Enzymatic $\omega$-Oxidation

II. FUNCTION OF RUBREDOXIN AS THE ELECTRON CARRIER IN $\omega$-HYDROXYLATION

(Received for publication, April 17, 1967)

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SUMMARY

Molecular oxygen and reduced diphosphopyridine nucleotide are required for the hydroxylation of octane, yielding n-octanol, in a soluble enzyme system from Pseudomonas oleovorans previously shown to hydroxylate fatty acids at the $\omega$ carbon atom. Hexane and decane are also oxidized, but at lower rates.

Procedures are described for the separation and partial purification of the three bacterial protein components required for fatty acid and hydrocarbon hydroxylation: rubredoxin (a red, nonheme iron protein containing no labile sulfide), a reduced diphosphopyridine nucleotide-rubredoxin reductase, and the $\omega$-hydroxylase. Electron transfer occurs from reduced diphosphopyridine nucleotide to rubredoxin, catalyzed by the reductase, and from reduced rubredoxin to cytochrome c. Evidence is presented that rubredoxin and the reductase function in a similar manner as electron carriers during substrate hydroxylation in the presence of the $\omega$-hydroxylase.

Since the discovery by Verkade et al. (1) of the biological oxidation of fatty acids at the $\omega$ carbon atom, many examples have been found in nature of such an oxidative attack at the methyl carbon atom of hydrocarbons (2, 3) as well as of fatty acids and their derivatives (4-9). In the study of a soluble enzyme system from Pseudomonas oleovorans which oxidizes octane (10, 11) and $\omega$-oxidizes a series of fatty acids (12, 13), we recently separated three protein components which participate in the initial hydroxylation reaction (14). These were tentatively identified as rubredoxin (a red protein containing nonheme iron but no labile sulfide), a DPNH-rubredoxin reductase, and a fatty acid $\omega$-hydroxylase or alkane 1-hydroxylase, which will be referred to as the "$\omega$-hydroxylase." The purpose of this paper is to describe the preparation of these three protein components and to demonstrate the role of rubredoxin as an electron carrier between reduced pyridine nucleotides and the $\omega$-hydroxylase. Rubredoxin was previously isolated from Clostridium pasteurianum and characterized by Lovenberg and Sobel (15) in 1965, and has also been purified from other anaerobes (16-21). The present work apparently provides the first example of a nonheme iron protein purified from an aerobic bacterium and indicates that participation in a hydroxylation reaction may be a physiological function of rubredoxin. Ferredoxins, which differ from rubredoxin in containing labile sulfide, have recently been shown to participate in steroid hydroxylation in adrenal mitochondrial fractions (22-24), in fatty acid desaturation in Euglena and spinach preparations (25), and apparently also in camphor oxidation in a bacterial enzyme system (26).

EXPERIMENTAL PROCEDURE

Materials

Tris-chloride and potassium phosphate were used routinely as buffers unless stated otherwise; no effect on enzyme activity was noted when sulfate was substituted for chloride, or sodium for potassium ions. Spinach TPNH-ferredoxin reductase, prepared by a modification of the method of Shin, Tagawa, and Arnon (27) and shown to be greater than 95% pure by polyacrylamide gel electrophoresis, was kindly furnished by Mr. Gordon P. Foust. Bovine serum albumin was obtained from Pentex, Inc.; Sephadex G-100, from Pharmacia; and horse heart cytochrome

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1 Proposed systematic name, NADH$_2$:rubredoxin oxidoreductase, EC 1.6.99.
c and the various pyridine nucleotides, from Sigma Chemical Company. 1-14C-Hexane, -octane, and -decane were purchased from Volk Radiochemical Company. The 14C-octane was at least 99% radiochemically pure as judged by gas-liquid chromatography. The effluent from the chromatograph was passed through a Nuclear-Chicago gas flow counter.

DEAE-cellulose, purchased from Sigma (0.95 meq per g), was washed with the following solutions in the order given: 0.5 N KOH, ethanolic 0.1 N HCl, and 0.5 N KOH. In each step, the DEAE-cellulose was suspended in the solution for about 15 min, filtered, and washed with water. The final material was freed of fine particles and washed with water to neutrality.

