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SEPARATION AND CHARACTERIZATION OF LONG-CHAIN ALCOHOL DEHYDROGENASE ISOENZYMES FROM *PSEUDOMONAS AERUGINOSA*

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SUMMARY

1. A NADP-dependent alcohol dehydrogenase (alcohol dehydrogenase I) from *Pseudomonas aeruginosa* was stabilized by glycerol and purified by chromatography on DEAE-cellulose and hydroxyapatite.

2. This purification also permitted the separation of other isoenzymes: (a) A second minor NADP-dependent alcohol dehydrogenase (alcohol dehydrogenase II). (b) A NAD-dependent alcohol dehydrogenase fraction (Fraction III).

3. Kinetic properties of alcohol dehydrogenase I, alcohol dehydrogenase II and Fraction III have been compared with those of alcohol:NAD^+ oxidoreductase (EC 1.1.1.1.) from yeast.

4. Alcohol dehydrogenase I and alcohol dehydrogenase II had a high affinity (low K_m values) for long-chain primary alcohols.

5. A study of Fraction III strongly suggested the presence of two enzymes, one for lower alcohols, the other one with a good affinity for higher alcohols. Further purification work on Fraction III resulted but in partial separation of these enzymes.

INTRODUCTION

The study of the alcohol dehydrogenases from *Pseudomonas aeruginosa* was undertaken in relation to their involvement in hydrocarbon metabolism, as this microorganism is able to grow on alkanes as its sole carbon source. It was first found that a constitutive NADP-dependent alcohol dehydrogenase able to utilize long chain alcohols was present in our strain[1] in agreement with previous results obtained with a different strain[2]. In the course of the purification of this enzyme from extracts of cells grown on glucose, other alcohol dehydrogenases were detected. The present paper reports the separation and purification of these enzymes and presents results concerning their specificity towards alcohols of different chain lengths.

MATERIALS AND METHODS

Microorganism

Pseudomonas aeruginosa 196 Aa, isolated by Traxler and Bernard[3] has been used throughout this study. Its identity has been confirmed in this laboratory by tests according to Stanier *et al.*[4].

Preparation of cell-free extracts

P. aeruginosa 196 Aa was grown in a 12-l Microferm New Brunswick fermentor, containing the basal medium[5] *plus* 1% (w/v) glucose, at 30 °C with stirring (800 rev./min) and forced aeration (0.8 $l \cdot min^{-1}$ per l of medium). Cells were harvested in the exponential phase of growth (6 h after inoculation) by centrifugation, washed with 10 mM phosphate buffer (pH 7.1) and kept at −30 °C until they were used.

Cells (37 g wet weight) were suspended in 51 ml of 10 mM phosphate buffer containing 7 mM β-mercaptoethanol (pH 7.2). The cells were disintegrated by sonic treatment (five bursts of 10 s at the maximal output of a Branson sonifier) and then centrifuged at 20 000 × *g* for 40 min at 4 °C. The supernatant was used as a crude starting material.

Enzyme assays

Problems due to the low water solubility of higher alcohols have been discussed previously and the methods for the assay of alcohol dehydrogenase activity have already been discussed[1]. The assay system contained, in a final volume of 3.0 ml: 2.8 ml of 0.05 M glycine buffer (pH 9.5) with the substrate usually present as a solution (unless otherwise stated the substrate was 2 mM 1-octanol), 0.23 mM NADP (or 1 mM NAD) and appropriate quantities of enzyme. The reaction was initiated by the addition of enzyme. A unit is defined as the amount of enzyme which catalyzes the conversion of 1 μmole of substrate per min at 30 °C.

Protein assays

Protein assays were done using the Folin method modified by Clark and Jakoby[6]. As these authors observed, the assay at 280 nm could not be used in the presence of β-mercaptoethanol because it strongly enhanced the enzyme absorption, and we made the same observation with our preparations. The following procedure was adopted: 50–200 μg of protein in 0.1–0.4 ml were precipitated by addition of 2 ml of 25% trichloroacetic acid. The preparation was allowed to rest for 15 min at 0 °C, and then was centrifuged 15 min at 10 000 × *g*. The precipitate was dissolved in 0.5 ml 1 M NaOH and protein determination was carried out with the Folin reagent[7].

