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Biochemical properties of alcohol dehydrogenase from *Drosophila lebanonensis*

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Purified *Drosophila lebanonensis* alcohol dehydrogenase (Adh) revealed one enzymically active zone in starch gel electrophoresis at pH 8.5. This zone was located on the cathode side of the origin. Incubation of *D. lebanonensis* Adh with NAD^+ and acetone altered the electrophoretic pattern to more anodal migrating zones. *D. lebanonensis* Adh has an M_r of 56000, a subunit of M_r of 28000 and is a dimer with two active sites per enzyme molecule. This agrees with a polypeptide chain of 247 residues. Metal analysis by plasma emission spectroscopy indicated that this insect alcohol dehydrogenase is not a metalloenzyme. In studies of the substrate specificity and stereospecificity, *D. lebanonensis* Adh was more active with secondary than with primary alcohols. Both alkyl groups in the secondary alcohols interacted hydrophobically with the alcohol binding region of the active site. The catalytic centre activity for propan-2-ol was 7.4 s^{-1} and the maximum velocity of most secondary alcohols was approximately the same and indicative of rate-limiting enzyme–coenzyme dissociation. For primary alcohols the maximum velocity varied and was much lower than for secondary alcohols. The catalytic centre activity for ethanol was 2.4 s^{-1}. With [$^2H_6$]ethanol a primary kinetic ^2H isotope effect of 2.8 indicated that the interconversion of the ternary complexes was rate-limiting. Pyrazole was an ethanol-competitive inhibitor of the enzyme. The difference spectra of the enzyme–NAD^+–pyrazole complex gave an absorption peak at 305 nm with ϵ_{305} 14.5×10^3 $M^{-1} \cdot cm^{-1}$. Concentrations and amounts of active enzyme can thus be determined. A kinetic rate assay to determine the concentration of enzyme active sites is also presented. This has been developed from active site concentrations established by titration at 305 nm of the enzyme and pyrazole with NAD^+. In contrast with the amino acid composition, which indicated that *D. lebanonensis* Adh and the *D. melanogaster* alleloenzymes were not closely related, the enzymological studies showed that their active sites were similar although differing markedly from those of zinc alcohol dehydrogenases.

INTRODUCTION

The biochemical characteristics of the alcohol dehydrogenases (EC 1.1.1.1) from different species of *Drosophila* are of topical interest, especially regarding population genetics and evolution (Vigue & Johnson, 1973; Clarke, 1975; Thompson *et al.*, 1977; David & van Herrewege, 1983).

Drosophila alcohol dehydrogenases contain two subunits, each with an M_r of about 27000 (Schwartz *et al.*, 1975; Thatcher, 1980; Juan & Gonzalez-Duarte, 1981), and have higher activity with secondary than with primary alcohols (Vigue & Johnson, 1973; Daggard, 1981; Juan & Gonzalez-Duarte, 1981; Oakeshott *et al.*, 1982).

Drosophila lebanonensis is a fruit fly species with a high ethanol tolerance and a better ability to utilize ethanol as a food resource than *D. melanogaster* (David *et al.*, 1979). Since phylogenetically *D. lebanonensis* and *D. melanogaster* are distantly related *Drosophila* species, it is of interest to study the biochemical characteristics of the *D. lebanonensis* Adh enzyme.

MATERIALS AND METHODS

Reagents

Starch was obtained from Connaught Medical Research Laboratories, Toronto, Canada. Acrylamide and *NN'*-methylenebisacrylamide were from Eastman Chemicals. Ampholines pH 3.5–9.5, 4–6, 5–8 and 9–11 were from LKB. Grade III NAD^+ and NADH were purchased from Sigma Chemical Co., and Grade I free acid NAD^+ from Boehringer. Cibacron Blue 3G-A was from Ciba–Geigy. Coomassie Blue was from Serva. Imidazole and pyrazole were from Fluka. Isobutyramide was from Eastman Chemicals. [$^2H_6$]Ethanol (99% ^2H) was from Norsk Hydro. [$^2H_8$]Propan-2-ol and [$^2H_{12}$]cyclohexanol were from Sigma. The verbenols were kindly given by Professor L. Skattebøl, University of Oslo, Norway and the bicyclo(2.2.1)heptanols were from Professor J. B. Jones, University of Toronto, Canada. Optical enantiomers of butan-2-ol and octan-2-ol were from Fluka, pentan-2-ol and heptan-2-ol were from Norse Laboratories, California, and hexan-2-ol, 3-methylbutan-2-ol and 1-phenylethanol were from Chemical Dynamics

Abbreviations used: Adh, alcohol dehydrogenase; Adh^F and Adh^S; fast and slow alcohol dehydrogenase (EC 1.1.1.1) from *Drosophila melanogaster*.