Methods

Rubredoxin Assay—A spectrophotometric assay for rubredoxin was devised on the basis of the observation that the reduced form of this nonheme iron protein readily reduces cytochrome c. TPNH and highly purified spinach TPNH-ferredoxin reductase were used to reduce rubredoxin, and the rate of the accompanying reduction of cytochrome c was determined by the increase in absorbance at 550 mp. Reaction mixtures containing 100 μmoles of Tris buffer (pH 7.5), 1.0 mg of bovine serum albumin, the spinach reductase (20 Kg of protein), and the sample of rubredoxin were preincubated for 5 min at 30°, and the reaction was initiated by the addition of 0.3 μmole of TPNH, bringing the final volume to 1.0 ml. Increasing the preincubation time to 15 min had no effect on the assay. In control experiments, rubredoxin was omitted in order to determine the rate at which the spinach reductase reduces cytochrome c directly. The rate of cytochrome c reduction, determined with a spectrophotometer equipped with a Gillford multiple sample absorbance recorder, is constant for at least 4 min under these conditions (Fig. 1) and is roughly proportional to the rubredoxin concentration (Fig. 2). At the higher rubredoxin concentrations, the spinach reductase becomes limiting. Although increasing the level of the reductase improves the linearity of the assay, insufficient amounts were available to do so routinely. Furthermore, when the spinach reductase is increased to higher levels, the rate of cytochrome c reduction in the absence of rubredoxin (cf. Fig. 1) increases sufficiently to interfere with the assay. The bacterial reductase does not reduce cytochrome c in the absence of rubredoxin, but was not used because it has not yet been purified extensively. Similar kinetics was observed when TPNH disappearance was followed at 340 μp.

One unit of rubredoxin is defined as that amount which catalyzes the reduction of 1.0 μmole of cytochrome c per min under these conditions, and the specific activity is defined as the number of units per mg of protein.

Assay for Bacterial Reductase—The rate of cytochrome c reduction at 30° in the procedure just described serves as an assay for the Pseudomonas DPNH-rubredoxin reductase provided that rubredoxin is present in excess and DPNH is substituted for TPNH as the final addition. The rate of the reaction is constant for at least 3 min and, as shown in Fig. 3, is proportional to the bacterial reductase concentration over the range used.

Assay for ω-Hydroxylase—The hydroxylase preparations were routinely assayed by determination of the oxidation products formed from radioactive octane. The following components were incubated in a final volume of 1.0 ml at 30°: 100 μmoles of Tris buffer (pH 7.5), 0.5 μmole of FeSO4, 0.2 μmole of DPNH, 1.0 μmole of 1-14C-octane (1 X 105 cpm) dissolved in 0.01 ml of ethanol, bacterial protein fractions containing rubredoxin and the reductase, and the hydroxylase sample to be assayed. Simi-
lar results were obtained when small amounts of acetone or detergents were used to solubilize the octane. Alternatively, TPNH and spinach TPNH-ferredoxin reductase could be substituted for DPNH and the bacterial reductase. The incubation period was 10 min unless otherwise indicated. The hydroxylase fraction was omitted from control experiments. The reaction was stopped with sulfuric acid, the mixture was extracted with 5 ml of diethyl ether, and the radioactive octanol and octanoate were separated by chromatography of the ether extract on alumina columns as described earlier (11). The rate of the reaction, as determined by the sum of the radioactivity appearing in octanol and octanoate, was found to be optimal at pH 7.5 in Tris buffer (Fig. 4) and constant for about 15 min (Fig. 5). Although the rate was not strictly linear with respect to the hydroxylase concentration (Fig. 6), the assay proved useful in determining the activity of this rather unstable enzyme.

