# Hydrocarbon Oxidation by a Bacterial Enzyme System. II. Cofactor Requirements for Octanol Formation from Octane*

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The oxidation of octane to *n*-octanol and octanoic acid has been demonstrated in cell-free, soluble enzyme preparations of a soil bacterium. Evidence has been obtained that the initial oxidative attack on octane requires the presence of either DPNH or DPN, whereas TPNH and TPN are relatively ineffective. The hydrocarbon oxidase system has been separated into two distinct enzyme fractions, both of which are required for octanol formation from octane. One of these fractions is stabilized by ascorbate and the other requires $Fe^{++}$ or $Fe^{+++}$ ions for activity.

Although the ability of a number of aerobic microorganisms to utilize paraffins and other hydrocarbons is well established (ZoBell, 1950; Davis and Updegraff, 1954; Beerstecher, 1954), the detailed mechanism of this unusual oxidative process is not known. In a previous paper evidence was presented that cell-free preparations of a *Pseudomonad* isolated from soil oxidize octane to *n*-octanol, which is then further oxidized to octaldehyde and octanoate (Baptist *et al.*, 1963). The over-all conversion of octane to octanoate was shown to require a pyridine nucleotide, and the presence of DPN-dependent octanol and octaldehyde dehydrogenases in the enzyme system was demonstrated.

It could not be concluded from these earlier findings, however, whether a pyridine nucleotide is also required in the initial oxidative attack on the hydrocarbon. As described below, an improved procedure for separating and determining both radioactive octanol and octanoate has been used to show that a pyridine nucleotide and $Fe^{++}$ ions are involved in the conversion of the hydrocarbon to the corresponding alcohol. The hydrocarbon oxidase system has been separated into two soluble fractions which are required for octanol formation from octane in the presence of the necessary cofactors.

## EXPERIMENTAL PROCEDURES

A *Pseudomonad* previously isolated in this laboratory was grown on hexane as sole carbon source as described earlier by Baptist *et al.* (1963). The packed cells obtained upon centrifugation were suspended in 1–1.5 volumes of 0.1 M $KHCO_3$ and passed through a French pressure cell, previously chilled to 0°, at an outlet pressure of about 15,000 psi. This broken-cell preparation was centrifuged at 80,000 × *g* for 30 minutes and the clear supernatant solution was used as a source of the hydrocarbon-oxidizing enzyme system.

*Preparation of Enzyme Fractions.*—The following operations were carried out at 0–4°. One volume of 5% streptomycin sulfate solution was added slowly with stirring to four volumes of bacterial extract and the mixture was centrifuged at 20,000 × *g*. To the supernatant solution an equal volume of saturated ammonium sulfate solution, adjusted to *p*H 7.2, was added over a 10-minute interval with stirring. After 20 minutes the precipitated protein was collected by centrifugation at 20,000 × *g*, dissolved in 0.1 M Tris buffer, *p*H 7.4, and diluted with buffer to a protein concentration of about 5 mg/ml. This solution was brought to 30% saturation in ammonium sulfate and the resulting precipitate collected as before and dissolved in 0.1 M Tris buffer, *p*H 7.4. This fraction is referred to as "A." The supernatant solution was brought to 45% saturation and the resulting precipitate was taken up in the same buffer to give fraction "B." As described below, these fractions were used to demonstrate the need for two distinct enzymes in the oxidation of octane to octanol.

Enzyme A was partially purified by the use of DEAE-cellulose. For this purpose 10 ml of the streptomycin-treated extract (containing 40–50 mg protein per ml) was diluted to 100 ml with 0.005 M Tris buffer, *p*H 8.0, and adsorbed on a 15 × 2-cm DEAE-cellulose column previously equilibrated with the same buffer. Fifty milliliters of the same buffer was passed over the column, followed by 50 ml of 0.02 M Tris buffer, *p*H 7.4, and enzyme A was eluted with 0.1 M Tris, *p*H 7.0. Ten-milliliter fractions were collected, and the enzyme was found in tubes 17–19. This preparation of A, assayed in the presence of excess B, had about ten times the specific activity of the streptomycin supernatant solution and was free of octanol dehydrogenase and DPNH oxidase activity.

The protein concentration of extracts was estimated by the procedure of Lowry *et al.* (1951) and that of partially purified enzyme preparations according to Warburg and Christian (1941–42).

