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Purification and Characterization of a Secondary Alcohol Dehydrogenase from a Pseudomonad

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Growth of *Pseudomonas* sp. NRRL B3266 in the presence of oleic acid resulted in the induction of two enzymes: oleate hydratase, which produced 10(*R*)hydroxyoctadecanoate, and hydroxyoctadecanoate dehydrogenase, which catalyzed the oxidized nicotinamide adenine dinucleotide-dependent production of 10-oxooctadecanoate. This latter enzyme was purified to homogeneity and shown to consist of two polypeptide chains of about 29,000 daltons each. The enzyme had a broad substrate specificity, catalyzing the dehydrogenation of a number of 18-carbon hydroxy fatty acids. The kinetic parameters for various 10- and 12-hydroxy fatty acids were similar (K_m ca. 5 μM and V_{max} ca. 50 to 200 μmol/min per mg of protein). The enzyme also catalyzed the dehydrogenation of unsubstituted secondary alcohols. The effectiveness of these alcohols as substrates was highly dependent on their hydrophobicity, the K_m decreasing from 9 mM for 4-heptanol to 7 μM for 6-dodecanol. Inhibition of the enzyme by primary alcohols also showed a dependence on hydrophobicity, the K_i decreasing from 350 mM for methanol to 90 μM for decanol.

The metabolism of oleic acid by *Pseudomonas* sp. NRRL B3266 involves two inducible enzymes, a hydratase that converts oleic acid to 10(*R*)hydroxyoctadecanoic acid (19–21) and a dehydrogenase that produces 10-oxooctadecanoic acid. The dehydrogenase has unique substrate specificity, functioning with a variety of hydroxy fatty acids and hydrophobic secondary alcohols. This enzyme has been purified to homogeneity, and its catalytic mechanism has been studied by steady-state kinetic analysis. Preliminary accounts of this work have been presented (W. Niehaus, Fed. Proc. **35**:1748, 1976; T. Frielle and W. Niehaus, Fed. Proc. **36**:794, 1977).

MATERIALS AND METHODS

Growth of organism and preparation of cell-extract. *Pseudomonas* sp. NRRL B3266 was grown on a medium consisting of 1% NZ amine, type ET (Sheffield), 1% yeast extract, 1% glucose, 0.5% potassium phosphate buffer (pH 6.8), 0.1% $MgSO_4$, and 0.03% oleic acid in a 110-liter New Brunswick fermentor. Typical yield was 10 g (wet weight) of cells per liter. The cell paste was stored frozen until use, when the cells were suspended in 4 volumes of 0.05 M potassium phosphate buffer (pH 6.8) at 4°C and broken by sonic oscillation (4 min for each 100 ml at 85 W) or by two passes through a Gaulin cell disruptor. The cell debris was removed by centrifugation at 16,000 × *g* for 30 min, leaving a very turbid supernatant fluid.

Enzyme purification. All operations were performed at 4°C. To the cell extract was added $(NH_4)_2SO_4$ to a concentration of 1 M. The extract was then stirred with 0.25 volume of 10-carboxydecyl-Sepharose prepared from cross-linked Sepharose 4B (Pharmacia) and 11-aminoundecanoic acid (Pfaltz and Bauer, Inc.) by the method of Schöpp et al. (24). After 1 h, the slurry was allowed to settle into a column, which was then washed with 3 column volumes of 1 M $(NH_4)_2SO_4$ in 0.05 M potassium phosphate buffer (pH 6.8). Enzyme was then eluted with 1 M ethanol in 0.8 M potassium phosphate buffer (pH 8.5) (24). The enzymatic activity, which was eluted in a rather large volume (10 column volumes), was concentrated by ultrafiltration with an Amicon PM 30 membrane, buffer-exchanged with 0.05 M potassium phosphate (pH 6.8) on a Sephadex G-25 column, and then concentrated further by precipitation by $(NH_4)_2SO_4$ (3.3 mol/kg). The precipitate was dissolved in a minimum volume of 1.0 M potassium phosphate (pH 6.8) and applied to a carboxydecyl-Sepharose column, 50% the size of the first column, that had been equilibrated with this buffer. The column was washed with 2 column volumes of 1 M potassium phosphate (pH 6.8) followed by a gradient of 1 column volume each of 1 and 0 M phosphate. Elution of the enzyme was completed with 0.025 M potassium phosphate (pH 6.8) (see Fig. 1). Active fractions were pooled and concentrated by ultrafiltration with an Amicon PM 10 membrane.