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Corp., NJ, U.S.A. All other chemicals used were the best commercial grades available. Alcohols with an alkyl group of more than four carbon atoms were dissolved in dioxan.

Enzymes

Drosophila lebanonensis Adh was purified as previously described by Juan & Gonzalez-Duarte (1980). Only one CM-Sepharose CL 6B column at pH 7.0 was used along with two Blue Sepharose CL 6B columns to remove proteinase activity which was higher than in other species. The half-life of the enzyme was 6–7 weeks at 4 °C in 20 mM-Tris/HCl, pH 8.6.

To prepare crude homogenates, 0.2 g of flies were blended in 0.46 ml of 20 mM-Tris/HCl, pH 8.6, and the slurry was centrifuged at 23000 *g* for 30 min at 4 °C.

Starch gel electrophoresis

Horizontal starch-gel electrophoresis and the conversion experiments were carried out as described earlier by Winberg *et al.* (1983).

Electrofocusing

Analytical thin-layer gel electrofocusing was performed in 7.5% acrylamide gels as described by Juan & Gonzalez-Duarte (1980). Gels were stained for alcohol dehydrogenase activity by incubation at 37 °C in 100 ml of 50 mM-Tris/HCl, pH 8.6, containing 1 ml of propan-2-ol, 20 mg of NAD^+, 10 mg of Nitro Blue Tetrazolium and 0.1 mg of *N*-methylphenazonium methosulphate. For protein staining, gels were fixed in 12.5% (w/v) trichloroacetic acid at 60 °C for 30 min before staining with 0.2% Coomassie Blue in ethanol/water/acetic acid (9:9:2, by vol.) for 30 min. The destaining solution was ethanol/water/acetic acid (5:13:2, by vol.).

Amino acid analysis

The amino acid composition of the protein was determined according to Juan & Gonzalez-Duarte (1981). Protein hydrolysates were analysed on a Chromaspek J 180 Rank Hilger automatic amino acid analyser equipped with a fluorimeter.

Determination of M_r

Determinations of M_r were performed as described by Juan & Gonzalez-Duarte (1980, 1981).

Metal analysis

This was performed by the Central Institute for Industrial Research, Oslo, in μg/ml amounts, with a simultaneous Jarrell–Ash 975 plasma spectrometer and enzyme solutions (0.1 mg/ml) in 2% HNO_3. All glassware was cleaned with Analar nitric acid and quartz triple-distilled water.

Kinetic measurements

These were carried out either with a fixed alcohol concentration of 1 mM or with varied alcohol concentrations and 0.5 mM-NAD^+ in a total volume of 3 ml of 0.1 M-glycine/NaOH buffer, pH 9.5, and 23.5 °C. Reaction was started by adding 20 μl of enzyme solution, and because of the position of equilibrium, by using a high pH of 9.5 the initial rate of NADH formation was accurately measured at 340 nm. Either a Pye–Unicam SP6-550 spectrophotometer coupled to a Kontron 1100 W+W recorder or a Gilford 260 spectrophotometer coupled to a Kontron 312 W+W recorder was employed.

The substrate specificity of the different alcohols was first determined by comparing the rate given by a 1 mM solution of each alcohol with that of 1 mM-ethanol. These experiments were performed in duplicate and the variation from the average was less than 5%. To determine the maximum velocity V_m ($1/\phi_0$) or the Michaelis constant K_m (ϕ_2/ϕ_0; Engel, 1981) for the large number of alcohols evaluated, a fixed concentration of 0.5 mM-NAD was used throughout. This is more than ten times the K_m (ϕ_1/ϕ_0) values determined and hence the measured values of V_m and K_m for the alcohols are within experimental error of the $1/\phi_0$ and ϕ_2/ϕ_0 values for an infinite concentration of the fixed substrate (Table 2 and Fig. 3). V_m for the different alcohols is the catalytic centre activity (molecules of product produced/s per enzyme active site). The determination of V_m and K_m from the experimental data was performed by using a double-reciprocal plot and linear regression on a Texas Instruments T1-51-III or Hewlett-Packard 32E calculator. Initial rate measurements were performed in duplicate with a reproducibility of 5%. The regression coefficient was greater than 0.99 for each plot.