The cause of the lack of linearity of the curve in Fig. 6 is not yet known; this is probably not due to the instability of the hydroxylase, however, for the addition of bovine serum albumin to the assay mixture or preincubation of the reaction mixture for as long as 60 min at 0° before the addition of octane was without effect on the reaction rate. Alternatively, the hydroxylase may be assayed by estimating the oxidation of 1-14C-

![Fig. 4. Octane hydroxylation as a function of pH.](image)

![Fig. 5. Octane oxidation as a function of time.](image)
but not by polyacrylamide gel electrophoresis at pH 7.5, when which had been equilibrated with 0.02 M buffer containing 0.10 M achieved by chromatography on a column of Sephadex G-100 homogeneous as judged by sedimentation in the ultracentrifuge solved in 10 ml of 0.02 M buffer to give a deep red solution. At the precipitate obtained at 0.40 to 0.60 saturation was dis-
frozen state for at least 1 month. Further purification was
activity of at least 0.76 in the spectrophotometric assay was used
buffer into 5 liters of 0.05 M KC1 in the same buffer. Those
column (10 x 10 cm) of DEAE-cellulose which had been equili-
gradient obtained by siphoning 5 liters of 0.50 M KC1 in 0.10 M
brownish fraction containing the reductase; this solution was

The solutions from Steps 1c and 2e (see Table I) were com-
bined and dialysed against 25 volumes of 0.02 M buffer for 4
with a change of the buffer at the end of 2 hours. The
solution was then diluted with cold water to a protein concentra-
tion of 20 mg per ml and submitted to chromatography on a
column (10 x 10 cm) of DEAE-cellulose which had been equili-
plus ampicillin in a minimal volume of 0.02 M buffer and shown to contain rubredoxin and a small amount of
the reductase.

The solutions from Steps 1c and 2e (see Table I) were com-
pared with solid ammonium sulfate. The fraction precipitating
at 0.30 saturation was discarded, and that precipitating at 0.30
to 0.40 saturation, which contained the a-hydroxylase, was
dissolved in a minimal amount of 0.02 M buffer containing 0.005
mercaptoethanol to give a reddish, faintly opalescent prepara-
This preparation was then stored in the frozen state. The
hydroxylase fraction was in some instances further purified by
gel filtration on a column of Sephadex G-100. Such preparations
had a visible spectrum indicating the absence of rubredoxin,
ferredoxin, and flavins, and the presence of a hemoprotein with
a Soret band at 414 nm in the oxidized form and 418 nm in the
reduced form (30). The supernatant solution from the hydro-
xylase fraction was brought to 0.60 saturation to give a pre-
cipitate, which was dissolved in a minimal volume of 0.02 M
buffer and stored in the frozen state. The
rubredoxin prepared in this manner and having a specific
activity of at least 0.76 in the spectrophotometric assay was used
in the experiment to be described in this paper unless stated
Preparations of this specific activity appeared to be
homogeneous as judged by sedimentation in the ultracentrifuge
but not by polyacrylamide gel electrophoresis at pH 7.5, when

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Protein</th>
<th>Total activity</th>
<th>Specific activity</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a. Extract of lyophilized cells.</td>
<td>21.6</td>
<td>318</td>
<td>0.015</td>
<td>39a</td>
</tr>
<tr>
<td>b. Streptomycin supernatant</td>
<td>19.2</td>
<td>249</td>
<td>0.013</td>
<td>31</td>
</tr>
<tr>
<td>c. Ammonium sulfate precipitate</td>
<td>11.0</td>
<td>248</td>
<td>0.023</td>
<td>31</td>
</tr>
<tr>
<td>(0.30-0.60 saturation)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2a. Sonic extract.</td>
<td>72.4</td>
<td>500</td>
<td>0.007</td>
<td>61a</td>
</tr>
<tr>
<td>b. Streptomycin supernatant</td>
<td>62.7</td>
<td>420</td>
<td>0.007</td>
<td>51</td>
</tr>
<tr>
<td>solution.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>e. Ammonium sulfate precipitate</td>
<td>12.3</td>
<td>420</td>
<td>0.034</td>
<td>51</td>
</tr>
<tr>
<td>(0.40-0.60 saturation)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| 3. Preparations 1e and 2e com-
| bined and dialyzed.              | 22.2    | 600            | 0.027            | 73    |
| 4. Eluate from first DEAE-
| cellulose column.                | 6.3     | 333            | 0.053            | 41    |
| 5. Ammonium sulfate precipi-
| tate (0.40-0.60 saturation)      |         |                |                  |       |
| eluate from second DEAE-
| cellulose column.                | 0.42    | 117            | 0.28             | 14    |
| 6. Sephadex G-100 effluent       | 0.11    | 84             | 0.76             | 10    |

* The sum of the rubredoxin in these two fractions is taken as the total initial amount.

about 10 protein bands were seen. Of these, rubredoxin appeared to be the major component and to represent about 10 to
20% of the total protein. The ratio of absorbance at 280 nm to
that at 495 nm is about 12.2 in such preparations.