Enzyme assays. Kinetic assays were run (in a 3.0-ml total volume) in 1-cm cuvettes by measuring absorbance changes at 340 nm with a Gilford optical density converter. The routine assay mixture consisted

of 0.05 M sodium pyrophosphate (adjusted to pH 9.0 with HCl), 0.2% Lubrol WX, 1.0 mM nicotinamide adenine dinucleotide (NAD), and 1.0 mM 12-hydroxyoctadecanoate. Pyridine nucleotides, except for thionicotinamide adenine dinucleotide and 3-aminopyridine adenine dinucleotide, which were gifts from B. M. Anderson, were obtained from Sigma Chemical Co. Of the fatty acid substrates, 12-hydroxyoctadecanoic acid was purchased from Pfaltz and Bauer and purified by recrystallization. The *R* and *S* isomers of 9- and 10-hydroxyoctadecanoate were prepared as previously described (19). *threo*-9,10-Dihydroxyoctadecanoic acid and 12-oxooctadecanoic acid were chemically synthesized from oleic acid and from 12-hydroxyoctadecanoic acid (8, 27). Primary and secondary alcohols were purchased from Aldrich Chemical Co., and from K & K Laboratories. All fatty acids and alcohols were added to reaction mixtures as solutions in freshly redistilled dimethyl sulfoxide. Dimethyl sulfoxide had no effect on kinetic parameters of the enzyme reactions over the range of concentrations used. The nonionic detergent Lubrol WX was a gift of ICI America, Inc.

Data analysis. All linear plots were generated from a least-squares regression analysis (Wang Laboratories no. 1000-2-ST3). All data points received equal statistical weight.

Physical characterization of the enzyme. For amino acid analysis, samples of protein were hydrolyzed under vacuum with constantly boiling HCl at 110°C for 24, 48, or 72 h. A parallel sample was oxidized with HCOOOH before hydrolysis for determination of cysteine and methionine. Hydrolysates were analyzed using a Spinco 120B automatic amino acid analyzer. Sedimentation equilibrium analyses were performed by Lewis Barnett, who followed the meniscus depletion method of Yphantis (29). Protein at a concentration of 0.5 mg/ml in 0.01 M potassium phosphate buffer (pH 6.8) was centrifuged in a Spinco model E ultracentrifuge at 24,630 and 21,740 rpm for 36 h at 4.5°C. Protein distribution was measured with interference optics, and molecular weight was calculated from a linear regression analysis of the data, using a value of $\bar{v} = 0.74$ calculated from the amino acid composition (11). Isoelectric focusing was performed by using an LKB 110-ml instrument with narrow range (pH 4 to 6) Ampholyte mixture. The pH gradient was established over a 24-h period before addition of the enzyme. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed by Philip Pekala, according to the method of Fairbanks et al. (13). Ovalbumin, bovine serum albumin, phosphorylase A, and β-galactosidase were used as molecular weight markers.

RESULTS

The outline and results of the purification of hydroxyoctadecanoate dehydrogenase from *Pseudomonas* sp. NRRL B3266 are summarized in Table 1. A 285-fold purification with a yield of 70% was obtained. Purification of enzymes from this organism was complicated by the presence of insoluble, difficultly sedimentable intracellular material. Until this material was removed, conventional procedures for protein fractionation such as protamine sulfate fractionation, ammonium sulfate fractionation, and ion-exchange chromatography were ineffective. We were able to effect the separation of the dehydrogenase by hydrophobic chromatography on carboxydecyl Sepharose by following the procedure of Schöpp et al. (24). The enzyme bound to the affinity column, which presumably mimicked the hydrophobic substrate while the majority of protein and insoluble material passed through. The dehydrogenase was then eluted by lowering the dielectric strength of the medium with ethanol. After concentration of the eluate, rechromatography on carboxydecyl Sepharose with elution by decreasing the ionic strength of the buffer afforded extensive purification with nearly quantitative recovery (Fig. 1).

No dehydrogenase activity was detectable in extracts of organisms grown in the absence of oleic acid.

Purity. Chromatography of the enzyme from the second carboxydecyl Sepharose column on Cellex D or on Sephadex G-150 demonstrated a single protein peak with constant specific catalytic activity. Electrophoresis of 50 μg of the enzyme on polyacrylamide gels at pH 8.9 produced a single band, stainable by Coomassie brilliant blue. Minor traces of contaminating protein that were observed upon electrophoresis of larger quantities of protein could be removed by conventional ion-exchange or gel permeation chromatography.

