The Enzymatic Hydroxylation of n-Octane by Corynebacterium sp. Strain 7E1C*

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SUMMARY

Cell-free extracts from sonically disrupted Corynebacterium sp. (7E1C) oxidized n-octane to 1-octanol and octanoic acid in the presence of NADH and O₂. The hydroxylation reaction, assayed by direct estimation of the reaction products, was found to be concentrated in the clarified S₁ supernatant fraction after centrifugation at 144,000 × g for 2 hours. By use of mass spectrometry it was shown that molecular oxygen is incorporated into the substrate during hydroxylation. The hydroxylation enzyme system was separated into two protein fractions, both of which were required for activity. One fraction, the S₃(25-40)D, which precipitated between 25 and 40% ammonium sulfate saturation, appeared “particulate” and contained cytochrome P₄₅₀. The participation of this hemoprotein in n-octane hydroxylation was established by inhibition, induction, and spectral studies. The S₃(60-100)D fraction, which precipitated between 60 and 100% ammonium sulfate saturation, was soluble, contained flavoprotein, and was functional in reducing cytochrome P₄₅₀ or cytochrome c in the presence of NADH. A tentative scheme for n-octane hydroxylation in the Corynebacterium 7E1C system is proposed.

In contrast to the hydroxylase from the Pseudomonas the n-alkane-hydroxylating system from liver microsomes contains a CO-binding hemoprotein, cytochrome P₄₅₀ (4). Cytochrome P₄₅₀ has been implicated in a number of biological hydroxylation reactions (9–13). Its function is not yet clear; however, it is believed to be involved in the terminal oxidation reaction of mixed function oxidases (14, 15).

The lack of information on the terminal oxidase of bacterial n-alkane-hydroxylating systems prompted us to investigate the problem with the use of cell-free extracts of Corynebacterium sp. In a preliminary communication (3), we reported that the hydroxylation of n-octane was catalyzed by Corynebacterium 7E1C by a multi-enzyme system. The system was separated into two distinct protein fractions, one containing cytochrome P₄₅₀, and the other having the spectrophotometric characteristics of a flavoprotein. This paper describes further the studies on the characterization of this enzyme system.

EXPERIMENTAL PROCEDURE

Culture Methods—Batch cultures of Corynebacterium sp. strain 7E1C (ATCC 19067) were prepared with the use of Medium L (16) to which n-octane (99 moles % pure) was supplied in the vapor phase (essentially air saturated with n-octane) as the sole source of carbon. Twenty-five 1-liter batches were placed on reciprocal shakers and incubated for 34 days at 28° and a total of 35 g of cells (wet weight) were obtained. After continuous flow centrifugation, and thorough washing in cold 0.067 M phosphate buffer, pH 7.4, the cells were resuspended in 150 ml of buffer and stored at −25°. For comparative studies on the induction of the hydroxylating enzyme system, 2% (w/v) sodium acetate in Medium L was used as the growth substrate. In such studies the inoculum culture had been transferred at least 10 times on a hydrocarbon-free medium; otherwise the procedure was identical with that used for the octane-grown cells.

Preparation of Cell-free Extracts—Unless otherwise stated, all subsequent operations were carried out at 4°. Cell-free extracts were obtained from the above resting cell suspensions by sonic oscillation, followed by differential centrifugation. During sonic treatment, the temperature of the cell suspension was maintained below 4° and the output of the sonifier was kept constant at 6.2 amp. The sonically disrupted cell suspension was centrifuged at 37,500 × g for 20 min to remove unbroken cells and cell debris. A second centrifugation at 144,000 × g for 2 hours yielded a clear orange supernatant, or S₁ fraction, which contained the active hydroxylation enzyme system. The S₁ fraction could be stored at −25° for 2 months without appreciable deterioration.
Mixtures of authentic 1-heptanol and 1-octanol were diluted in 25 and 40% ammonium sulfate saturation; the S3(60-100)D, a SB fraction was resolved into two protein fractions by ammonium sulfate precipitation, and both fractions were required for appreciable loss of hydroxylating activity. The hydroxylase in the procedure was used for the calibration curve for heptanoic acid and octanoic acid. The acids were separated on Column B as described.