RESULTS

Requirement for DPNH and Oxygen in Hydroxylation Reaction—It was previously found that octane hydroxylation in the presence of enzyme Fractions A and B was enhanced by the addition of a boiled juice, which could be replaced by pyridine nucleotides, and that DPNH and DPN were about equally effective in this regard (11). This requirement was established for the step in which octanol is formed, and is therefore distinct from the known requirement of DPN by octanol dehydrogenase and octaldehyde dehydrogenase, the combined action of which yields octanoate (10). The experiments recorded in Table II with charcoal treated enzyme preparations clearly indicate that DPPh is superior to DPN, TPN, or TPNH in the hydroxylation reaction. A portion of the DPPh is known to be converted to DPN by an oxidase in the enzyme preparations, and the latter nucleotide then serves in the further oxidation of most of the octanol to octanoate. The sum of the radioactive octanol and octanoate formed is a measure of the effectiveness of DPPh in the hydroxylation reaction, which is apparently the rate-limiting step. The oxidative attack on the hydrocarbon is greatly reduced under anaerobic conditions, as shown in Table III. Although the direct incorporation of oxygen has not yet been shown with
O2, it appears likely that molecular oxygen is the source of the
oxygen atom of octanol. The finding that both DPPh and O2 are involved in this reaction strongly suggests that it is catalyzed by a mixed function oxidase.
TABLE II

Pyridine nucleotide requirement for octane hydroxylation

The standard assay conditions were used, with Fractions A and B which had been stirred with charcoal for 30 min (0.1 and 1.4 mg of protein, respectively), except that the octane was added as 0.01 ml of 0.1% Triton X-100 rather than in alcohol solution to avoid the rapid reduction of DPN due to alcohol dehydrogenase in the enzyme preparations. The incubation time was 30 min.

<table>
<thead>
<tr>
<th>Pyridine nucleotide added</th>
<th>Products formed</th>
<th>Octanol</th>
<th>Octanoate</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>0</td>
<td>0.4</td>
</tr>
<tr>
<td>DPNH</td>
<td></td>
<td>1.6</td>
<td>13.7</td>
</tr>
<tr>
<td>DPN</td>
<td></td>
<td>0</td>
<td>0.9</td>
</tr>
<tr>
<td>TPNH</td>
<td></td>
<td>0</td>
<td>1.2</td>
</tr>
<tr>
<td>TPN</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

TABLE III

Oxygen requirement

A typical complete reaction mixture containing Fractions A and B (0.5 and 2.3 mg of protein, respectively) in a final volume of 1.0 ml was placed in a Thunberg tube and frozen in a Dry Ice bath. (The Fraction A was less active than the preparation used for the experiments in Table II.) The tubes were evacuated with a water aspirator, and, prior to thawing and subsequent incubation for 15 min at 28°C, the aerobic tube was opened to the atmosphere.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Products formed</th>
<th>Octanol</th>
<th>Octanoate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aerobic</td>
<td></td>
<td>0.32</td>
<td>4.0</td>
</tr>
<tr>
<td>Anaerobic</td>
<td></td>
<td>0</td>
<td>0.28</td>
</tr>
</tbody>
</table>

TABLE IV

Requirements for catalytic reduction of cytochrome c by rubredoxin

The complete reaction mixture contained rubredoxin (2.0 µg of protein in Experiment 2; 1.5 µg of protein in the other experiments), either the spinach reductase or the less purified bacterial reductase (20 µg of protein in each case); and other components as described in the text for the spectral assay of rubredoxin. Where indicated, TPNH was replaced by DPNH at the same concentration.