Inhibition experiments were carried out with either 0.5 mM-NAD^+ and varied alcohol concentrations or with 8 mM-butan-2-ol and varied NAD^+ concentrations in a total volume of 3 ml of either 0.1 M-glycine/NaOH buffer, pH 9.5, or 0.1 M-Tris/HCl buffer, pH 8.5.

Spectra measurements

Absorption and difference spectra were determined by using a 8450A UV/VIS Hewlett–Packard spectrophotometer coupled to a 7470 A Hewlett–Packard plotter.

Fluorescence emission spectra were determined using a MPF-2A Hitachi/Perkin–Elmer recording spectrophotofluorimeter coupled to a QPD-33 Hitachi recorder.

Spectrophotometric titrations

These were performed at 23.5 °C using a Beckman DU-7 spectrophotometer. The enzyme and 10 mM-pyrazole was titrated at 305 nm with NAD^+ in 0.1 M-Tris/HCl buffer, pH 8.5. The titration experiments were performed in duplicate, with a reproducibility within 3%. The linear parts of the titration curves were analysed by linear regression, using a Hewlett–Packard 32E calculator. The regression coefficients were better than 0.99.

Rate assay

This was based on the active site concentrations determined in titration experiments. The rate assay (done in duplicate and with a reproducibility within 5%) was carried out at 23.5 °C with a varied concentration of enzyme and a fixed concentration of 0.5 mM-NAD^+ and 100 mM-ethanol in a total volume of 3 ml of 0.1 M-glycine/NaOH buffer, pH 9.5. At these high substrate concentrations, the velocity v at a given enzyme concentration is approx. 94% of the maximum velocity for ethanol. The velocity v (ΔA_{340}/min) is a linear function of [e], the active site concentration in the cuvette. As $v = k \cdot [e]$, the rate constant k (ΔA_{340}/min per μM enzyme active sites) was obtained from a plot of v against [e]. The regression coefficients were greater than 0.99.

RESULTS

Electrophoresis

After starch gel electrophoresis, purified alcohol dehydrogenase from *D. lebanonensis* showed mainly one active zone, located cathodically to *D. melanogaster* Adh^S-5 (Fig. 1). However, after analytical electrofocusing both protein and activity staining of the purified alcohol dehydrogenase from *D. lebanonensis* showed three zones, with over 90% of the protein and activity in the most cathodal zone (Fig. 2). The isoelectric points of these three zones were 7.95, 7.76 and 7.50, and in crude extracts 7.95, 7.50 and 7.15. In the case of the *D. melanogaster* alleloenzymes, NAD^+ along with a ketone such as acetone can convert the main active zone Adh-5 to the more anodal forms Adh-3 and Adh-1 (Winberg *et al.*, 1983). As shown in Fig. 2 this was also the case with *D. lebanonensis* Adh.

Amino acid composition and M_r values

The amino acid composition of the alcohol dehydrogenase from *D. lebanonensis* is given in Table 1, which for comparison also shows the data for the alleloenzyme Adh^S of *Drosophila melanogaster* (Thatcher, 1980).

Sephadex gel permeation chromatography resulted in an M_r for the native enzyme of 56000 ± 5000. SDS/polyacrylamide-gel electrophoresis revealed that *D. lebanonensis* Adh has a subunit M_r of 28000 and is a dimer.