Fig. 1. Calibration curves for the quantitative estimation of 1-octanol and octanoic acid by vapor phase chromatography. Mixtures of authentic 1-heptanol and 1-octanol were diluted in diethyl ether to the concentration ranges comparable to those of the enzymatic samples. Samples of 5 μl were chromatographed on Column A as described. The peak areas of 1-heptanol and 1-octanol were measured on the chromatogram by means of a compensating polar planimeter. The area ratios (1-heptanol to 1-octanol) were then plotted as a function of molar ratios. The same procedure was used for the calibration curve for heptanoic acid and octanoic acid. The acids were separated on Column B as described.

ciable loss of hydroxylating activity. The hydroxylase in the S3 fraction was resolved into two protein fractions by ammonium sulfate precipitation, and both fractions were required for activity (3). The S3(25-40)D, a red fraction, precipitated between 25 and 40% ammonium sulfate saturation; the S3(60-100)D, a greenish yellow fraction, precipitated between 60 and 100% saturation. Both ammonium sulfate fractions were very labile and had to be used within 2 days. Protein concentrations were determined by the biuret method of Gornall, Bardawill, and David (17).

Assay for n-Octane-hydroxylating Activity—The n-octane-hydroxylating activity in cell-free extracts of Corynebacterium 7E1C was measured by the direct estimation of the reaction products, i.e. 1-octanol and octanoic acid. The enzyme assay was carried out in 50-ml Erlenmeyer flasks sealed with rubber stoppers. The standard reaction mixture of 5.0 ml contained 0.355 mmole of phosphate buffer, pH 7.4, 0.05 ml of n-octane (99.85 mole % pure), 5 μmoles of NADH, and enzyme. The flasks were incubated on a rotary shaker (300 rpm) at 30°C. The reaction was stopped after 20 min by addition of 0.5 ml of concentrated HCl. At this time 1 pmole of 1-heptanol (0.1 ml of 0.01 M heptanol in ethanol) or 1 μmole of 1-heptanoic acid (0.1 ml of a 0.01 M aqueous solution of sodium heptanoate), or both, was added to the reaction vessel as an internal standard. The mixture was then extracted three times with a total volume of 12 ml of diethyl ether. The ether extracts were concentrated under N2 to approximately 20 μl and the reaction products were quantitatively determined by vapor phase chromatography.

Unless stated otherwise, enzymatic activities were expressed as millimicromoles of 1-octanol and octanoic acid formed per min per mg of protein at 30°C.

Separation and Quantitative Estimation of 1-Octanol—A Perkin-Elmer model 154C vapor fractorimeter, fitted with a flame ionization detector, was used for all routine gas chromatographic analyses. Unless otherwise indicated, 1-octanol and 1-heptanol were separated on a 6-foot stainless steel column packed with 15% di-2-ethylhexyl sebacate on 100 to 120 mesh Gas-Chrom P (Column A). The operational conditions were the following: column temperature, 135°C; carrier gas (helium) flow rate, 60 ml per min.

Between 2 and 5 μl of the concentrated ether samples were injected. 1-Octanol was quantitatively determined by comparing its normalized peak area to the peak area of the internal standard (1-heptanol). A calibration curve had been previously obtained by chromatographing known mixtures of 1-heptanol and 1-octanol and by plotting peak area ratios against molar ratios. As shown in Fig. 1, this plot is a straight line, indicating that (a) the detector response towards both 1-heptanol and 1-octanol is a linear function of concentration, and (b) the relative detector response per mole of 1-heptanol is 0.94 (detector response per mole of 1-octanol = 1.00). The factor 0.94 was, therefore, used to normalize the peak area of 1-octanol to that of the standard. The amount of product is given by equation

\[
x = \frac{(8-\text{OH})(0.94)}{(7-\text{OH})}
\]

where x — micromoles of octanol; 8- OH — area of the 1-octanol peak; 7-OH = area of the 1-heptanol peak; and 0.94 = normalization factor.