<table>
<thead>
<tr>
<th>Reductase source and conditions</th>
<th>Cytochrome c reduced</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Spinach reductase</td>
<td></td>
</tr>
<tr>
<td>Complete system</td>
<td>4.3</td>
</tr>
<tr>
<td>No reductase</td>
<td>0</td>
</tr>
<tr>
<td>No rubredoxin</td>
<td>1.0</td>
</tr>
<tr>
<td>No cytochrome c</td>
<td>0</td>
</tr>
<tr>
<td>No TPNH</td>
<td>0</td>
</tr>
<tr>
<td>2. Spinach reductase</td>
<td></td>
</tr>
<tr>
<td>Complete system</td>
<td>5.8</td>
</tr>
<tr>
<td>No TPNH; DPNH added</td>
<td>0.7</td>
</tr>
<tr>
<td>3. Pseudomonas reductase</td>
<td></td>
</tr>
<tr>
<td>Complete system</td>
<td>0.3</td>
</tr>
<tr>
<td>No TPNH; DPNH added</td>
<td>1.5</td>
</tr>
</tbody>
</table>

TABLE V

Requirements for Cytochrome c Reduction by Rubredoxin—As described above, the reduction of cytochrome c by catalytic amounts of reduced rubredoxin serves as a means of assaying the latter protein. The data in Table IV show that this reaction requires the presence of a reductase as well as a reduced pyridine nucleotide as the primary source of electrons. Although TPNH and spinach TPNH-ferredoxin reductase are used routinely for this purpose, DPNH and the less purified Pseudomonas DPNH-rubredoxin reductase may also be employed (cf. Fig. 3). The spinach reductase, unlike the bacterial reductase, reduces cytochrome c slowly in the absence of rubredoxin, as shown in Table IV. The superiority of DPNH to TPNH with the bacterial reductase in the experiment shown would be predicted from the $K_m$ values, which are $7.2 \times 10^{-7}$ and $7.0 \times 10^{-4}$ M, respectively. On the other hand, DPNH is much less effective than TPNH with the spinach reductase.

**Requirement for Three Enzyme Components in Hydroxylation Reaction**—On the basis of the visible spectrum of one of the three partially purified protein fractions required for the hydroxylation reaction, as well as the spectral changes resulting from reduction by DPNH or TPNH in the presence of the appropriate reductase, it was tentatively concluded that rubredoxin is the active component of that fraction (14). The data in Table V confirm this conclusion by showing that a homogeneous preparation of rubredoxin (having a specific activity of $3.4 \times 10^4$ in the spectral assay) is fully active in octane hydroxylation; detailed evidence for the purity of such samples of rubredoxin will be presented in a subsequent paper. The other required components (Experiment 1) are DPNH and the bacterial hydroxylase and reductase fractions. Of particular interest is the failure of TPNH to substitute for DPNH when the Pseudomonas reductase is used, whereas TPNH is much superior to DPNH when the spinach reductase is present. Thus the pyridine nucleotide specificities...
The rate of oxidation of the various radioactive alkanes was determined at known saturating concentrations in the usual assay in the presence of Fractions A and B (0.1 and 1.0 mg of protein, respectively). The final alcohol concentration in the reaction mixtures was 3.0%. No effect on the rate of octane oxidation was noted with alcohol concentrations from 1 to 5%. The rates are expressed in terms of substrate oxidized per min per mg of total protein. Unincubated controls were included in order to correct for any traces of polar products present as impurities in the 14C-hydrocarbon substrates.

for hydroxylation are the same as already described for cytochrome c reduction. It may be noted that the omission of Fe++ ions resulted in only a slight decrease in activity, in contrast to results obtained earlier with relatively crude enzyme preparations.

Substrate Specificity—Although the bacterial cultures are routinely grown on hexane as the major carbon source, radioactive octane has been used routinely as the substrate for hydroxylation because of its commercial availability. More recently, other alkanes have been tested, as shown in Table VI. At known saturating concentrations of each, octane appears to be oxidized most rapidly, but hexane and decane are also relatively good substrates. The apparent Km value of hexane for hydroxylation is somewhat higher than the values found with octane and decane.