Analytical polyacrylamide gel electrophoresis

The polyacrylamide gel electrophoresis was adapted from Davis[8]. No sample or spacer gel were used. Acrylamide monomer concentration was 7%. The pH of the Tris–glycine phosphate buffer was 8.3. During electrophoresis the gels were cooled to at least 18 °C. The alcohol dehydrogenase activity on the gels was detected by the technique of Grell *et al.*[9], slightly modified in the following conditions: the developing solution contained 2.8 ml of glycine buffer (0.05 M, pH 9.5) with either 1-octanol (2 mM) or ethanol (1 M), 0.2 ml of NAD or NADP (12.5 mg/ml), 0.12 ml of phenazine

methosulfate solution (1 mg/ml) and 0.15 ml of nitroblue tetrazolium (10 mg/ml). When 1-octanol was used as the developing substrate, gels were allowed to stay 30 min in the glycine buffer containing only 1-octanol before other constituents were added. Purple spots of the reduced formazan were usually visible within 40 min or less after incubation at 25 °C was started.

Chromatographic techniques

All operations were carried out in the cold room at 4 °C. Chromatographic columns from Whatman were used and the flow rate of the eluent was adjusted by a peristaltic pump (Desaga). The concentration gradient in the elution buffer was generated by allowing the more concentrated solution to flow into a constant volume mixer connected to the column. This system results in an exponential (convex) gradient[10]. Concentrations of the eluting salts in the effluent fractions from chromatographic columns were measured with a conductivity meter. Solutions of enzyme were concentrated on a Diaflo concentrating unit (Amicon corporation, Lexington, Mass.) equipped with an UM. 20. E membrane.

Reagents

Alcohols were purchased from Fluka or Baker and their purity was checked by gas chromatography. This analysis showed for some of them the presence of one of the nearest lower homologs as a minor (less than 1%) impurity.

Diethyl amino ethyl cellulose was the D.E.52 preparation from Whatman. Hydroxyapatite was prepared by the method of Tiselius *et al.*[11] using the procedure described by Bernardi[12].

NAD-dependent ethanol dehydrogenase from yeast (alcohol:NAD^+ oxidoreductase, EC 1.1.1.1.) was purchased from Sigma. NAD and NADP were from Boehringer. All other reagents used were of analytical grade.

RESULTS

Purification and separation of the enzymes

Stabilization of the enzymes

Crude extracts are not stable at 0 °C. They lose 60% activity in 5 days unless glycerol 20% (v/v) is added. The enzymes stored at −8 °C in 20% glycerol with 7 mM β-mercaptoethanol keep more than 50% activity after 3 weeks. We used this property for the purification and separation of the different alcohol dehydrogenases. Unless otherwise specified all purification operations were performed at 2–5 °C. The activities and yields are summarized in Table I.

Ultracentrifugation

The crude extract obtained as described above was ultracentrifuged 90 min at 160 000 × *g* in a MSE Superspeed 65 ultracentrifuge.

Addition of glycerol and precipitation of DNA

15.3 ml of a 10 mM phosphate buffer containing 7 mM β-mer-captoethanol in 80% glycerol (v/v) (pH 7.2) was added to 46 ml of the supernatant. Then 6 ml of a 15% (w/v) solution of streptomycin sulfate was added and the solution was gently stirred for 20 min. The precipitate obtained by a 40 min centrifugation at 20 000 × *g* was discarded and the supernatant was kept for the next operation.

TABLE I

PURIFICATION OF *Pseudomonas aeruginosa* ALCOHOL DEHYDROGENASE I

	Vol. (ml)	*Total activity (units)*	*Spec. act.* (units/mg)*	*Overall yield (%)*	*Overall purification*
Crude extract	51.4	112	0.068**	—	—
Ultracentrifugation supernatant	46	116	0.078	100	1.15
Glycerol + streptomycin sulfate + supernatant	64	88	0.066	76	0.97
DEAE-cellulose effluent	130	68	0.348	61	5.11
Hydroxyapatite effluent	210	56	2.94***	50	43.2

* For 1-octanol (see Materials and Methods).

** The second NADP-dependent alcohol dehydrogenase represents in crude extracts at least 15% of the total activity. The actual values for yield and purification of alcohol dehydrogenase I are thus higher than the presented values, calculated with respect to the total activity of the crude extract, indicate.

*** Measured at 0.23 mM NADP. At saturating NADP concentration (1 mM) the specific activity was 3.8. A maximum specific activity of 10.6 (purification × 120) was obtained in another purification experiment including an $(NH_4)_2SO_4$ fractionation (30–70%) just before DEAE-cellulose chromatography but the yield was only 9%.