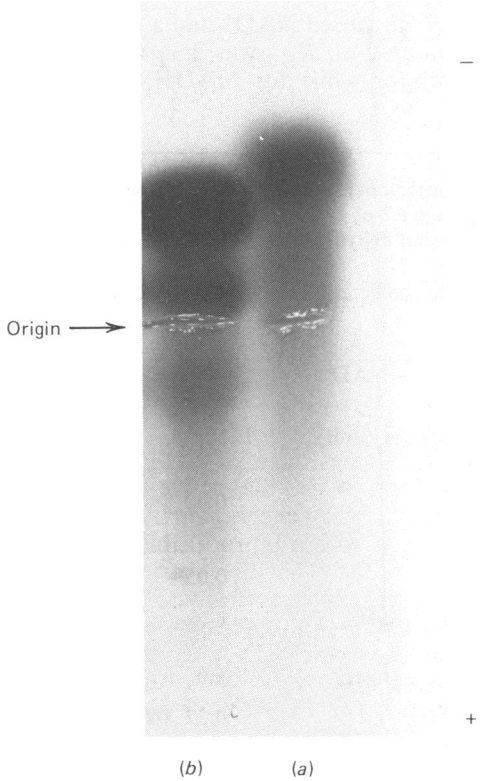

Fig. 1. Starch gel electrophoresis in 0.1 M-Tris/HCl buffer, pH 8.5, stained for alcohol dehydrogenase activity, of *D. lebanonensis* Adh (*a*) and *D. melanogaster* Adh^S (*b*)

Metal analysis

Plasma emission spectra indicated that *D. lebanonensis* Adh did not contain stoichiometric amounts of more than 0.2 mol of zinc or any other metal/mol of enzyme.

Substrate stereospecificity

The results of the initial rate measurements with varied ethanol and NAD^+ are shown in the primary plots of Fig. 3. Secondary plots of the intercepts and the slopes are also shown. The intercepts and slopes are the values of the kinetic coefficients in eqn. (1) and are given in Table 2. In Table 2, which correlates the binding sites concentration with enzyme activity, the catalytic centre activity is 2.4 s^{-1} for ethanol and 7.4 s^{-1} for propan-2-ol. For the dimeric native enzyme the turnover number or molecular activity is twice this. Table 2 and Fig. 3 also show that the values obtained with 0.5 mM-NAD^+ and varied alcohols approximate to those for an infinite concentration of NAD^+ and the alcohol.

Table 3(*a*) shows the percentage activity of a 1 mM solution of various saturated and unsaturated alcohols relative to 1 mM-ethanol. The values of V_m (molecules of product produced/s per enzyme active site), K_m and V_m/K_m are summarized in Table 3(*b*). Alcohol concentrations ranging from 0.05 to 100 mM were normally used for the initial rate measurements. The upper limit was set by the low water solubility of the larger alcohols. In the initial rate measurements with butan-1-ol, propan-2-ol and butan-2-ol, substrate activation occurred at concentrations greater than 25 mM. For the other alcohols no substrate activation or inhibition was observed. The striking constancy of V_m for secondary alcohols emphasizes the common rate-determining step for these alcohols.

Isotope effects

Table 4 lists the kinetic isotope effects on V_m^H/V_m^D and $(V_m/K_m)^H/(V_m/K_m)^D$. The alcohols used were ethanol, [$^2H_6$]ethanol, propan-2-ol, [$^2H_8$]propan-2-ol, cyclohexanol and [$^2H_{12}$]cyclohexanol. The V_m, K_m and V_m/K_m values for these alcohols are summarized in Table 3(*b*).

Inhibition

Pyrazole is an ethanol-competitive inhibitor of the enzyme. K_I was 16.8 μM and 14.1 μM at pH 8.5 and 9.5. As these values were obtained with saturating concentrations of NAD^+, they reflect $K_{EO,I}$ values (Theorell *et al.*, 1969).

Imidazole showed pure competitive inhibition against varied ethanol and (*R*)-(+)-1-phenylethanol (Fig. 4*a*). However, with butan-1-ol, propan-2-ol, butan-2-ol or hexan-2-ol as the varied substrate, competitive inhibition with stimulation occurred (Fig. 4*b*).

Isobutyramide is an ethanol- and butan-2-ol-competitive inhibitor of the enzyme. At pH 9.5 the $K_{EO,I}$ values are 14.7 mM and 17.4 mM.

2-Chloroethanol and 2,2,2-trifluoroethanol are ethanol-competitive inhibitors of the enzyme. At pH 9.5, $K_{EO,I}$ was 5.6 mM with 2-chloroethanol and 2.5 mM with trifluoroethanol. Like these two primary haloalcohols, the two secondary alcohols 2-methylpentane-2,4-diol and 2,3-dimethylbutan-2-ol were either poor or non-substrates. Used as inhibitors, concentrations up to 2 mM of the latter alcohols did not affect ethanol oxidation.