DISCUSSION

Since the oxidative attack at the methyl carbon atom of fatty acids and hydrocarbons requires both molecular oxygen and DPNH, it appears that this enzyme system may be classified as a "mixed function oxidase" (31) or "monooxygenase" (32), and that the over-all reaction may tentatively be written as follows.

\[
\text{Fatty acid (or n-alkane) + DPNH + H}^+ + O_2 \rightarrow \omega\text{-hydroxy fatty acid (or n-alcohol) + DPNH}^+ + H_2O
\]

The identity of the properties of the enzyme systems and in the cofactor requirements for hydrocarbon and fatty acid hydroxylation suggests that a single hydroxylase may act on both types of substrates. The data presented in this paper show that C8 is the optimal chain length for alkanes, whereas among the fatty acids tested previously those having 8 to 18 carbon atoms were all oxidized, but C8 was found to be the optimal chain length (13).

Wakabayashi and Shimazono (8) have reported that the ω oxidation of fatty acids in liver microsomes is also an aerobic reaction and requires a reduced pyridine nucleotide, TPNH, being superior to DPNH. We have recently found that a rat liver microsomal preparation which ω-hydroxylates deconate and laurate is even more active with octane as the substrate, but differs from the bacterial ω-hydroxylase in being readily inhibited by carbon monoxide (33).

In contrast to the hydroxylation reaction which we are proposing to account for the initial oxidative attack on hydrocarbons and at the ω carbon atom of fatty acids, Senes et al. (34-37) have reported that, as the first step in oxidation, paraffins undergo anaroe dehydrogenation by DPN or pyocyanin in extracts of a strain of Pseudomonas aeruginosa. These workers (38, 39) have further reported the isolation from their reaction mixtures of a compound which appeared to have the infrared spectrum of heptene. We have been unable, however, to detect an alkane dehydrogenase by looking for the octane-dependent reduction of DPN under anaroe conditions in the presence of enzyme preparations from P. oleovorans. Furthermore, enzyme Fractions A and B which we have prepared from P. aeruginosa (kindly furnished by Dr. Senes) are interchangeable with comparable fractions from P. oleovorans in catalyzing the hydroxylation reactions described in the present paper. It may be noted that the energetics for alkane dehydrogenation are highly unfavorable, since the Δ"G" value for the reaction heptane + DPN" → heptene + DPNH + H" has been estimated as about +16.6 kcal per mole (2). Additional evidence against olefins as free intermediates in hydrocarbon oxidation is provided by the observation of Huybregtse and van der Linden (40) that the main pathway of oxidation of 1-octene by a species of Pseudomonas is via the saturated end of the molecule to give 7-heptenoic acid. On the other hand, the possibility that an enzyme-bound alkene is formed as a transient intermediate by the action of the ω-hydroxylase cannot be ruled out at this time.

As reported previously, rubredoxin is bleached to a colorless form on reduction by dithionite or by TPNH in the presence of highly purified spinach TPNH-ferredoxin reductase (14). The reduction of cytochrome c by rubredoxin in the presence of TPNH and the spinach reductase or of DPNH and the bacterial reductase further indicates the ability of this nonheme iron protein to act as an electron carrier, and the following evidence shows that rubredoxin and the reductase function in a similar manner in the hydroxylation reaction sequence: (a) both rubredoxin and a reductase, as well as the ω-hydroxylase, are required for substrate hydroxylation to occur; (b) the specificity of reduced pyridine nucleotides for hydroxylation is the same as for cytochrome c reduction in the presence of the appropriate reductase; (c) a homogeneous preparation of rubredoxin retains all of the activity in the hydroxylation system previously attributed to a partially purified preparation; and (d) under comparable conditions when rubredoxin is the limiting component, the rate of electron transfer from DPNH to cytochrome c as an artificial acceptor (2.1 μmole per min per mg of rubredoxin) is more than rapid enough to account for the rate of electron transfer from DPNH to oxygen in the presence of the hydroxylase (0.2 μmole per min per mg of rubredoxin).

Our results, therefore, indicate that rubredoxin functions as an...
electron carrier in bacterial \(\alpha\)-hydroxylation as shown in Scheme 1.

Acknowledgments—The authors are indebted to Mr. Egils T. Lode for devising some of the enzyme purification procedures, and to Mrs. Irene Unger and Miss Aina Jakobsens for technical assistance.

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