Assuming that 1-heptanol and 1-octanol are extracted from the reaction mixture to the same extent, the internal standard technique gives quantitative results regardless of extraction losses that occur during the preparation of the sample (18).

Separation and Quantitative Estimation of Octanoic Acid—Octanoic acid and heptanoic acid were separated on a 10-foot stainless steel column, packed with 0.25% Carbowax 20 M and 0.4% isophthalic acid on 200 μ glass beads (19) (Column B). The operational conditions were the following: column temperature, 140°C; helium flow rate, 80 ml per min.

Between 2 and 5 μl of the concentrated ether extracts of the reaction mixtures were chromatographed on Column B. The quantitative determination of octanoic acid was obtained also by comparing the peak area of the product to that of the standard. The calibration curve in Fig. 1 shows that the relative detector response per mole of heptanoic acid is 0.78 (detector response per mole of octanoic acid = 1.00). Knowing the amount of standard added (1 μmole), the amount of product is calculated by the following equation

\[
x = \frac{(8-\text{COOH})(0.78)}{(7-\text{COOH})}
\]

where x = micromoles of octanoic acid; 8-COOH = area of the octanoic acid peak; 7-COOH = area of the heptanoic acid peak; and 0.78 = normalization factor.

Carbon Monoxide Inhibition Studies—Mixtures of CO-air and N2-air were prepared in 2-liter suction flasks. The reaction vessels were then flushed with 1 liter of the gas mixture and immediately sealed. These operations were carried out at 4°C.
**RESULTS**

**Coenzyme Requirements**—The hydroxylation of n-octane by the cell-free extract of Corynebacterium 7ElC requires NADH as a coenzyme (3). Maximal activity was recorded when 1 mM NADH was added to the reaction mixture. No activity was observed with heated enzyme nor when the high speed supernatant, S₂₀, was assayed in the absence of coenzymes. Very little activity was observed when either NAD or NADPH was substituted for NADH at the 1 mM level. The addition of ferrous ions (5 × 10⁻⁴ M) appeared to inhibit the reaction. Neither FNN nor FAD affected the n-octane-hydroxylating activity of the S₂₀ fraction.

**Product Formation as Function of Time and Protein Concentration**—The enzymatic formation of 1-octanol and octanoic acid as a function of time is shown in Fig. 2. The formation of 1-octanol is a linear function of time during the first 10 to 15 min. At 20 min the concentration of 1-octanol reaches its peak. After this time, its concentration decreases. On the other hand, the rate of formation of octanoic acid is constant over the time period studied. The curves of Fig. 2 indicate that (a) the over-all hydroxylation reaction is no longer significant after 20 min, and (b) 1-octanol is converted to octanoic acid. The alcohol and aldehyde dehydrogenases, which presumably catalyze the latter reaction, are still active after a 60-min incubation interval. Fig. 2 shows that, at 20 min, the concentration of 1-octanol is maximal and approximately 4 times the concentration of octanoic acid. This relationship is characteristic of all preparations that were studied. Consequently, a simplified assay was developed based on the measurement of 1-octanol only after 20 min of incubation. This was used routinely to determine n-octane-hydroxylating activity of cell-free extracts.

Fig. 3 shows the effect of enzyme concentration on the amount of 1-octanol and octanoic acid formed after the 20-min incubation interval. This experiment was repeated several times. A linear relationship between enzyme concentration and amount of reaction product formed was never observed. This finding is...
FIG. 2. Enzymatic formation of 1-octanol and octanoic acid from n-octane as a function of time. The S3 fraction was used in this study and identical reaction mixtures were incubated under standard assay conditions for different time intervals. The concentration of the reaction products formed (in micromoles) was plotted against time. The curve for the total end product was obtained by adding the amounts of 1-octanol and octanoic acid formed.