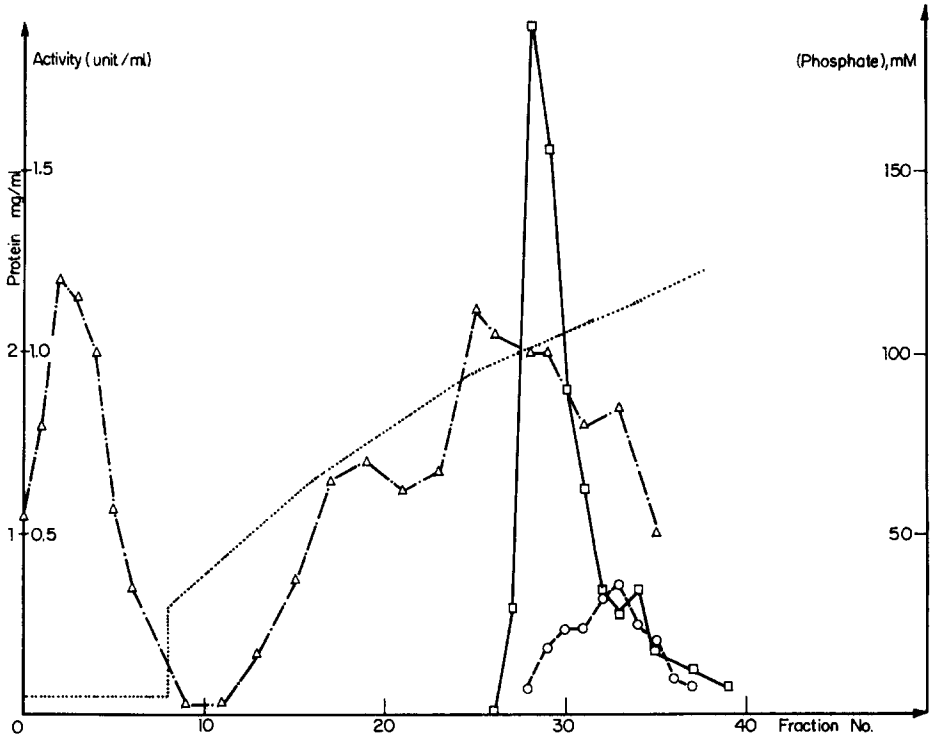

Fig. 1. DEAE-cellulose chromatography of streptomycin sulfate supernatant. The preparation after being twice dialyzed against 5 mM phosphate buffer contained 88 units of NADP-dependent alcohol dehydrogenase in a volume of 64 ml containing 20.8 mg of protein per ml. This preparation was applied to a 2.54 cm × 33.5 cm DEAE-cellulose column. 10 ml fractions were collected. The elution rate was 45 ml/h. △—·—△, protein concentration (mg/ml); □—□, NADP-dependent and ○---○, NAD-dependent alcohol dehydrogenase activities in units/ml; ·····, phosphate (mM) in effluent.

DEAE-cellulose chromatography

The supernatant was then dialyzed against a 5 mM phosphate buffer*. The precipitate which often appeared at this step was removed by a centrifugation identical to the previous one. The preparation (63.5 ml) was then applied to a DEAE-cellulose column that had been equilibrated with 5 mM phosphate buffer, as indicated in the legend of Fig. 1. After the sample had been adsorbed on the top of the column, a few ml of 5 mM phosphate buffer were added and elution was begun with a convex phosphate gradient in a concentration range from 30 to 400 mM phosphate. The fractions were analyzed for protein and dehydrogenase activity and the results are shown in Fig. 1. The bulk of NADP-dependent alcohol dehydrogenase activity was eluted at phosphate concentrations between 95 and 120 mM. This step usually resulted in a 5- to 6-fold purification and a recovery of 70–80% (Table I and 2nd footnote).