*Determination of Enzyme Activity.*—An improved assay procedure was devised in which octanol and octanoate, the two major products accumulating in the octane-oxidizing enzyme system, were separated and estimated. Reaction mixtures containing buffer, octane-1-$C^{14}$ (50,000 cpm per μmole) dissolved in ethanol or acetone, enzyme, and suitable cofactors in a final volume of 1 ml were incubated at 28°. No inhibition was observed when alcohol or acetone was used to solubilize the octane. Similar results were obtained when the substrate was dispersed in the presence of small amounts of detergents. Enzyme was omitted from control experiments. The reaction was stopped by the addition of 0.4 ml of 10% $H_2SO_4$. Five milliliters of petroleum ether (bp 30–60°) was then added, and the tubes were tightly stoppered and shaken vigorously on a mechanical shaker for 5 minutes. If necessary, the tubes were centrifuged to separate the

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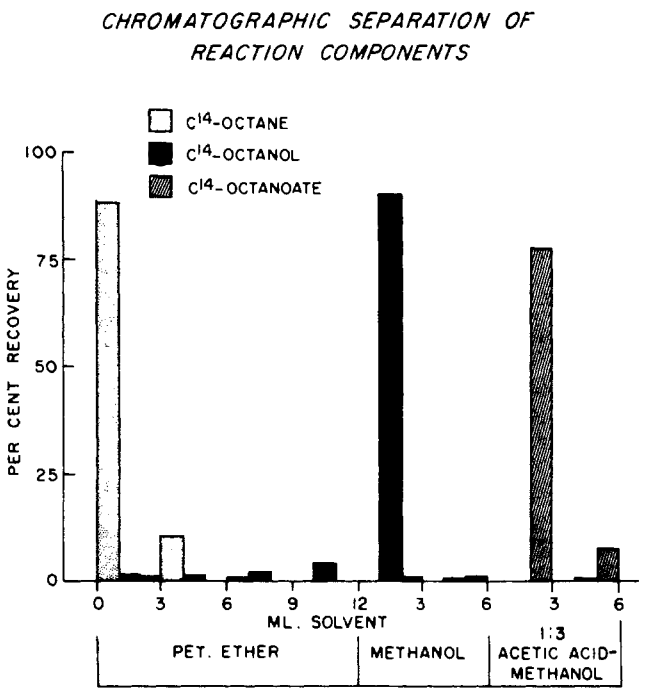


FIG. 1.—Chromatography of radioactive octane, octanol, and octanoic acid. In separate experiments, each compound was applied to an alumina column and eluted with the solvents indicated in the manner described in the text. The values shown are the average of duplicate experiments.

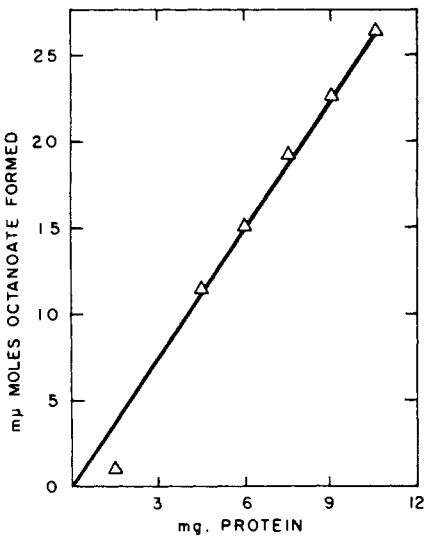


FIG. 2.—Octanoate formation as a function of protein concentration. Reaction mixtures containing 90 μmoles potassium phosphate buffer, pH 7.0, 0.2 μmole DPNH, 4 μmoles ascorbic acid, 1.0 μmole octane-1-$C^{14}$ in 0.05 ml ethanol, and varying amounts of enzyme (0–50% ammonium sulfate fraction) in a total volume of 1 ml were incubated 20 minutes. No significant amount of radioactive octanol accumulated in these reaction mixtures; this enzyme preparation was less active than many.

aqueous and organic layers. Three milliliters of the petroleum ether layer was then passed over a column of 500 mg of alumina (Merck Reagent Aluminum Oxide) in a tube of 8-mm diameter. The column was washed with 10 ml of petroleum ether to remove the octane; octanol was eluted with 3 ml of absolute methanol, and octanoic acid was eluted with 3 ml of 25% glacial acetic acid in absolute methanol. The radioactivity of the eluates was determined with a Packard liquid scintillation spectrometer.