Isoelectric point. Upon isoelectric focusing of the crude cell extract, dehydrogenase activity was localized in a narrow band centered at pH 4.5.

Molecular weight. The molecular weight as determined by sedimentation equilibrium anal-

TABLE 1. *Purification of pseudomonas hydroxyoctadecanoate dehydrogenase*

Purification step	Vol (ml)	Total protein (mg)	Sp act (U/mg of protein)	Total activity (U)	Recovery (%)	Purification factor (fold)
16,000 × *g* Supernatant	1,200	12,000	0.7	8,400	100	1
Carboxydecyl Sepharose no. 1	3,000	800	8.4	6,700	80	12
Carboxydecyl Sepharose no. 2	50	30	200	6,000	71	285

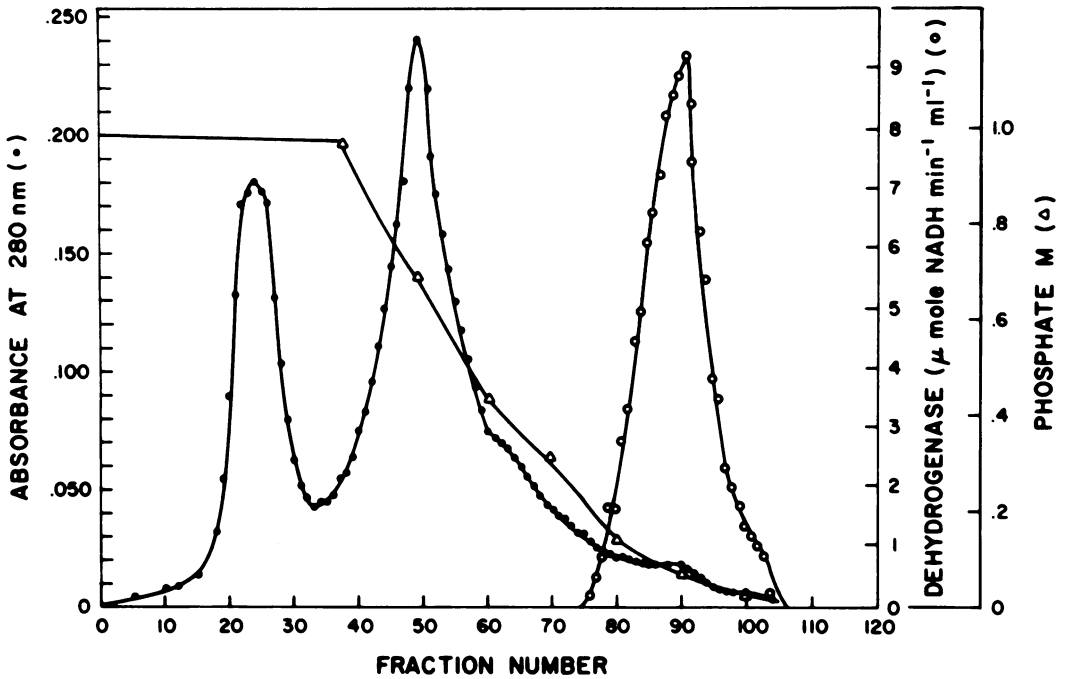

FIG. 1. *Purification of hydroxyoctadecanoate dehydrogenase by hydrophobic chromatography on carboxydecyl Sepharose. Ten-milliliter fractions were collected. Symbols: ●, absorbance at 280 nm; ○, dehydrogenase activity; △, concentration of potassium phosphate (pH 6.8).*

ysis was 5.7×10^4. Replicate analyses at different rotor speeds agreed to within 3%. The molecular weight as determined by gel permeation chromatography on a calibrated column of Sephadex G-150 was 6×10^4. The molecular weight as determined by electrophoresis in sodium dodecyl sulfate-polyacrylamide gel columns in the presence of 2-mercaptoethanol was 2.9×10^4, indicating that the enzyme contains two subunit polypeptide chains. It is not known at present whether the polypeptide chains are identical.