FIG. 3. Effect of protein concentration on the amount of 1-octanol and octanoic acid formed. Varying concentrations of S3 fraction were added to the reaction mixtures and incubated under standard assay conditions for 20 min. The amounts of product formed (in micromoles) were plotted against the total protein concentration (in milligrams) used in the assay. The total end product curve was obtained by adding the amounts of 1-octanol and octanoic acid formed.

FIG. 4. Mass spectra of 1-octanol formed from the enzymatic reaction in a normal atmosphere (A) and in an 18O-enriched atmosphere (B). The ion abundance of both spectra is normalized to that of the ion at m/e 41 (\(\text{CH}_2=\text{CH}-\text{CH}_2\)).

not unusual for unpurified enzyme preparations and may also indicate that the hydroxylation reaction is catalyzed by a multi-enzyme system.

Incorporation of 18O into n-Octane in Enzymatic Conversion to 1-Octanol—The S3 fraction was incubated in normal atmosphere and in 18O-enriched atmosphere, as described under “Experimental Procedure.” 1-Octanol was extracted from the reaction mixtures and separated by means of preparative vapor phase chromatography. The identity of the enzymatic product with 1-octanol was established by retention time and by infrared spectrophotometry. The infrared spectra of the enzymatic product formed in an atmosphere of 18O2 and of authentic 1-octanol were identical.

The mass spectrum of 1-octanol obtained from the enzymatic oxidation of n-octane was in good agreement with the data reported by Friedel, Schultz, and Sharkey (24). Since 1-octanol gives a very weak parent peak, the oxonium ion \(\text{CH}_2=\text{CH}-\text{CO}^+\) (at m/e 31) was selected to show 18O incorporation. As shown in Fig. 4A, the oxygen containing fragment at m/e 31 has a relative abundance of 38% whereas the ion at m/e 33 is insignificant (relative abundance 2%). In contrast, the mass spectrum of 1-octanol obtained from the 18O-enriched mixture (Fig. 4B) shows a marked increase in the abundance of the ion at m/e 33 whereas the relative abundance of the m/e 31 ion is greatly decreased. These results are consistent with the presence of 18O in the hydroxyl group of octanol. The unexpectedly high intensity of the m/e 31 peak (relative abundance 11%, Fig. 4B) can be attributed to some unlabeled octanol that was present in the mixture before the reaction was started. In addition, it is likely that a \(\text{H}-\text{C}=\text{O}\) ion contributes to the m/e 29 peak (Fig. 4A).

Thus, in the mass spectrum of octanol formed in 18O-enriched atmosphere, one observes a decrease of the m/e 29 peak, accompanied by an increase of the m/e 31 peak. These findings unequivocally indicate that molecular oxygen is incorporated into n-octane in the enzymatic conversion of 1-octanol.

Requirement of Two Enzyme Fractions for Hydroxylation of n-Octane—Attempts at purifying the hydroxylating enzymes by use of ammonium sulfate precipitation resulted initially in the loss of the activity. Subsequently, it was established that two enzyme fractions are required for the n-octane hydroxylation. As shown in Table I, neither the fraction that precipitates between 25 and 40% ammonium sulfate saturation, nor that which precipitates between 60 and 100% saturation, is active alone. However, the activity is reconstituted when these two fractions are combined. Optimal hydroxylation rates were observed when
the S3(25-40)D and the S3(60-100)D were combined in a 1:2.5 proportion on a protein basis. The intermediate ammonium sulfate fraction, S3(40-60)D, is active by itself. However, its specific activity is approximately one-half of that observed for the combined fractions.