Cibacron Blue and NADH are NAD^+- competitive

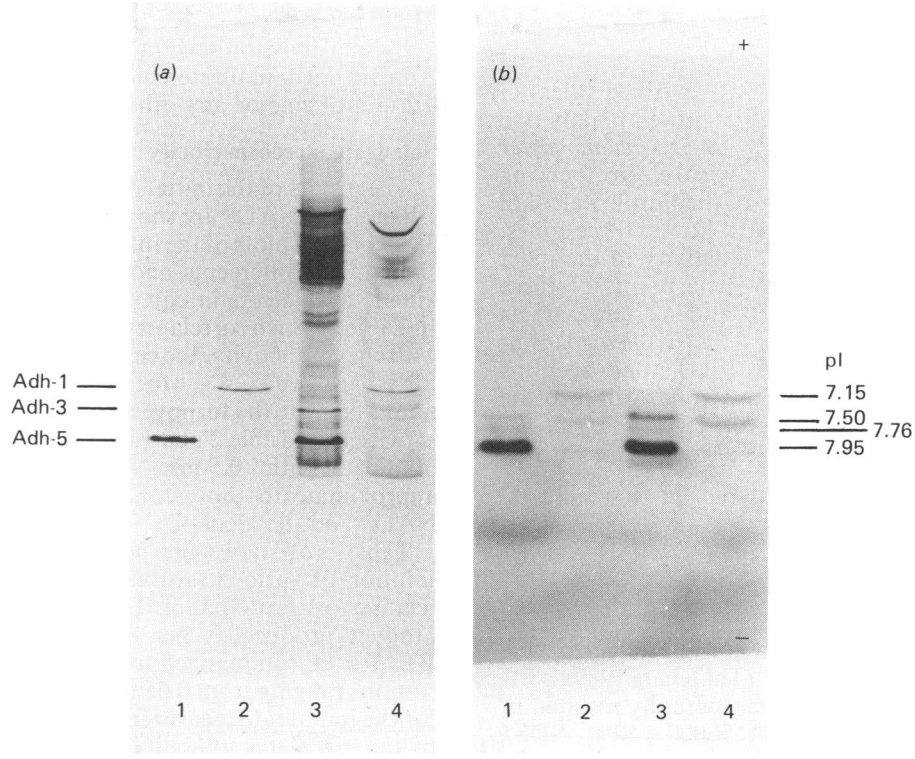

Fig. 2. Thin-layer gel electrofocusing in polyacrylamide gel of *D. lebenonensis* Adh

(*a*) Gel fixed and stained for protein as described in the Materials and methods section. (*b*) Gel incubated for alcohol dehydrogenase activity. 1 and 3, pure Adh and crude homogenate; 2 and 4, pure Adh and crude homogenate preincubated with 50 mM-NAD^+ and 150 mM-acetone for 4 h at room temperature.

Table 1. Amino acid analysis of *D. lebanonensis* alcohol dehydrogenase

The number of residues/enzyme subunit was calculated from the analytical data in column 1 by dividing by 4.55, which gave the best fit for an enzyme subunit of M_r 27800.

Amino acid	Amount recovered (nmol)	Content (residues/subunit)	Adh^S†
Asx	117.2	25.7	29
Thr	89.3	19.6	27
Ser	59.2	13.0	9
Glx	93.8	20.6	17
Pro	54.5	11.9	11
Gly	73.0	16.0	20
Ala	101.3	22.3	21
Val	93.2	20.5	22
Met	–	1*	0
Ile	94.8	20.8	23
Leu	103.0	22.6	27
Tyr	14.8	3.3	6
Phe	45.0	9.8	9
His	32.4	7.1	4
Lys	69.3	15.2	18
Arg	43.3	9.5	5
Trp	–	4*	4
Cys	–	2*	2

* Data from Vilageliu *et al.* (1982).
† Sequence composition from Thatcher (1980).

inhibitors. With varied NAD^+ and a fixed concentration of 8 mM- butan-2-ol, $K_{E,I}$ was 2.3 μM at pH 9.5 for the former inhibitor while for NADH, K_I was 2.9 μM at pH 8.5.