Hydroxyapatite chromatography

Fractions from the preceding step were combined, dialyzed several times

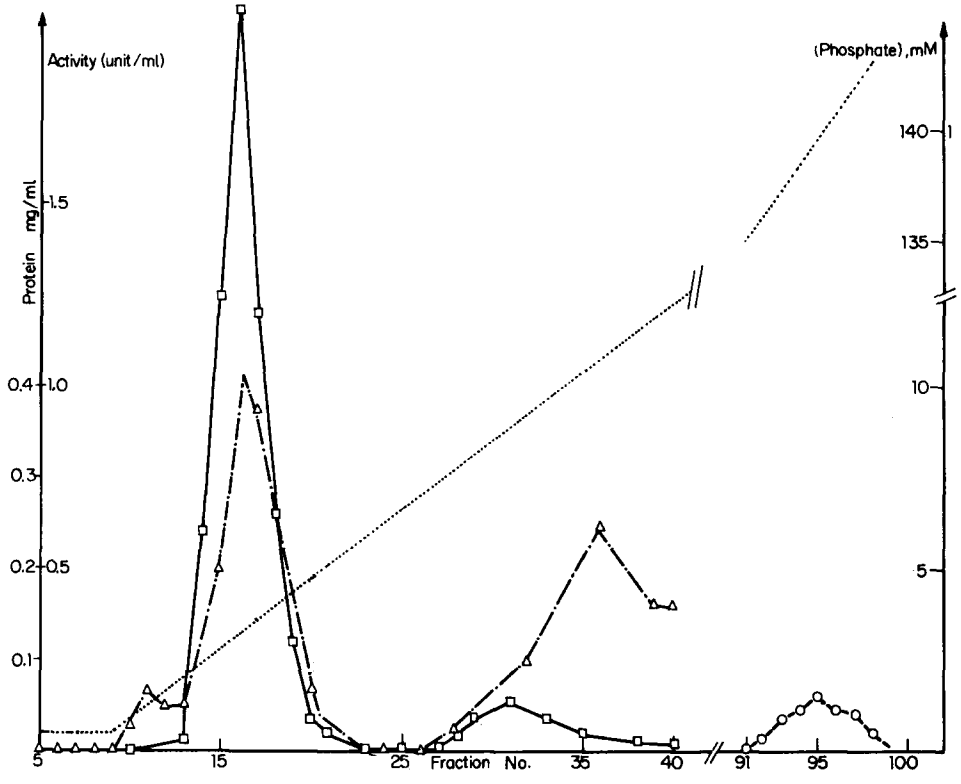

Fig. 2. Hydroxyapatite chromatography. The preparation from DEAE-cellulose chromatography (Fractions 27–33 in Fig. 1) were dialyzed twice against a 1 mM phosphate buffer and concentrated to a volume of 44 ml, 42.5 ml of which were applied to a 2.54 cm × 29 cm hydroxyapatite column. The preparation contained 64 units of NADP-dependent alcohol dehydrogenase and 4.2 mg of protein per ml. 10 ml fractions were collected. The elution rate was 32 ml/h. △—·—△, protein concentration (mg/ml); □—□, NADP-dependent and ○---○, NAD-dependent alcohol dehydrogenase in units/ml. ·····, phosphate (mM) in effluent.

* Unless otherwise noted, from this point on, all buffers used were potassium phosphate in 20% glycerol at pH 7.2 and contained 7 mM β-mercaptoethanol.

against a 1 mM phosphate buffer and concentrated by ultrafiltration. The resulting solution (42.5 ml) was applied to a hydroxyapatite column previously equilibrated with the same buffer. The column was washed with 35 ml of equilibration buffer, then a convex gradient ranging from 1 to 55 mM phosphate was started. When the 55 mM concentration was reached, a second steeper convex gradient from 55 to 400 mM was started. The results are given in Fig. 2.

The bulk of the major NADP-dependent alcohol dehydrogenase (called alcohol dehydrogenase I in this paper) was eluted at a phosphate concentration ranging from 3.5 to 5.5 mM, resulting in a 9- to 15-fold purification and a 80–90% recovery.

Between 7.5 and 12.5 mM another minor NADP-dependent alcohol dehydrogenase (called alcohol dehydrogenase II) was eluted. A slight separation of alcohol dehydrogenase I and alcohol dehydrogenase II is already visible in the elution pattern from DEAE-cellulose shown in Fig. 1.

No further activity appeared until the phosphate concentration reached 135 mM. A NAD-dependent alcohol dehydrogenase then appeared and was completely eluted at about 150 mM phosphate. It will be referred to as Fraction III throughout this text.

Each of these fractions was dialyzed against a 10 mM phosphate buffer, concentrated and kept at −8 °C before use.

Acrylamide gel electrophoresis

Each of the three peaks eluted from the hydroxyapatite column showed only one band of activity on acrylamide gel electrophoresis. Fraction III showed a relatively wide band (Fig. 3).

Samples of the streptomycin sulfate supernatant showed two bands with NADP as the coenzyme and one wide band with NAD (Fig. 3).