**Inhibition Studies**—Table II shows the effect of varying concentrations of carbon monoxide on the n-octane hydroxylation activity of the S3 fraction. The hydroxylation activity is completely inhibited by CO-air, 50:50 (v/v) mixtures and is still strongly inhibited by CO-air, 5:95 (v/v) mixtures. These results suggest the participation of a hemoprotein in the hydroxylation reaction. The data of Table II also show that the hydroxylation of n-octane is inhibited under reduced oxygen pressure. Table III shows the effect of other inhibitors on the hydroxylation activity of the S3 fraction. Heavy metals (Cu++, Hg++, p-chloromercuribenzoate) inhibit the hydroxylation effectively at a concentration of 10⁻³ M. Of the other inhibitors, cyanide, azide, arsenite, and EDTA are ineffective inhibitors at a concentration of 10⁻⁴ M. No inhibition was observed with SKF-525 (10⁻³ M) which is known to inhibit cytochrome P-450 in the N-demethylation reaction of certain drugs by rat liver microsomes (25).

Cytochrome c and 2,6-dichloroindophenol produced approximately 50% inhibition at a concentration level of 10⁻² M. These results suggest that cytochrome c and DC1 can react with the enzymes involved and drain electrons from the hydroxylation system. Ferricytochrome c inhibits n-octane hydroxylation even when NADH is present in excess concentration.

**Spectral Studies on Hydroxylation Enzyme Fractions**—As reported earlier (3) one of the two protein fractions that was required for hydroxylation contains cytochrome P-450. Fig. 5 shows the difference spectra of the S3(25-40)D fraction. The dithionite-reduced minus oxidized preparation (solid line) is characterized by a broad absorption peak at 429 μm with a shoulder at 440 to 445 μm region and a weak absorption peak at 555 μm. Addition of CO to the reduced preparation brings about a marked change in the Soret region; a broad peak appears at 450 μm and a small, sharp peak at 425 μm. No changes are observed at higher wave lengths. These observations, and the characteristic absence of α- and β-bands, are consistent with the presence of cytochrome P-450. Cytochrome P-450 in the S3(25-40)D fraction was converted readily to the P-420 form by exposure to potassium deoxycholate (3). The absorption at 425 μm is due to the presence of a small amount of cytochrome P-420. The concentration of cytochrome P-450, calculated

### Table I

| Enzyme fraction | Total protein concentration (mg/5 ml) | Specific activitya (mmol/min/mg) |
|-----------------|--------------------------------------|---------------------------------|
| Experiment 1    |                                      |                                 |
| S3(25-40)D      | 44.3                                 | 0                               |
| S3(60-100)D     | 38.0                                 | 0                               |
| S3(25-40)D + (60-100)D | 40.7 | 1.61             |
| Experiment 2    |                                      |                                 |
| S3(40-60)D      | 44.0                                 | 0.91                            |

a Millimicromoles of product formed per min per mg of total protein (30%).

### Table II

| Gas phase                          | Specific activitya (mmol/min/mg) | Inhibitionb (%) |
|------------------------------------|----------------------------------|-----------------|
| N2-air, 50:50                      | 3.52                             | 100             |
| CO-air, 50:50                      | 0                                | 91              |
| N2-air, 10:90                      | 3.72                             | 5               |
| CO-air, 10:90                      | 0.35                             | 5               |
| N2-air, 5:95                       | 3.90                             | 5               |
| CO-air, 5:95                       | 0.64                             | 84              |
| Air                                | 4.26                             |                 |
| N2                                | 0.45                             |                 |

a Millimicromoles of 1-octanol formed per min per mg of protein (30%).

b Inhibition is normalized to the activity of the control in which N2 was substituted for an equal volume of CO.

