When the enzymatic digests were treated with NaOH prior to the addition of hydroxylamine, no radioactivity was recovered in Dowex 1 eluates.

These findings support other evidence, already mentioned, that the end product of the aromatization reaction is benzoyl-CoA and that the various intermediates are also thioesters.

Properties of Enzyme System

The relation between rate and enzyme concentration, the time course of the reaction, the activity as a function of pH, and $K_m$

---

**Table IX**

<table>
<thead>
<tr>
<th>Assay</th>
<th>Cyclohexene-1-carboxylate</th>
<th>Cyclohexene-2-carboxylate</th>
<th>Cyclohexene-3-carboxylate</th>
<th>Benzolic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Based on radioactivity collected from gas-liquid chromatography</td>
<td>1.0</td>
<td>1.20</td>
<td>0.53</td>
<td>0.46</td>
</tr>
<tr>
<td>Based on radioactivity after recrystallization</td>
<td>1.0</td>
<td>1.20</td>
<td>0.05</td>
<td>0.46</td>
</tr>
</tbody>
</table>

---

**Table X**

<table>
<thead>
<tr>
<th>Carrier acid</th>
<th>Cyclohexene-1-carboxylate</th>
<th>Cyclohexene-2-carboxylate</th>
<th>Cyclohexene-3-carboxylate</th>
<th>Benzolic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. None</td>
<td>1.0</td>
<td>1.33</td>
<td>0.68</td>
<td></td>
</tr>
<tr>
<td>2. Cyclohexene-1-carboxyl-CoA</td>
<td>1.0</td>
<td>0.28</td>
<td>0.28</td>
<td></td>
</tr>
<tr>
<td>3. Cyclohexene-2-carboxyl-CoA</td>
<td>1.0</td>
<td>0.73</td>
<td>0.23</td>
<td></td>
</tr>
<tr>
<td>4. Cyclohexene-3-carboxyl-CoA</td>
<td>1.0</td>
<td>0.87</td>
<td>0.23</td>
<td></td>
</tr>
</tbody>
</table>

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The incubation mixtures contained 50 μmoles of potassium phosphate buffer (pH 7.4), 0.09 μmole of FAD, 0.5 μmole of phenazine methosulfate, 0.16 μmole of cyclohexanecarboxylic-CoA-7-14C, and 1.2 mg of mitochondrial enzyme, in a total volume of 0.75 ml. Where indicated, 0.10 μmole of one of the isomers of cyclohexanecarboxyl-CoA was present. Incubations were carried out for 4 min at 37°C.
Aromatization of Cyclohexanecarboxylic Acid

Origin CONHOH

FIG. 2. Radioactive scan of hydroxamates obtained by treating reaction products with hydroxylamine and separation by paper chromatography. The incubation mixture contained 50 μmoles of potassium phosphate buffer (pH 7.4), 0.09 μmole of FAD, 0.5 μmole of phenazine methosulfate, 0.72 μmole of cyclohexanecarboxyl-CoA-1-14C, and 1.2 mg of enzyme, in a total volume of 80 ml. Incubation was carried out as described in the text. The ferric chloride-positive areas are indicated by the dotted outlines.

FIG. 3. Pathways of metabolism of cyclohexanecarboxyl-CoA.

values were determined for the mitochondrial enzyme with the two substrates cyclohexanecarboxyl-CoA and cyclohexene-1-carboxyl-CoA.

The rate of consumption as a function of enzyme concentration was linear and essentially identical for both substrates. The pH optimum for both substrates was 7.5.

The disappearance of substrate with respect to time was not linear, but decreased perceptibly within the first 2 min. This was attributed to the fact that large changes in substrate concentration were necessary for analytical accuracy, and the activity of the enzyme was such that changes of this magnitude could only be produced when the initial substrate concentration was close to the \( K_m \) for the substrate.

Since the rate of disappearance of substrate was not constant with respect to time, evaluation of the \( K_m \) with the usual reciprocal plot (28) would have required measurements of initial rates, measurements which would have been tedious and inaccurate with the available assay. The \( K_m \) values were, therefore, determined by plotting \( \ln (S_0/S) \) against \( (S - S_0) \), rather than by the usual reciprocal plots (27). \( S_0 = \) initial substrate concentration, and \( S = \) substrate concentration at the end of the incubation.) This method requires measurement of only initial and final substrate concentrations. Data were obtained by carrying out incubations for fixed times with varying substrate concentrations. The method of graphing was obtained from the integrated Michaelis-Menten equation. The slope of the line is \( 1/K_m \). The \( K_m \) values found were 9.1 × 10^{-5} for cyclohexanecarboxyl-CoA and 3.4 × 10^{-5} for cyclohexene-1-carboxyl-CoA.