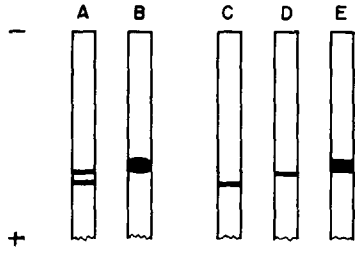

Fig. 3. Analytical disc electrophoresis on polyacrylamide gels. The substrate used was 1 M ethanol. Similar results could be obtained with 2 mM 1-octanol. The coenzyme used is indicated for each sample. A, supernatant from streptomycin sulfate precipitation with NADP; B, supernatant from streptomycin sulfate precipitation with NAD; C, alcohol dehydrogenase I with NADP; D, alcohol dehydrogenase II with NADP; E, Fraction III with NAD.

Study of the characteristics of the purified preparations

The kinetic constants (apparent K_m and V values) were determined with different alcohols for the three different fractions and are given in Table II. To compare with these results we also measured these constants for the NAD-linked ethanol dehydrogenase from yeast.

Characteristics of alcohol dehydrogenase I

Alcohol dehydrogenase I shows lower K_m values for the long chain alcohols[1]. In

TABLE II

KINETIC CONSTANTS OF THE DIFFERENT ALCOHOL DEHYDROGENASES STUDIED

Kinetic constants (apparent K_m and V values) were measured at NADP (or NAD) concentrations of 1 mM. Maximal velocities are given in percentage of the maximal velocity for ethanol for each fraction.

Substrates	*Alcohol dehydrogenase I*		*Alcohol dehydrogenase II*		*Fraction III*		*Alcohol dehydrogenase from yeast*	
	K_m *(mM)*	V	K_m *(mM)*	V	K_m *(mM)*	V	K_m *(mM)**	V^*
Ethanol	1 300	100	1 100	100	2	100**	6	100
1-Propanol					1.8	50**	30	
1-Butanol	13	66			1.0	50**	400	85
1-Pentanol					12	190		
1-Hexanol	0.79	66	1.2	66	5	125	≈ 1 000§	
1-Heptanol	0.43	50						
1-Octanol	0.14	42.5	0.21	23	1.5	100		
1-Decanol	0.12	33	0.2	12.5	0.45	33		
1-Dodecanol	0.08	10	0.2	5.9				
1-Tetradecanol	0.04	1.7						
1-Hexadecanol		0.7***						

* Measured at 30 °C in 0.05 M glycine buffer (pH 9.5).

** Fig. 5 shows that Fraction III has two K_m values for lower alcohols. The maximal velocities given in this table are those corresponding to the lower K_m.

*** Because it had a small activity with glycerol, the enzyme preparation had to be dialyzed before the determination of activity with 1-hexadecanol could be done.

§ 1-Hexanol was not soluble enough to allow an accurate determination of this high K_m.

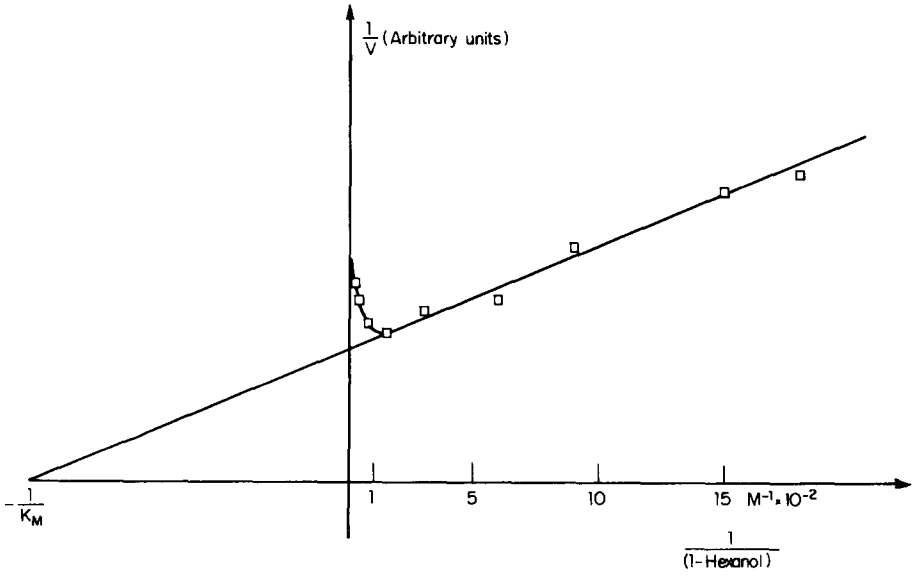

Fig. 4. Double-reciprocal plot of alcohol dehydrogenase I activity *vs* 1-hexanol concentration. The NADP concentration was 1 mM.

return, the maximal velocities decrease with the length of the alcohols. With 1-hexanol, which is soluble enough, we found an inhibition by excess substrate (Fig. 4).