### Table III

| Addition                     | Concentration (M) | Inhibition (%) |
|------------------------------|-------------------|----------------|
| None                         | 0                 | 0              |
| CuCl2                        | 10⁻⁴              | 97             |
| HgCl₂                        | 10⁻³              | 98             |
| p-Chloromercuribenzoate       | 10⁻⁴              | 51             |
| KCN                          | 5 x 10⁻⁴          | 10             |
| KCN                          | 10⁻³              | 13             |
| Azide                        | 10⁻²              | 20             |
| Arsenite                     | 10⁻³              | 5              |
| EDTA                         | 10⁻⁴              | 3              |
| SKF-525                      | 10⁻⁵              | 0              |
| Cytochrome c                 | 10⁻³              | 67             |
| DC1                          | 10⁻⁴              | 45             |

Fig. 5. Difference spectra of the cytochrome P-450 component in the S3(25-40)D fraction. ---, dithionite-reduced minus oxidized; ----, CO-dithionite-reduced minus oxidized.
from the extinction coefficient of 91 mm$^{-1}$ cm$^{-1}$ (26), was 0.25 mmol per mg of protein. The $S_{25-40}D$ fraction is essentially free from other hemoproteins as revealed by spectral analyses.

Although the cytochrome P-450 fraction is obtained from a high speed supernatant, it is unlikely that it is a soluble hemoprotein. It was observed that a lipid-soluble, red carotenoid pigment was always present in this fraction, suggesting that lipid was present in the preparation. Moreover, this $S_{25-40}D$ fraction could be partially clarified by the addition of detergents (deoxycholate and Triton X-100).

The second fraction, $S_{60-100}D$, required for n-octane hydroxylation, appears to contain flavoprotein. This fraction is greenish yellow in color and the difference spectrum obtained for this fraction has been published previously (3). The characteristic bleaching attributed to flavins (450 nm region) was noted after the addition of dithionite. The component responsible for dithionite bleaching was extractable by acid, and the acid-extractable material revealed a typical flavin spectrum. With the use of the extinction coefficient of 11.3 mm$^{-1}$ cm$^{-1}$ (reduced minus oxidized at 450 nm), the acid-extractable flavin content was calculated to be 1.51 mmol per mg of protein (3). Similar dithionite bleaching studies conducted directly on the $S_{60-100}D$ fraction revealed a flavin content of 1.72 mmol per mg of protein which strongly suggests that the predominant bleaching at 450 nm (approximately 88%) is undoubtedly due to a flavoprotein component. Although the presence of a relatively large concentration of flavin in a fraction obligately required for n-octane hydroxylation, would not directly implicate its involvement in this reaction, it is difficult, however, to postulate a mechanism as to how NADH (a required oxidation-reduction component for n-alkane hydroxylation) might function, if not via a flavoprotein intermediary.

A trace amount of a hemoprotein component was also noted in the $S_{60-100}D$ fraction. This hemoprotein was concentrated in the intermediate ammonium sulfate precipitate ($S_{40-60}D$ fraction) and was subsequently identified as cytochrome $a$. This cytochrome appeared to be a constitutive component in Corynebacterium 7E1C since it was found in approximately equal concentrations in both octane- and acetate-grown cells.

**Enzymatic Reduction of Cytochrome P-450—** In view of the possible function of cytochrome P-450 in n-alkane hydroxylation and of the NADH requirement of this reaction, it was interesting to establish whether cytochrome P-450 could be reduced by NADH. CO does not react with the oxidized P-450 but combines readily with the reduced form of the hemoprotein, giving the characteristic peak at 450 nm. By using this property of cytochrome P-450, it was possible to follow its reduction upon addition of NADH, NADH, and of the NADH requirement of this reaction, it was interesting to establish whether cytochrome P-450 could be reduced by NADH. CO does not react with the oxidized P-450 but combines readily with the reduced form of the hemoprotein, giving the characteristic peak at 450 nm. By using this property of cytochrome P-450, it was possible to follow its reduction upon addition of NADH partially reduced the cytochrome. Complete reduction of cytochrome P-450 is brought about by the addition of NADH in the presence of catalytic amounts of the $S_{60-100}D$ fraction. As shown by the curve of Fig. 6B (---) the reduction of NADH partially reduced the cytochrome. Complete reduction of cytochrome P-450 is brought about by the addition of NADH in the presence of catalytic amounts of the $S_{60-100}D$ fraction (Fig. 6B, ---). Indeed, the CO peak of the enzymatically reduced cytochrome P-450 (Fig. 6B, ---) is comparable to the CO peak obtained by dithionite reduction (Fig. 6A, ---).