Table 2. Kinetic coefficients for the oxidation of ethanol and propan-2-ol by NAD^+ and *Drosophila lebanonensis* alcohol dehydrogenase at 23.5 °C and pH 9.5

The kinetic coefficients (Engel, 1981) are those in the initial rate equation:

$$\frac{e}{v} = \phi_0 + \frac{\phi_1}{[NAD^+]} + \frac{\phi_2}{[alcohol]} + \frac{\phi_{12}}{[NAD^+][alcohol]} \quad (1)$$

Kinetic parameter (unit)	Ethanol	Propan-2-ol
ϕ_0 (s)	0.42	0.14
ϕ_1 (μM·s)	4.5	3.8
ϕ_2 (mM·s)	2.5	0.16
ϕ_{12} (mM^2·s)	0.059	0.0027
$\frac{\phi_1}{\phi_0} = K_{m(NAD^+)}$ (μM)	11	28
$\frac{\phi_2}{\phi_0} = K_{m(alcohol)}$ (mM)	6.0	1.1
$\phi_1^{-1} = k_1$ ($M^{-1}\cdot s^{-1}$)	$0.22\cdot10^6$	$0.26\cdot10^6$
$\frac{\phi_{12}}{\phi_1\phi_2} = k_{-1}(s^{-1})$	5.1	4.6
$\frac{\phi_{12}}{\phi_2} = K_{E,NAD^+}$ (μM)	23	17
$\phi_0^{-1} = V_m$ (s^{-1})	2.4	7.4