Amino acid composition. The corrected amino acid composition of hydroxyoctadecanoate dehydrogenase is presented in Table 2. From these data, an average hydrophobicity of 1,100 calories (ca. 4,600 J) per residue was calculated by the method of Bigelow (6). This value is not appreciably greater than the mean value published by Bigelow for over 150 proteins. Thus, although hydroxyoctadecanoate dehydrogenase must have a hydrophobic binding site for substrates, the protein molecule as a whole is no more hydrophobic than other typical globular proteins.

Extinction coefficient. Using the amino acid analysis data to quantitate protein, we determined that the extinction coefficient at 280 nm was 0.5 ml mg^{-1} cm^{-1}.

Catalytic properties. For the assay of the dehydrogenase, we routinely used the commercially available substrate 12-hydroxyoctadecanoate, although the enzyme has a wide substrate specificity (see below). The Michaelis constants for 12-hydroxyoctadecanoate, 12-oxooctadecanoate, oxidized NAD (NAD^+), and reduced NAD (NADH) are presented in Table 3. The Michaelis constants for the fatty acids were well below their critical micellar concentrations and presumably represented interaction of the enzyme with individual fatty acid molecules. However, analogous to unsubstituted fatty acids, the solution possibly also contained a significant proportion of dimeric and oligomeric species (26). Routine assays were facilitated by the inclusion of 0.2% Lubrol WX, a nonionic detergent (16-cetylpolyoxyethylene ether). Partition of the fatty acid substrate between free solution and detergent micelles resulted in about a 10-fold increase in the apparent K_m with no change in the maximum velocity over a wide range of enzyme concentrations. The interaction of substrates and products with detergents is undergoing further study in our laboratory. The enzyme catalyzed the dehydrogenation of 12-hydroxyoctadecanoate in the presence of detergent at pH 9.0 and 25°C with a turnover of about 12,000 mol/min per 57,000 g of protein.

The enzyme exhibited a fairly broad pH op-

TABLE 2. *Amino acid composition of hydroxyoctadecanoate dehydrogenase*

Amino acid	Mol/57,000 g of protein			
	24 h	48 h	72 h	Corrected
Lysine	46	46	44	46
Histidine	11	11	9	11
Arginine	7	6	6	6
Aspartate	66	66	66	66
Threonine	44	42	37	48
Serine	18	16	12	20
Glutamate	44	48	46	46
Proline	29	26	29	28
Glycine	32	32	35	33
Alanine	59	59	59	59
Half-cystine[a]	—[b]	—	—	7
Valine	35	32	35	34
Methionine[c]	—	—	—	10
Isoleucine	29	32	32	32
Leucine	39	46	44	45
Tyrosine	11	11	8	11
Phenylalanine	26	26	31	28
Tryptophan[d]	—	—	—	3

[a] Determined as cysteic acid after performic acid oxidation.

[b] —, Not determined.

[c] Determined as methionine sulfone after performic acid oxidation.

[d] Determined spectrophotometrically (12).

TABLE 3. *Michaelis constants for fatty acid and nucleotide substrates measured at pH 9.0 in the absence of detergent*[a]

Substrate	K_m (M)
12-Hydroxyoctadecanoate	4.0×10^{-6}
12-Oxooctadecanoate	3.0×10^{-5}
NAD^+	5.0×10^{-5}
NADH	2.5×10^{-5}

[a] Concentrations of nonvaried substrates: NAD, 1×10^{-3} M; NADH, 2×10^{-4} M; 12-hydroxyoctadecanoate, 5×10^{-5} M; 12-oxooctadecanoate, 8×10^{-5} M.

timum for dehydrogenation of 12-hydroxyoctadecanoate (maximum about pH 10) and reduction of 12-oxooctadecanoate (maximum about pH 7).

The equilibrium constant for the dehydrogenation of 12-hydroxyoctadecanoate was determined at pH 8.0, by the method of Cleland (9) (Fig. 2). In a cuvette at 25°C were mixed 50 mM potassium phosphate buffer (pH 8.0), 12-hydroxyoctadecanoic acid (10 μM), 12-oxooctadecanoic acid (20 μM), and NADH (20 μM). The concentration of NAD^+ was varied from 4.0 to 20 μM. The reaction was initiated with the enzyme, and the total change in NADH concentration was monitored at 340 nm. The intersection of the experimental curve with the axis drawn through zero absorbance change indicates the concentration of NAD^+ that yielded a thermodynamic equilibrium mixture with the above concentrations of the other reactants. Using this value (9.7 μM NAD^+), we calculated an apparent equilibrium constant of $4.1 \pm 0.3 \times 10^{-8}$, corresponding to K_{eq} (pH 7.0) of 0.41 ± 0.03. Comparable values for K_{eq} were calculated from the concentration of NADH present at the cessation of reaction when NAD^+ plus hydroxyoctadecanoate or NADH plus oxooctadecanoate were the only reactants present at zero time.