The separation of known radioactive compounds on the column is shown in Figure 1. Octane-1-$C^{14}$ was obtained from the Volk Radiochemical Company. The radioactive octanoic acid used in the experiment had been isolated as a product of the enzymatic oxidation of octane, and the octanol was produced by reducing the octanoic acid with lithium aluminum hydride according to the general procedure of Nystrom and Brown (1947). In other experiments nonradioactive octaldehyde, determined by a colorimetric assay (Lappin and Clark, 1951), was found to be eluted with octane in the petroleum ether wash. Since the radioactive aldehyde does not accumulate in enzyme reaction mixtures except in the presence of trapping agents such as hydroxylamine, the sum of the radioactivity in octanol and octanoate was taken as a measure of hydrocarbon oxidase activity.

The radioactive octanol formed from $C^{14}$-labeled octane in typical enzyme preparations was identified previously by recrystallization to constant radioactivity as the dinitrobenzoate, and radioactive octanoate was identified by reversed-phase column chromatography on siliconized Super-Cel (Baptist *et al.*, 1963). The identity of the "octanol" fraction obtained by alumina chromatography of a similar enzyme reaction mixture was established by gas-liquid chromatography of this fraction on a column of 9% silicone SE 30 on Chromosorb W. Under the conditions employed the radioactive material had the same retention time as authentic *n*-octanol. The octanol peak was collected and found to contain the expected number of counts, whereas no radioactivity was detected prior or subsequent to the appearance of the octanol peak. The identity of the "octanoate" fraction from alumina chromatography of a complete reaction mixture was shown by column chromatography on silicic acid. The octanoic acid fraction, eluted by 5:95 ether-benzene, was found to contain the expected amount of radioactivity. As noted previously (Baptist *et al.*, 1963), an acid somewhat more polar than octanoate is formed in trace amounts in enzyme reaction mixtures. This acid is not eluted from alumina under the conditions employed, however, nor is it eluted from silicic acid with octanoic acid. Furthermore, this more polar acid is present in amounts too small to interfere with the assay procedure even if it were detected.

In numerous experiments in which $Fe^{++}$ ions, pyridine nucleotides, or one of the two necessary enzyme fractions was omitted from the usual incubation mixtures, or when either of the enzyme fractions had become inactive on storage, no radioactivity was detected in the "octanol" or "octanoate" fractions from the alumina column. Thus it may be concluded that the column efficiently separates octanol and octanoate from octane. As an indication that saturating amounts of substrate and cofactors are employed and that the sum of the radioactivity in octanol and octanoate is a reliable measure of the hydrocarbon-oxidizing activity of the enzyme system, the total radioactivity so measured is linear with protein concentration, as described below. No attempts have as yet been made to examine the "octane" fraction from the alumina column for the presence of trace amounts of intermediates, such as a hydroperoxide.

## RESULTS

*Product Formation as a Function of Time and Enzyme Concentration.*—A linear relationship between the amount of enzyme employed and octane oxidation products formed could be shown with partially purified preparations, but not with crude extracts. Figure 2 shows the relationship between protein concentration

TABLE I

NUCLEOTIDE REQUIREMENT FOR OCTANOL FORMATION

The reaction mixtures contained 100 μmoles Tris buffer, pH 7.4, 0.5 μmole $FeSO_4$, 0.65 μmole carrier octanol, 1.0 μmole octane-1-$C^{14}$ in 0.01 ml ethanol, charcoal-treated enzyme (1.4 mg protein), and 0.2 μmole pyridine nucleotide, as indicated, in a total volume of 1 ml, and were incubated for 20 minutes. The enzyme preparation (0–60% ammonium sulfate fraction) had been stirred with activated charcoal (0.4 mg per mg protein) for 30 minutes at 0° and then centrifuged.

| Cofactor Added | Octanol Formed (cpm) | Octanoic Acid Formed (cpm) |
|---|---|---|
| None | 17 | 0 |
| DPNH | 1628 | 893 |
| DPN | 899 | 864 |
| TPNH | 416 | 65 |
| TPN | 357 | 43 |