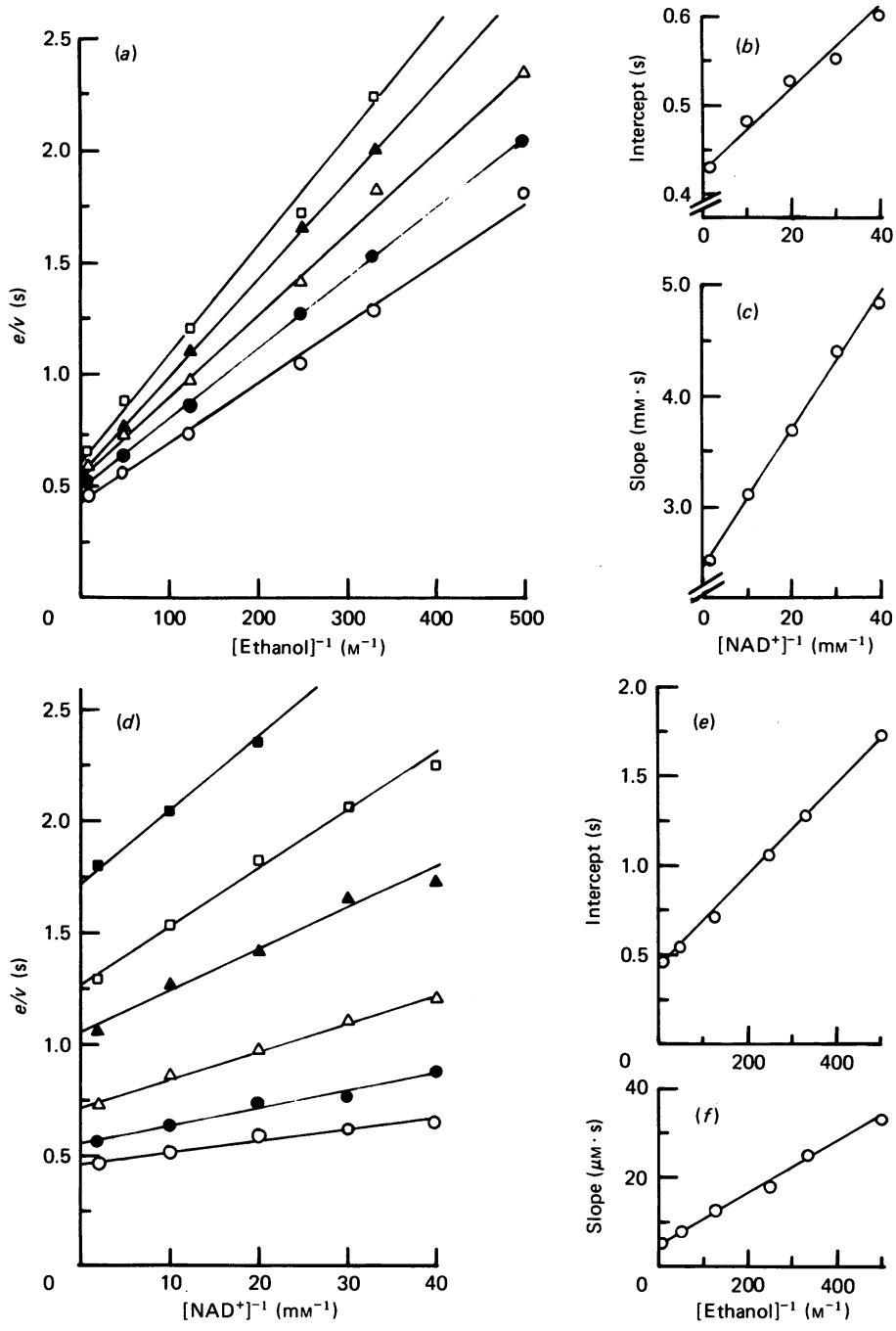

Fig. 3. Primary and secondary plots at pH 9.5 and 23.5 °C

(*a*) Variation of the reciprocal of the specific initial rate (v/e) with the reciprocal of the ethanol concentration for several constant NAD^+ concentrations (μM): ○, 500; ●, 100; △, 50; ▲, 33; □, 25. (*b*) and (*c*) Variation of the intercepts and slopes of the double-reciprocal plots in (*a*) with the reciprocal of the NAD^+ concentration. (*d*) Variation of the reciprocal of the specific initial rate (v/e) with the reciprocal of the NAD^+ concentration for several constant ethanol concentrations (mM): ○, 100; ●, 20; △, 8; ▲, 4; □, 3; ■, 2. (*e*) and (*f*) Variation of the intercepts and slopes of the double reciprocal plots in (*d*) with the reciprocal of the ethanol concentration.

Spectra

Addition of pyrazole to NAD^+ or to enzyme in 0.1 M-Tris/HCl buffer, pH 8.5, caused no spectral change. The absorption spectrum for a mixture of NAD^+ (13 μM) and the enzyme (10 μM) was the sum of the individual spectra (Fig. 5). Addition of 10 mM-pyrazole to the mixture of enzyme and NAD^+ produced the ternary enzyme–NAD^+–pyrazole complex ($K_{EO,I}$ is 16.8 μM). This gave an increase in absorption between 275 and 340 nm, while the difference spectrum of the ternary enzyme–NAD^+– pyrazole complex minus the binary enzyme–NAD^+ complex showed a peak at 305 nm (Fig. 5).

Table 3. Activity and kinetic coefficients for the oxidation of primary and secondary alcohols by NAD^+ and *Drosophila lebanonensis* alcohol dehydrogenase at 23.5 °C and pH 9.5

(*a*) Substrate specificity determined with 1 mM-alcohol and 0.5 mM-NAD^+ in a total volume of 3 ml of 0.1 M-glycine/NaOH buffer. (*b*) Kinetic coefficients.