Substrate specificity. The enzyme was highly specific for NAD^+ as coenzyme, exhibiting less than 2% of the V_{max} activity with 0.2 mM oxidized NAD phosphate ($NADP^+$), thionicotinamide adenine dinucleotide, acetylpyridine adenine dinucleotide, or 3-aminopyridine adenine dinucleotide. The substrate specificity for the secondary alcohol was quite broad, however. A number of hydroxy fatty acids had similar K_m and V_{max} values (Table 4). Ricinelaidic acid, having a *trans* double bond beta to the hydroxyl group, was a good substrate, whereas ricinoleic acid, with a *cis* double bond, was not a substrate but a competitive inhibitor. The vicinal hydroxyl groups of *threo-* and *erythro-*9,10-dihydroxy-octadecanoic acids were poorer substrates for dehydrogenation, and good kinetic data were not obtained for the *erythro* isomer due to solubility problems. The stoichiometry of dehydrogenation of dihydroxy fatty acids and identity of the products are being investigated currently in this laboratory. A free carboxylate group on the substrate is not required; 12-hydroxyoctadecanoic acid methyl ester and 1,12-dihydroxyoctadecane will function as substrates. Kinetic data are not available due to low solubility.

Unsubstituted secondary alcohols also served as substrates for the dehydrogenase (Table 4).

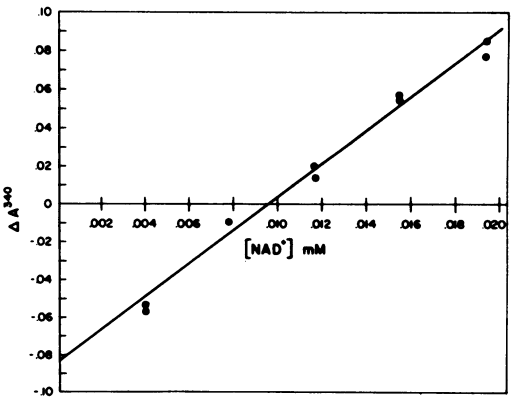

FIG. 2. *Determination of the equilibrium constant for hydroxyoctadecanoate dehydrogenase.* ΔA^{340}, *Change in absorbance at 340 nm.*

The structural requirements of the substrate appeared to be a minimum of two linear methylene groups on each side of the carbinol, because 2-decanol and 2-nonanol were not dehydrogenated. The cyclic secondary alcohols cyclohexanol, cyclooctanol, and cyclododecanol were not substrates. The kinetic parameters K_m and V_{max} for these secondary alcohols were related to the hydrophobicity of the substrates, as expressed by the water-octanol partition coefficient (Fig. 3). The linear free-energy plot relating K_m and P (octanol-water partition coefficient) has a slope of −1.1, corresponding to 780 calories (ca. 3,300 J) per mol for each additional CH_2 group in the substrate. This value is rather large, indicating that the enzyme-substrate interaction was particularly sensitive to hydrophobic interactions (14).

TABLE 4. *Michaelis constants and maximal velocities for various-hydroxy fatty acids and secondary alcohols*[a]

Substrate	K_m (M)	V_{max} (μmol/min per mg of protein)
9(*R*)Hydroxyoctadecanoate	7.0×10^{-6}	40
9(*S*)Hydroxyoctadecanoate	5.5×10^{-6}	40
10(*R*)Hydroxyoctadecanoate	2.5×10^{-6}	150
10(*S*)Hydroxyoctadecanoate	4.0×10^{-6}	100
12(*R*)Hydroxyoctadecanoate	4.0×10^{-6}	200
12(*R*)hydroxy-*trans*-octadec-9-enoate[b]	4.5×10^{-6}	140
threo-9,10-Dihydroxyoctadecanoate[c]	2.5×10^{-5}	40
4-Heptanol	9.0×10^{-3}	15
4-Octanol	1.0×10^{-3}	20
5-Nonanol	2.5×10^{-4}	40
5-Decanol	8.0×10^{-5}	100
5-Undecanol	2.0×10^{-5}	120
6-Dodecanol	7.0×10^{-6}	220

[a] NAD concentration, 1×10^{-3} M. Assayed at pH 9.0 in the absence of detergent.
[b] Ricinelaidic acid.
[c] Racemic mixture.