(of a 0–50% ammonium sulfate fraction) and octanoate formation. In this system, as in most relatively crude preparations, the dehydrogenases which convert octanol to octanoate were apparently present in excess and no octanol-$C^{14}$ accumulated. The enzyme preparation used in this experiment was somewhat less active than usual. Typical yields of products, as stated previously (Baptist *et al.*, 1963), were about 8–32% in the presence of 10 mg protein. For example, in Table I, in the experiment in which DPNH was present, the yield of radioactive products was 36% calculated per 10 mg protein. Occasionally a crude extract was obtained in which octanol was the major product accumulating from octane-1-$C^{14}$ oxidation. Figure 3 shows the time course of octanol and octanoate formation in such an extract.

*Nucleotide Requirement for Octanol Formation.*—Evidence for the presence of DPN-linked octanol and octaldehyde dehydrogenases in the bacterial enzyme preparations was presented previously, and it was reported that treatment of crude extracts with charcoal greatly diminished octanoate formation. Such preparations could be reactivated with DPN, TPNH, or a boiled juice (Baptist *et al.*, 1963). Since octanol was not measured by the assay procedure then being used, it was not clear whether a pyridine nucleotide is required for octane oxidation as well as in the reactions which convert octanol to octanoic acid. Experiments were therefore carried out in which the column assay procedure described above was used to measure both octanol and octanoate (Table I). Charcoal treatment of the enzyme preparation markedly decreased octane oxidation, but activity was restored completely by the addition of DPNH, somewhat less by DPN, and only partially by TPN or TPNH. In several other experiments not reported here DPN and DPNH were found to be equally active, and TPN or TPNH about one-seventh as effective. These results clearly indicate that a pyridine nucleotide participates in the conversion of octane to octanol.

Because of these findings DPNH was included in the reaction mixtures in subsequent experiments. Attempts to distinguish between oxidized and reduced DPN as the cofactor for octanol formation have so far been unsuccessful, presumably because these forms are interconverted by DPNH oxidase and octanol and octaldehyde dehydrogenases known to be present in the hydrocarbon oxidase system.

*Requirement for Two Enzyme Fractions for Octanol Formation.*—Attempts to purify the hydrocarbon-oxidizing enzyme system by ammonium sulfate fractionation resulted in apparent loss of activity. For example, fraction A usually had about 10% of the original activity while fraction B had none. The addition of B to A, however, resulted in 4- to 10-fold stimulation of the activity of A alone. This effect was even more striking when enzyme A was prepared by DEAE chromatography or by fractionation twice with ammonium sulfate. As shown in Table II, hydrocarbon-oxidizing activity was then found to be almost completely dependent on the presence of both enzyme fractions. Neither A nor B could be replaced by pyridine nucleotides, boiled juice, ascorbate, iron salts, $H_2O_2$, or catalase. Attempts to demonstrate the formation by either fraction of a stable intermediate compound which could then be utilized by the other fraction have so far not proved successful.

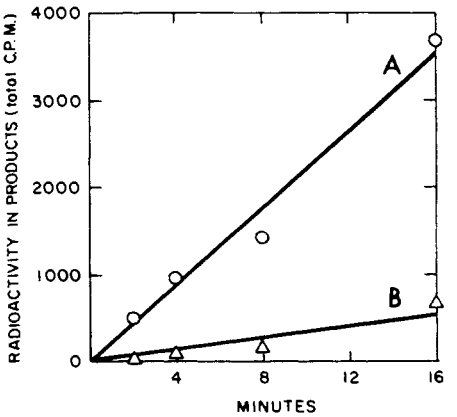


FIG. 3.—Octanol and octanoate formation as a function of time. The reaction mixtures contained 50 μmoles Tris buffer, pH 7.4, 0.2 μmole DPNH, 0.5 μmole $FeCl_3$, 0.8 μmole octane-1-$C^{14}$ in 0.04 ml ethanol, and bacterial extract (5 mg protein) in a total volume of 1 ml and were incubated at 28° for the time intervals indicated. Curve A, octanol formation; Curve B, octanoate formation.