Table II shows clearly that this enzyme, which is specific (low K_m values) for higher alcohols is also active with ethanol, but that the K_m for this alcohol is much too high to have a physiological significance (1.3 M). The specific activity of this enzyme in crude extracts is 1.5-fold greater for cells grown on glucose than grown on hydrocarbons.

This enzyme can also utilize α,ω diols at a higher rate than corresponding mono-alcohols. The maximal velocities and K_m values decrease with increasing chain lengths up to 1,10 decanediol for which physiological K_m values are obtained ($K_m = 10^{-4}$ M). A very low activity is observed with 2-octanol.

The K_m for NADP of alcohol dehydrogenase I measured with 2 mM 1-octanol as the substrate is 0.15 mM. NAD cannot replace NADP as the coenzyme.

Characteristics of alcohol dehydrogenase II

Results similar to those obtained with alcohol dehydrogenase I were obtained with alcohol dehydrogenase II. The most important differences appear when alcohols higher than 1-hexanol are assayed. The maximal velocities decrease faster than with alcohol dehydrogenase I whereas K_m values no longer decrease after 1-octanol.

The K_m for NADP of alcohol dehydrogenase II measured with 2 mM 1-octanol as substrate is 0.048 mM. NAD cannot replace NADP as the coenzyme.

Characteristics of Fraction III

The results obtained with fraction III strongly suggest the presence of two enzymes:

The variations of the K_m and V values for different alcohols presented in Table II clearly indicate the existence of two active sites with different specificities.

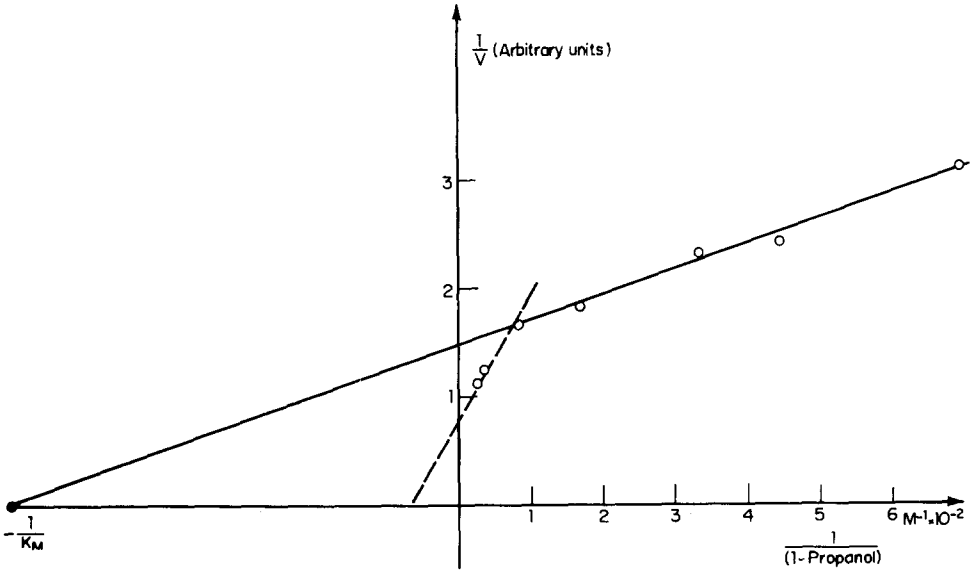

Fig. 5. Double-reciprocal plot of Fraction III activity *vs* 1-propanol concentration. The NAD concentration was 1 mM.

The determination of the K_m for lower alcohols using the Lineweaver and Burk[13] representation gives two intersecting straight lines (Fig. 5).

Analytical disc electrophoresis did not separate the enzymes well but showed a wide activity band (Fig. 3), although two distinct but very close bands were often visible at early stages of colour development.