Initial velocity studies in absence of product. When hydroxyoctadecanoate was the variable substrate at various fixed concentrations of NAD^+, double-reciprocal plots of the data converged and intersected above the x coordinate. A similar pattern was observed when NAD^+ was varied at fixed concentrations of hydroxyoctadecanoate. Kinetic constants were calculated by replotting these data (9).

Inhibition by primary alcohols. The series of primary alcohols from methanol through decanol are competitive inhibitors with respect to hydroxyoctadecanoate. The longer-chain alcohols are more potent inhibitors, and a linear free-energy relationship exists between log K_i and log P (14) (Table 5), (Fig. 4). The slope of the plot is −0.9, corresponding to a free energy change of 640 calories (ca. 2,700 J) per mol for each additional CH_2 group in the substrate. Although the inhibition by primary alcohols was not as sensitive to hydrophobic interactions as was the substrate interaction (Fig. 3), it still falls into the group of interactions that Hansch classified as most sensitive to hydrophobic effects

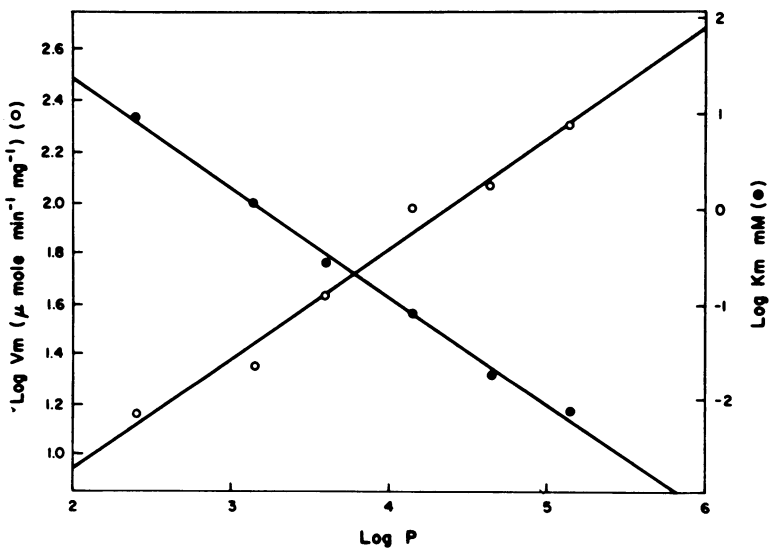

FIG. 3. *Kinetic parameters V_{max} (○) and K_m (●) as a function of octanol-water partition coefficient (P) for 4-heptanol, 4-octanol, 5-nonanol, 5-decanol, 5-undecanol, and 6-dodecanol.*

TABLE 5. *Inhibition constants for various primary alcohols in the absence of detergent*[a]

Alcohol	K_i (M)
Methanol	3.5×10^{-1}
Ethanol	1.3×10^{-1}
1-Propanol	3.5×10^{-2}
1-Butanol	2.0×10^{-2}
1-Pentanol	7.0×10^{-3}
1-Hexanol	1.3×10^{-3}
1-Heptanol	4.0×10^{-4}
1-Octanol	1.5×10^{-4}
1-Nonanol	1.3×10^{-4}
1-Decanol	9.0×10^{-5}

[a] Inhibition is competitive with respect to 12-hydroxyoctadecanoate. Substrate concentrations: 12-hydroxyoctadecanoate, 5×10^{-6} M to 2.5×10^{-5} M; NAD, 1×10^{-3} M.

(14). The deviation from linearity seen with nonanol and decanol (Fig. 4) was most probably due to the presence of oligomeric species of these alcohols in the aqueous solution. Although the concentrations required for 50% inhibition were appreciably below the limits of water solubility (5), significant interaction between alcohol molecules probably occurred, as described for longer-chain carboxylic acids (26). When the inhibition studies were performed in the presence of the nonionic detergent Lubrol WX, there was a break from linearity between hexanol and heptanol, representing the partitioning of the alcohols between aqueous solution and detergent micelles.