*Stabilization of Fraction B by Ascorbate.*—Whole cells and crude extracts retained activity for at least a month when kept frozen, but after partial purification with ammonium sulfate enzymatic activity was rapidly lost upon storage. Preliminary tests showed that ascorbate was effective in preserving the activity of

TABLE II

REQUIREMENT FOR TWO ENZYME FRACTIONS FOR HYDROCARBON OXIDATION

The reaction mixtures contained 50 μmoles Tris buffer, pH 7.4, 0.2 μmole DPNH, 0.5 μmole $FeCl_3$, 1 μmole carrier *n*-octanol where indicated, 0.8 μmole octane-1-$C^{14}$ in 0.04 ml ethanol, and enzyme fractions in a total volume of 1 ml and were incubated for 20 minutes.

| Experiment | Enzyme Fractions Present | Carrier Octanol Added | Radioactivity in Products (cpm) | |
|---|---|---|---|---|
| | | | Octanol | Octanoic Acid |
| 1[a,b] | A | − | 0 | 75 |
| | B | − | 13 | 0 |
| | A + B | − | 2123 | 302 |
| 2[b,c] | A | + | 0 | 0 |
| | B | + | 2 | 8 |
| | A + B | + | 835 | 75 |
| | A + B | − | 58 | 1230 |

[a] Enzyme A prepared by DEAE chromatography (0.5 mg protein). [b] Usual B fraction (2.0 mg protein). [c] Usual A fraction carried through an additional fractionation with ammonium sulfate, 0–26% saturation (2.0 mg protein).

TABLE III

EFFECT OF ASCORBATE IN STABILIZING ENZYME FRACTIONS

Fractions A and B were prepared as described in the text, dissolved in 0.1 M Tris buffer, *p*H 7.4, or Tris buffer containing 0.01 M ascorbate, and stored in an ice-bath for the intervals indicated before being assayed. The reaction mixtures were like those in Table II except that 0.3 μmole octane-1-$C^{14}$ was used. A and B were added in the amount of 9.4 and 2.4 mg, respectively.

| Enzyme Preparation | Radioactivity in Products (cpm) | | | | | |
|---|---|---|---|---|---|---|
| | 0 hours | | 21 hours | | 40 hours | |
| | Octanol | Octanoate | Octanol | Octanoate | Octanol | Octanoate |
| A | 0 | 13 | | | 35 | 0 |
| B | 23 | 218 | | | 0 | 0 |
| A + B | 33 | 1610 | 0 | 20 | 17 | 7 |
| A in ascorbate | 0 | 15 | | | 0 | 0 |
| B in ascorbate | 23 | 160 | | | 8 | 62 |
| A in ascorbate + B in ascorbate | 38 | 1668 | 37 | 1510 | 0 | 842 |
| A in ascorbate + B | | | 0 | 37 | 10 | 35 |
| A + B in ascorbate | | | 20 | 1208 | 10 | 910 |

ammonium sulfate fractions, and the data in Table III indicate that the stabilizing effect of ascorbate is specifically on fraction B. Other experiments have shown that once preparations have lost activity it is not restored by ascorbate, metal ions, or boiled juice.

*Metal Requirement for Octanol Formation.*—One of the possible mechanisms for the conversion of octane to octanol is a direct attack (hydroxylation) by an "activated oxygen." Since many oxygen transferases and some mixed-function oxidases have been shown to have a requirement for $Fe^{++}$ ions (cf. Mason, 1957), the possible involvement of this metal in octanol formation was investigated. The addition of ferrous ions to our usual bacterial enzyme preparations gave no consistent stimulation of hydrocarbon oxidase activity. However, preincubation of the enzyme system with iron-binding agents such as *o*-phenanthroline, α,α'-dipyridyl, or 8-hydroxyquinoline at a concentration of $5 \times 10^{-3}$ M inhibited enzyme activity by about 90%. More direct evidence for a metal requirement was obtained with enzyme fractions inactivated by dialysis, as summarized in Table IV. The results indicate that less than 10% of the activity remained after dialysis but that this could be restored to about 60% that of the undialyzed preparation by $5 \times 10^{-4}$ M $FeSO_4$ or $FeCl_3$. In other experiments it was shown that higher concentrations of these salts produced no further stimulation and that addition of boiled juice to iron-activated preparations produced no further increase in activity. Apparently the enzyme preparation is partially denatured on dialysis, since the original activity is not entirely restored. Experiments showing that $Mg^{++}$, $Ca^{++}$, and $Al^{+++}$ ions are also effective in restoring hydrocarbon oxidase activity are summarized in Table V. In other experiments it was shown that $5 \times 10^{-4}$ M $Cu^{++}$, $Zn^{++}$, $Ni^{++}$, and $Co^{++}$ are ineffective in substituting for iron.