DISCUSSION

The enzymatic conversions observed in this investigation are summarized in Fig. 3. These studies have firmly established the dehydrogenation of cyclohexanecarboxyl-CoA to benzoyl-CoA by way of cyclohexene-1-carboxyl-CoA. The first step in the aromatization of the cyclohexane ring is thus similar to the reaction catalyzed by acyl-CoA dehydrogenase. The β,γ- and γ,δ-unsaturated acids are as readily aromatized as the α,β isomer, but trapping experiments indicate that they are not normal intermediates in the dehydrogenation of cyclohexanecarboxylate. Also, it is not clear whether the cyclohexene-2- and -3-carboxylates are directly converted to benzoate by the aromatizing enzyme. The β,γ-unsaturated compound...
seems to isomerize readily to cyclohexene-1-carboxylate,
and this indirect route may be the major one in the conversion of this isomer. As to the intervening steps between the monoene and the completely aromatized ring, it seems reasonable to postulate that they include formation of a cyclohexadiene intermediate. On incubation of cyclohexene-1-carboxyl-CoA, one does, in fact, observe the transient appearance of a broad peak between 295 and 320 mλ, the appropriate region for absorption by the thiol ester of a cyclohexadiene-1,3-carboxylic acid (22, 28, 29). Because of the extreme ease of autoxidation of such diene systems, further investigation of this finding was not considered practical.

The enzymes from acetone powders of whole liver and liver mitochondria catalyze the formation of benzoxy-CoA from both cyclohexene- and cyclohexene-carboxyl-CoA. Numerous attempts to fractionate the aromatizing system into separate activities catalyzing, respectively, monoene formation and the further dehydrogenation of the monoene gave no indications for the presence of more than one enzyme. In view of the limited enzyme purification achieved, these results are not to be regarded as conclusive.

The aromatizing system for cyclohexene-carboxyl-CoA resembles the many known flavoprotein dehydrogenases which catalyze the introduction of double bonds into aliphatic and hydroaromatic compounds. Examples of such flavoproteins are the fatty acyl-CoA dehydrogenases (30) and the enzymes which catalyze the desaturations of ring A of 3-ketosteroids (31, 32). These flavoproteins are in all cases concerned with the introduction of double bonds conjugated to a carboxylic group.

The formation of a conjugated double bond system by a cell-free preparation has been shown only in a few instances. A reaction similar to benzene formation, the desaturation of Δ^{1}-cholestenol to Δ^{14}-cholestanol, is catalyzed by microsomal rather than by mitochondrial enzymes (33, 34). The sterol diene is formed only under aerobic conditions, but it may be a dehydrogenase reaction also since it does not require a reduced pyridine nucleotide. The conjugated double bond system of the carotenoids appears to arise by a similar mechanism. In a cell-free tomato homogenate, carotenoid synthesis requires oxygen (35), but studies with Mycobacterium species indicate that in this process ferricyanide can replace oxygen (36). The cofactor requirements for these reactions are not known.

The biochemical significance of the aromatization reaction studied here is difficult to assess because cyclohexene-carboxylic acid is probably not a normal metabolite. It is possible that the desaturation reactions studied in this investigation reflect a lack of specificity of one or more known dehydrogenating enzymes (e.g., a fatty acyl dehydrogenase or a steroid-desaturating enzyme). It thus appears to be generally true that flavin-linked dehydrogenases catalyze the entry of double bonds in positions α,β to a carbonyl group, and possibly the formation of conjugated dienes from monoenes, and that these reactions involve oxygen as electron acceptor only secondarily. On the other hand, a direct interaction of substrate with oxygen seems to occur during introduction of isolated double bonds or double bonds of nonconjugated diene system (mono- and polyunsaturated fatty acids). These differences can be correlated with the presence or absence of polarizable carbonyl groups in the vicinity of the carbon atoms from which hydrogen is to be removed.

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Aromatization of Cyclohexanecarboxylic Acid
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