DISCUSSION

The enzyme isolated from *Pseudomonas* sp. NRRL B3266 is unique among alcohol dehydrogenases in that it exhibits specificity for hydrophobic secondary alcohols with a minimum of two to four unbranched methylene groups on each side of the carbinol. The simplest substrate for this enzyme is 3-heptanol. The physiologically significant substrate for this inducible enzyme, 10(*R*)hydroxyoctadecanoic acid, is not an appreciably better substrate than a simple secondary alcohol such as 6-dodecanol (Table 4). Hydrophobic effects on enzyme-substrate interactions, although more pronounced for this enzyme, are recognized for a number of alcohol dehydrogenases. A hydrophobic pocket exists near the active site in liver alcohol dehydrogenase (7). The hydrophobic dependence of the interactions of a large number of substrates and inhibitors with liver alcohol dehydrogenase has been summarized by Hansch (15). His approach is based on the postulate that the effect of substituents on a rate or equilibrium constant can be factored as a first approximation as follows (15):

$$\int_X \log k = \int_X \Delta G_{\text{hydrophobic}} + \int_X \Delta G_{\text{electronic}} + \int_X \Delta G_{\text{steric}}$$

The operator $\int_X$ implies that the effect of substituent X on the rate or equilibrium constant can be factored into the effect of X on hydrophobic, electronic, and steric characteristics of a parent compound. The octanol-water partition coefficient, P, is used to model hydrophobic characteristics and an extensive table of log P values has been published (18).

Using this approach, Hansch has quantitated the increase in the binding of inhibitors by alcohol dehydrogenase with the increase in the number of methylene units added to a parent molecule, using the data of Theorell (25) and Anderson (1, 2, 16). The mean value for the slope of the relationship between log K_i and log P was 0.79 ± 0.19, which falls into the category of biochemical responses that have intermediate dependence upon the hydrophobicity of the ligand (14). The interaction of yeast alcohol dehydrogenase with benzyl alcohols falls into the same category because its mean slope, correlating binding to hydrophobicity, is 0.62 ± 0.14 (17).

Alcohol dehydrogenases from *Pseudomonas* species linked to NAD^+ (3, 4, 22), $NADP^+$ (28), or other electron acceptors (28) catalyze the dehydrogenation of hydrophobic primary alcohols arising from hydrocarbon oxidation. Although the importance of hydrophobic interaction is obvious. Quantitative correlations cannot readily be made from published data.

Hydroxyoctadecanoate dehydrogenase falls into another category of biological systems that are more sensitive to hydrophobic changes in substrates and inhibitors (14). Because the substrates or inhibitors are unbranched, aliphatic secondary or primary alcohols, electronic and

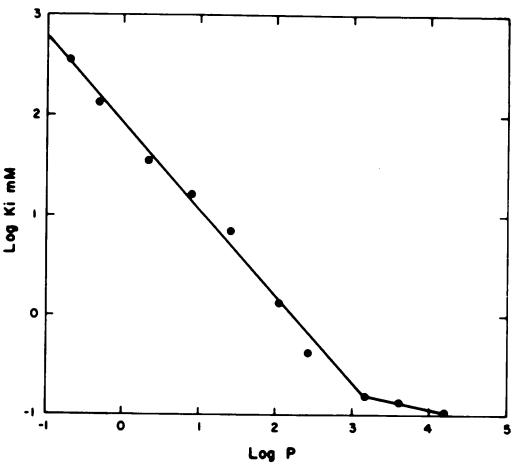

FIG. 4. *Constants for the competitive inhibition of dehydrogenation of hydroxyoctadecanoate by primary alcohols (methanol through decanol) as a function of the octanol-water partition coefficient (P).*

steric differences are of negligible importance, and the linear relationship between log K_m or log K_i and log P ensues. It is expected that eventually, as the substrate or inhibitor is made more and more hydrophobic, a point is reached where further increases in lipophilic character actually result in a decrease in biochemical response (15). Observation of this transition is complicated by a concurrent decrease in water solubility of the hydrophobic molecules. Thus, the break in the linear relationship between log K_i and log P for the primary alcohols (Fig. 4) may represent the inversion point P_0 (23), or may simply reflect association between the longer-chain alcohols to form dimers and oligomers in aqueous solution (26). The secondary alcohol substrates have less of a tendency to aggregate in aqueous solution, due to the nonterminal position of the polar hydroxyl group (26), and thus do not show a break in log K_m versus log P through 5-dodecanol. A linear free-energy relationship between log V_{max} and log P, as seen in Fig. 4, was not described by Hansch and Dunn (14). The physical significance of this linear relationship is not understood.

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