Separation of the two assumed enzymes has been attempted first by chromatography on a second hydroxyapatite column but no resolution was achieved. Another trial using chromatography on a second DEAE-cellulose column (starting elution at 90 mM phosphate with a gradient of 0.1 mM phosphate/ml) resulted in two overlapping peaks as shown in Fig. 6. The two fractions obtained were collected and con-

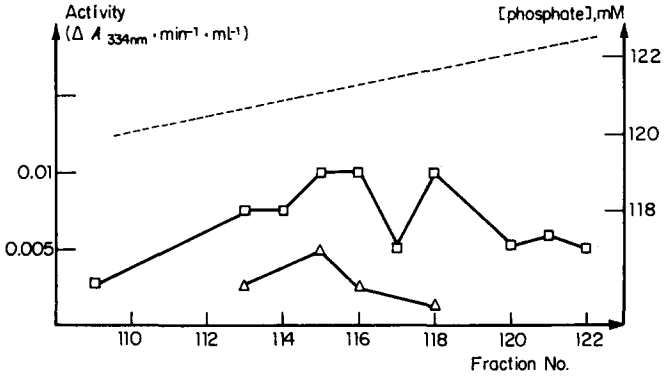

Fig. 6. DEAE-cellulose chromatography of Fraction III. Fraction III from hydroxyapatite chromatography was applied to a 1.5 cm × 30 cm DEAE-cellulose column which had been equilibrated with 5 mM phosphate buffer. The elution rate was 18 ml/h and 2 ml fractions were collected. The first fraction and the second fraction of Table III were constituted by pooling Fractions 113–116 and Fractions 117–120, respectively. □—□, NAD-dependent alcohol dehydrogenase activity assayed with 1 M ethanol; △—△, NAD-dependent alcohol dehydrogenase activity assayed with 30 mM 1-hexanol; ---------, calculated phosphate gradient (mM).

TABLE III

PARTIAL SEPARATION OF FRACTION III IN TWO COMPONENTS POSSESSING DIFFERENT SUBSTRATE SPECIFICITIES

The concentrations of 1-octanol and ethanol are 2 mM and 2.5 mM, respectively.

	Fraction III	*First fraction of the second DEAE-cellulose chromatography*	*Second fraction of the second DEAE-cellulose chromatography*
Specific activity for 1-octanol / Specific activity for ethanol	1.1	4.95	0.75

centrated. The ratios of the specific activities with 2 mM 1-octanol and 2.5 mM ethanol were measured for the different preparations. The results given in Table III indicate that a partial separation of two different enzymes has taken place. Fraction III is also active with α,ω diols. NADP cannot replace NAD as a coenzyme.

One of the most important characteristics of the different enzymes studied here has been represented in Fig. 7 which shows the variation of log $(1/K_m)$ with the num-