**Induction of Cytochrome P-440—** The $S_{25-40}D$ fraction contains 0.25 mmol of cytochrome P-450 per mg of protein. The comparable fraction obtained from acetate-grown cells contains only 0.04 mmol per mg of protein of the CO-binding hemoprotein (in this case all the cytochrome was present in the P-420 form). Thus a 6-fold increase in concentration is induced by growth in the presence of n-octane. A similar induction of cytochrome P-450 occurs in the liver microsomes of phenobarbital-treated animals (13, 27).

The n-octane-hydroxylating activities of enzyme fractions obtained from octane- and acetate-grown cells were tested. The results of these experiments are shown in Table IV. Experiment 1 shows that the high-speed supernatant from acetate-grown cells (S40) is neither active by itself nor when combined with the cytochrome P-450 fraction, $S_{25-40}D$, obtained from n-octane-induced cells. This indicates that the flavoprotein component of the hydroxylating system is not present in non-induced cells. However, the S40 fraction shows some activity when combined with the flavoprotein fraction from the n-octane-induced cells, $S_{60-100}D$. Experiment 2 shows that this activity is concentrated in the $S_{25-40}D$ fraction. As mentioned above, the latter fraction contains low levels of cytochrome P-420 and is spectrally free of the P-450 form. The capability of obtaining reconstitutive activity with the P-420 form of cytochrome is in contrast with the observations of several investigators (27-29) who have shown that microsomal cytochrome P-420, obtained by treatment with detergents, sulfhydryl reagents, and lipolytic or proteolytic enzymes, is catalytically inactive. It is possible that the cytochrome P-420, obtained solely by sonic
disruption of acetate-grown cells and precipitation by ammonium sulfate, allowed this form of the cytochrome to retain some activity.

**NADH Oxidation by S₃ and Derived Ammonium Sulfate Fractions**—The S₃(60–100)D fraction is catalytically active in transferring electrons from NADH to the oxidized cytochrome P-450 (Fig. 6B). It was also observed (Table III) that cytochrome c and DCI inhibit the hydroxylation of the n-octane, suggesting that they may compete with cytochrome P-450 as electron acceptors. In view of these observations, it would be expected that the S₃(60–100)D fraction would catalyze the transfer of electrons from NADH to artificial electron acceptors, cytochrome c and DCI. That this actually does occur can be seen from the following results which compare the activities for NADH oxidation (with various acceptors) by the S₃ and the three derived ammonium sulfate fractions. All fractions were completely devoid of NADH oxidase activity but possessed DCI, cytochrome c, and ferricyanide reductase activities. Of all the fractions assayed, the S₃(60–100)D fraction exhibited the highest specific activities for NADH oxidation with these three electron acceptors. On the basis of a 2-electron change, and in terms of millimicromoles per min per mg of protein (25°), the S₃(60–100)D fraction exhibited specific activities of 335, 22.5, and 485 with the use of DCI, cytochrome c, and ferricyanide, respectively, as electron acceptors for NADH oxidation. Although the activities were distributed throughout all three derivative ammonium sulfate fractions, highest specific activity for the NADH “diaphorase-type” activity was consistently found in the S₃(60–100)D fraction. Since this fraction is required for n-octane hydroxylation, it suggests that a functional NADH flavoprotein component is most probably associated with the n-alkane hydroxylation reaction.