Use was made of the inhibitory effect of 8-hydroxyquinoline to establish which of the two enzyme fractions involved in hydrocarbon oxidation is metal-requiring. This reagent was shown to be strongly inhibitory when preincubated with the complete enzyme system for 10 minutes, but to inhibit only about 20% when added at the same time as the other components of the assay incubation mixture. The data in Table VI show that preincubation of fraction A with 8-hydroxyquinoline resulted in extensive loss of activity whereas fraction B was not appreciably affected by such treatment.

TABLE IV

STIMULATION OF OCTANOL FORMATION BY FERROUS AND FERRIC IONS

Reaction mixtures containing 50 μmoles Tris buffer, *p*H 7.4, 0.8 μmole octane-1-$C^{14}$ in 0.04 ml ethanol, 0.2 μmole DPNH, and enzyme (0–60% ammonium sulfate fraction; 10 mg protein) in a total volume of 1 ml were incubated for 20 minutes. The enzyme preparation had been dialyzed for 17 hours against 80 volumes of 0.1 M Tris buffer, *p*H 7.4, containing 0.01 M ascorbate, with a change of buffer solution at 5 hours.

| Enzyme Preparation | Metal Salt Added | Radioactivity in Products (cpm) | |
|---|---|---|---|
| | | Octanol | Octanoate |
| Undialyzed | None | 77 | 4280 |
| Dialyzed | None | 3 | 375 |
| | $FeSO_4$ ($1 \times 10^{-4}$ M) | 20 | 1040 |
| | $FeSO_4$ ($5 \times 10^{-4}$ M) | 90 | 2520 |
| | $FeCl_3$ ($1 \times 10^{-4}$ M) | 17 | 940 |
| | $FeCl_3$ ($5 \times 10^{-4}$ M) | 93 | 2475 |

## DISCUSSION

It may be concluded from the results presented that DPNH (or DPN) and $Fe^{++}$ (or $Fe^{+++}$) ions participate in the initial oxidative attack at a methyl group of the

TABLE V

STIMULATION OF DIALYZED PREPARATIONS BY VARIOUS METAL IONS

The reaction mixtures contained 50 μmoles Tris buffer, *p*H 7.4, 0.2 μmole DPNH, 0.3 μmole octane-1-$C^{14}$ in 0.04 ml ethanol, and fractions A and B (1.1 and 1.4 mg protein, respectively). The enzyme fractions had been dialyzed for 48 hours against 50 volumes Tris-ascorbate, with a change of buffer solution at 24 hours. Reagent grade metal salts were added as indicated.

| Metal Salt Added | Final Concentration (M) | % Maximal Activity |
|---|---|---|
| None | | 0 |
| $FeSO_4$ | $5 \times 10^{-4}$ | 100 |
| $FeSO_4$ | $1 \times 10^{-4}$ | 35 |
| $MgCl_2$ | $5 \times 10^{-4}$ | 48 |
| $MgCl_2$ | $1 \times 10^{-4}$ | 6 |
| $CaCl_2$ | $5 \times 10^{-4}$ | 90 |
| $CaCl_2$ | $1 \times 10^{-4}$ | 17 |
| $Al_2(SO_4)_3$ | $5 \times 10^{-4}$ | 84 |
| $Al_2(SO_4)_3$ | $1 \times 10^{-4}$ | 70 |

TABLE VI

EVIDENCE THAT FRACTION A REQUIRES METAL IONS

The reaction mixtures contained 50 μmoles Tris buffer, pH 7.4, 0.2 μmole DPNH, 0.8 μmole octane-1-$C^{14}$ in 0.04 ml ethanol, and enzyme preparations in a total volume of 1 ml, and were incubated for 10 minutes. A and B were each added in the amount of 3 mg protein.