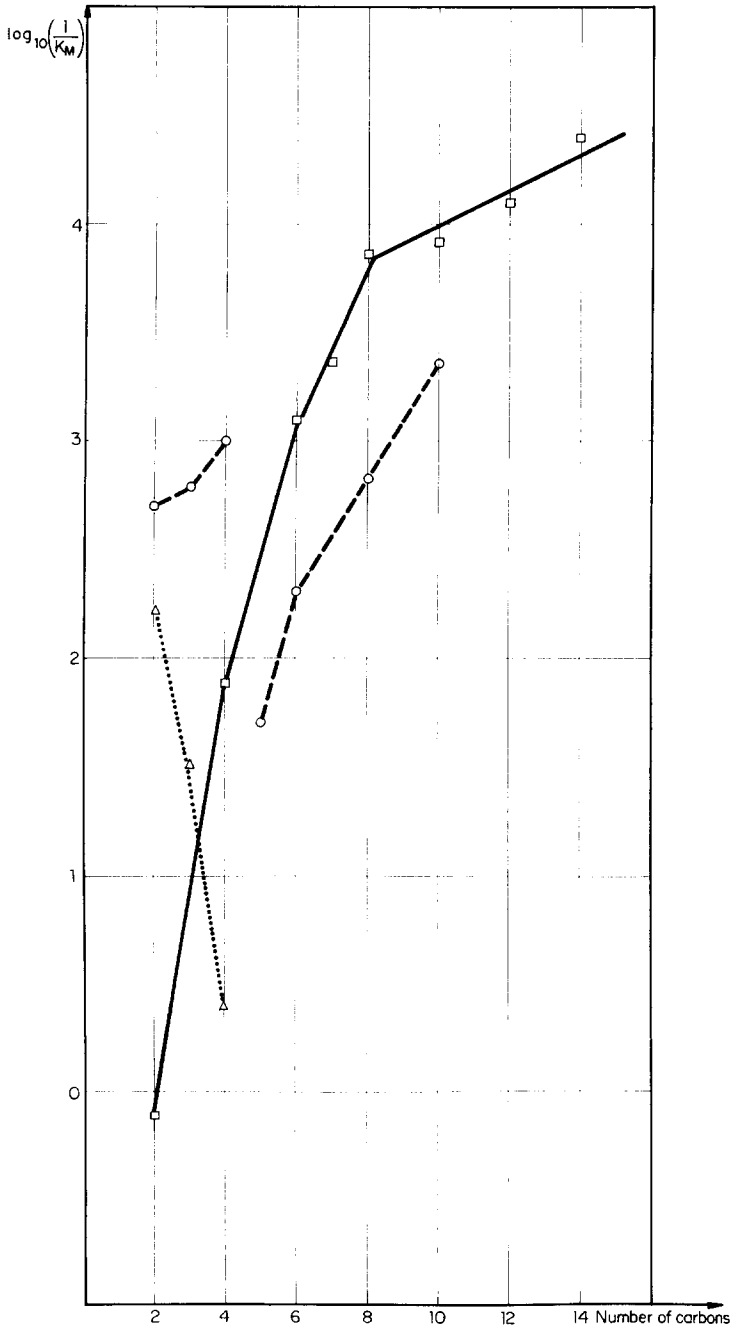

Fig. 7. Variations of log $(1/K_m)$ *vs* number of carbons of the alcohol. □—□, alcohol dehydrogenase I; ○---○, Fraction III; △...△, NAD-dependent ethanol dehydrogenase from yeast.

ber of carbons of the alcohol for alcohol dehydrogenase I, Fraction III and yeast alcohol dehydrogenase (the results concerning alcohol dehydrogenase II have been omitted for reasons of clarity).

DISCUSSION

Three alcohol dehydrogenases with high affinity for long chain alcohols have been found in *P. aeruginosa* 196 Aa extracts (alcohol dehydrogenase I, alcohol dehydrogenase II and a third one, NAD-specific, contained in Fraction III) in addition to an ethanol dehydrogenase present in Fraction III.

The study of the properties of the most abundant isoenzyme, alcohol dehydrogenase I, in comparison with those of yeast ethanol dehydrogenase clearly indicates the differences between the two types of enzymes. Although both are active with ethanol, the K_m values of alcohol dehydrogenase I for this alcohol are quite high. Characteristically, lower and lower K_m values are obtained with alcohols of increasing chain lengths for this enzyme (whereas for ethanol dehydrogenase, an opposite relationship is observed). The low water solubility of long chain alcohols makes more imperative the necessity of a low K_m for an enzyme utilizing these substrates. These observations suggest the presence of a hydrophobic site on the enzyme capable of binding the hydrocarbon chain. The occurence of inhibition by excess substrate observed with 1-hexanol but only scarcely with ethanol or 1-butanol may also indicate the existence of two neighbouring sites on the enzymes. Inhibition by excess substrate has already been observed, especially for horse liver alcohol dehydrogenase, and has been partially explained by the formation of a ternary "abortive complex" Enzyme–NADH–alcohol[14,15]. This mechanism may account for the results described here but the presence of an hydrophobic site, already postulated for horse liver alcohol dehydrogenase[14,16,17] is also a possible explanation.

An examination of Fig. 7 shows that the K_m of alcohol dehydrogenase I decreases rapidly from ethanol to 1-octanol and more slowly beyond this alcohol. In this way, a kind of mapping of the active site is obtained which indicates the size of its hydrophobic portion. Alcohol dehydrogenase II is an enzyme similar to alcohol dehydrogenase I. From the kinetic values obtained, it seems that the specificity of alcohol dehydrogenase II is directed towards alcohols of somewhat shorter chain length than that of alcohol dehydrogenase I.

A study of the maximal velocities obtained for alcohol dehydrogenase I shows that they decrease with increasing chain lengths of the substrates. It has been reported[18] for the long chain alcohol dehydrogenases of yeast that maximal velocities increase with increasing chain length of the alcohol. In the present study the opposite was observed for the three *Pseudomonas* enzymes studied.

The purity of the different enzymes obtained is not known but gel electrophoresis of alcohol dehydrogenase I preparations revealed several other important protein bands and it is expected that the completely pure enzyme would have a specific activity several fold higher.

The simultaneous presence of both NADP-linked and NAD-linked isoenzymes in glucose-grown cells demonstrated here, completes the reports of NAD-dependent[19] and NADP-dependent[2] constitutive alcohol dehydrogenases in other *Pseudomonas aeruginosa* strains and suggests the generality of the presence of these isoenzymes,

possibly in variable relative proportions, in this species. The possible relationship of these enzymes to the capacity of *Pseudomonas aeruginosa* to grow on paraffins is presently investigated.

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