**DISCUSSION**

The enzymatic hydroxylation of n-octane in *Corynebacterium* 7E1C requires NADH and oxygen. The results of the °O incorporation experiment establish conclusively that oxygen is incorporated into n-alkane during hydroxylation. The process can be described by the following reaction

\[
R-\text{CH}_2 + \text{NADH} + \text{H}^+ + \text{O}_2 \rightarrow \\
R-\text{CH}_2\text{OH} + \text{NAD}^+ + \text{H}_2\text{O}
\]

where NADH is the electron donor and O₂ the electron acceptor. Thus the conversion of n-octane to 1-octanol is catalyzed by a mixed function oxidase (30). This term was previously proposed for the a-hydroxylating system of *P. oleovorans*; however, there was no direct evidence to indicate that oxygen is incorporated into n-alkane during hydroxylation. The process can be described by the following reaction

R—CH₄ + NADH + H⁺ + O₂ → 
R—CH₂OH + NAD⁺ + H₂O

NADH Oxidation by S₃ and Derived Ammonium Sulfate Fractions

| Experiment | Fraction | Protein concentration (mg/5.0 ml) | Specific activity (mmol/mg/min) |
|------------|---------|----------------------------------|-------------------------------|
| 1          | S₃(25–40)D | 31                               | 0                             |
|            | S₃(60–100)D | 28                               | 0                             |
|            | S₃(60–100)D | 31                               | 28                            |
|            | S₃(60–100)D | 31                               | 0                             |
|            | S₃(60–100)D | 28                               | 0                             |
|            | S₃(60–100)D | 37                               | 0.45                          |
| 2          | S₃ac     | 37                               | 0.35                          |
|            | S₃(60–100)D | 38                               | 0.16                          |
|            | S₃(60–100)D | 38                               | 0.44                          |

* Millimicromoles of 1-octanol formed per min per mg of total protein (30°).

* S₃ac (fraction from acetate-grown cells) precipitated between 40 and 60% ammonium sulfate concentration.

* S₃ac precipitated between 25 and 40% ammonium sulfate concentration.
There also appears to be a good correlation between hydroxylating activity and cytochrome P-450 concentration in induced cells. 

(d) Cytochrome P 450 can be completely reduced by NADH in the presence of the S$_6$(60-100)D flavoprotein fraction (Fig. 6B). This observation, together with the known NADH requirement for n-octane hydroxylation, suggests that cytochrome P-450 is a natural electron carrier in the hydroxylation system of Corynecracterium. It would be coincidental that two similar oxidation-reduction components, having the same pyridine nucleotide specificity for different electron transport systems, would concentrate in one of the two fractions required for n-octane hydroxylation.

The second fraction that is required for n-octane hydroxylation in Corynecracterium 7E1C, the S$_6$(60-100)D, contains flavoprotein (S). The function of this fraction in the overall hydroxylation system is suggested by its capability of reducing flavoprotein (3). The function of this fraction in the overall hydroxylation system even in the presence of an excess of NADH (Table III); this suggests that the NADH-cytochrome c reductase activity.

The data on NADH oxidation indicate that the S$_6$(60-100)D fraction from Corynecracterium 7E1C has a similar NADH-cytochrome c reductase activity. Moreover, cytochrome c was shown to inhibit the overall hydroxylation reaction even in the presence of an excess of NADH (Table III); this suggests that the NADH-cytochrome c reductase in Corynecracterium may also have NADH-cytochrome c reductase activity.

Presently it is not known whether the n-octane hydroxylation system in Corynecracterium 7E1C requires a nonheme iron protein for catalytic activity. Accordingly, the following tentative scheme for n-alkane hydroxylation in Corynecracterium 7E1C is proposed (where fp = flavoprotein, and cyt = cytochrome):

\[
\text{NADH} \rightarrow \text{fp} \rightarrow ? \rightarrow \text{cyt} \rightarrow \text{P-450} \rightarrow \text{R-CH}_2OH + \text{H}_2O
\]

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