| Experiment No. | Components Added | Final Concentration of 8-Hydroxyquinoline (M) | Radioactivity in Products (cpm) | |
|---|---|---|---|---|
| | | | Octanol | Octanoic Acid |
| 1 | A | 0 | 8 | 900 |
| 2 | B | 0 | 8 | 3 |
| 3 | A + B | 0 | 42 | 4770 |
| 4[a] | A + B | $1 \times 10^{-3}$ | 52 | 3800 |
| 5 | A + B[b] | $1 \times 10^{-3}$ | 35 | 3240 |
| 6 | A[b] + B | $1 \times 10^{-3}$ | 0 | 133 |
| 7 | A[b] + B[b] | $2 \times 10^{-3}$ | 15 | 97 |

[a] 8-Hydroxyquinoline added at beginning of assay. [b] Preincubated with $5 \times 10^{-3}$ M 8-hydroxyquinoline for 10 minutes at 28°.

substrate. Whether octanol or octanoate is the major product accumulating in a particular enzyme preparation depends upon the amount of octanol and octaldehyde dehydrogenases present, DPN being in adequate supply for these further oxidative reactions because of the action of a DPNH oxidase in the preparations. On the other hand, as reported previously, octaldehyde does not accumulate unless suitable aldehyde-binding agents are added. The failure of octanol to accumulate when $Fe^{++}$ ions or pyridine nucleotide is omitted cannot be attributed to product inhibition since in many experiments (cf. Fig. 3) octanol is formed in much larger amounts than octanoate. Furthermore, the addition of $10^{-3}$ M carrier octanol does not block the conversion of $C^{14}$-octane to radioactive octanol. This amount of carrier octanol is in considerable excess over the amounts of radioactive octanol formed in the experiments described above.

Intermediates in octanol formation have so far not been detected upon incubating fraction A or B alone with $C^{14}$-octane and the necessary cofactors. On the other hand, the formation of a labile intermediate such as a hydroperoxide cannot be ruled out at this time. The finding that metal ions such as $Mg^{++}$, $Ca^{++}$, and $Al^{+++}$ partially replace $Fe^{++}$ was unexpected and raises the question of whether iron plays some role other than participation in an oxidation-reduction sequence.

There appears to be general agreement that the oxidative attack by bacteria on paraffins having seven or more carbon atoms is at a methyl, rather than methylene, group. The conversion of heptane to heptanoic acid (as well as shorter chain acids) in cultures of *Pseudomonas aeruginosa* has been described by Senez and Konovaltschikoff-Mazoyer (1956). Stewart *et al.* (1959) have identified cetyl palmitate as the product of hexadecane oxidation in cultures of a gram-negative coccus, and Stewart and Kallio (1959) have reported that waxes produced from other paraffins have a primary alcohol moiety with the same carbon skeleton, whereas the acid moiety may have a different chain length. Kester and Foster[1] have recently obtained evidence that a *Corynebacterium* strain oxidizes normal paraffins containing 10–14 carbon atoms to the corresponding dicarboxylic acids and converts fatty acids to the corresponding ω-hydroxy and dicarboxylic acids.

[1] Personal communication from Dr. J. W. Foster describing studies carried out in his laboratory by A. S. Kester (1961).

The conversion of octane to octanol and octanoate in our cell-free enzyme preparations is thus in accord with the oxidative pathway known to occur in intact bacteria.

Whereas the enzymatic pathway for hydrocarbon metabolism under study in this laboratory is an aerobic one, Azoulay and Senez (1958) have described an anaerobic reaction by which paraffins undergo dehydrogenation in extracts of a strain of *Pseudomonas aeruginosa* in the presence of pyocyanin. Chouteau *et al.* (1962) have recently reported that resting cells of this microorganism incubated anaerobically with heptane produce 1-heptene, which was identified by its infrared spectrum.

Several recent reports indicate that flavins may participate in a number of similar enzymatic reactions involving pyridine nucleotides and oxygen. Katagiri *et al.* (1962) have established an FAD requirement for the conversion of salicyclic acid to catechol, and Fulco and Bloch (1962) have described an FAD requirement for fatty acid desaturation in *Mycobacterium phlei*. Previously, Conrad *et al.* (1961) found that FMN participates in the lactonization of diketocamphane. Although earlier attempts to show a role of flavin in hydrocarbon oxidation were negative, we have recently found instances in which enzyme fractions A and B treated with Sephadex have greater activity when boiled juice or FAD is added, as well as DPNH and $Fe^{++}$ ions.[2] Studies are in progress to determine whether one of these enzyme fractions is a flavoprotein.

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