	(*a*)	(*b*)		
Substrate	Rate relative to ethanol	V_m $(1/\phi_0)$ (s^{-1})	K_m (ϕ_2/ϕ_0) (mM)	V_m/K_m $(1/\phi_2)$ $(s^{-1}\cdot mM^{-1})$
Methanol	0	–	–	–
Ethanol	100	2.3	6.2	0.37
[$^2H_6$]Ethanol	–	0.81	10.4	0.08
Propan-1-ol	314	3.2	1.9	1.68
Butan-1-ol	446	4.3	1.9	2.3
Pentan-1-ol	119	1.8	4.1	0.44
Hexan-1-ol	133	4.0	7.4	0.54
Heptan-1-ol	76	–	–	–
Octan-1-ol	25	–	–	–
2-Methylpropan-1-ol	136	1.6	2.7	0.59
S-(−)-2-Methylbutan-1-ol	287	2.0	0.95	2.1
2,2-Dimethylbutan-1-ol	76	–	–	–
3-Methylbutan-1-ol	43	–	–	–
3,3-Dimethylbutan-1-ol	9	–	–	–
4-Methylpentan-1-ol	303	1.7	0.81	2.1
S-(+)-2-Aminobutan-1-ol	14	–	–	–
R-(−)-2-Aminobutan-1-ol	2	–	–	–
2-Chloroethanol	4	–	–	–
2,2,2-Trifluoroethanol	0	–	–	–
2-Propyn-1-ol	4	–	–	–
2-Propen-1-ol	280	–	–	–
2-Buten-1-ol	275	–	–	–
3-Buten-1-ol	304	–	–	–
trans-2-Hexen-1-ol	108	–	–	–
5-Hexen-1-ol	120	–	–	–
Nerol	51	–	–	–
Geraniol	53	–	–	–
Farnesol	29	–	–	–
L-Histidinol	0	–	–	–
Propan-2-ol	998	7.2	1.2	6.0
[$^2H_8$]Propan-2-ol	–	7.2	2.3	3.1
R/*S*-Butan-2-ol	1432	7.1	0.39	18.2
R-(−)-Butan-2-ol	1390	7.6	0.38	20.0
S-(+)-Butan-2-ol	1259	6.8	0.48	14.2
R/*S*-Pentan-2-ol	1665	7.3	0.42	17.4
R-(−)-Pentan-2-ol	1624	7.6	0.28	27.1
R/*S*-Hexan-2-ol	1809	8.0	0.31	25.8
R-(−)-Hexan-2-ol	2238	8.0	0.16	50.0
S-(+)-Hexan-2-ol	1815	8.0	0.31	25.8
R/*S*-Heptan-2-ol	1223	8.0	1.42	5.6
R-(−)-Heptan-2-ol	1685	7.4	0.66	11.2
S-(+)-Heptan-2-ol	75	1.7	6.5	0.26
R-(−)-Octan-2-ol	1931	8.1	0.50	16.2
S-(+)-Octan-2-ol	68	0.77	6.4	0.12
Pentan-3-ol	1342	–	–	–
R/*S*-Hexan-3-ol	890	–	–	–
R/*S*-Heptan-3-ol	1158	–	–	–
Heptan-4-ol	161	4.3	6.6	0.65
R/*S*-3-Methylbutan-2-ol	1381	–	–	–
R-(−)-3-Methylbutan-2-ol	1301	8.0	1.02	8.0
S-(+)-3-Methylbutan-2-ol	1539	8.0	0.62	12.9
R/*S*-3,3-Dimethylbutan-2-ol	299	–	–	–
2-Methyl-pentan-2,4-diol	3	–	–	–
2,3-Dimethylbutan-2-ol	0	–	–	–
1-Aminopropan-2-ol	2	–	–	–
3-Butyn-2-ol	1383	–	–	–
3-Penten-2-ol	1595	–	–	–
4-Penten-2-ol	1674	–	–	–
1-Penten-3-ol	1440	–	–	–

Table 3. (*cont.*)

Substrate	(*a*) Rate relative: to ethanol	(*b*) V_m $(1/\phi_0)$ (s^{-1})	K_m (ϕ_2/ϕ_0) (mM)	V_m/K_m $(1/\phi_2)$ $(s^{-1}\cdot mM^{-1})$
1-Hexen-3-ol	1668	–	–	–
1-Hexyn-3-ol	1300	–	–	–
Cyclohexanol	1314	7.4	0.72	10.3
[$^2H_{12}$]Cyclohexanol	–	7.4	1.9	3.9
trans-4-Methylcyclohexanol	953	7.1	1.1	6.5
cis-4-Methylcyclohexanol	1100	7.1	1.0	7.1
3-Methyl-2-cyclohexanol	2088	8.2	0.28	29.3
R-(+)-*cis*-Verbenol	854	7.1	1.2	5.9
S-(−)-*cis*-Verbenol	94	–	–	–
R-(+)-*trans*-Verbenol	162	–	–	–
S-(−)-*trans*-Verbenol	140	–	–	–
S-(−)-*trans*-Bicyclo(2.2.1)-heptanol	1054	6.7	0.85	7.9
R-(+)-*trans*-Bicyclo (2.2.1)-heptanol	812	6.4	1.5	4.3
R/S-*trans*-Bicyclo(2.2.1)-heptanol	795	6.3	1.6	3.9
S-(−)-*cis*-Bicyclo(2.2.1)-heptanol	427	6.6	3.8	1.7
R/S-Borneol	24	–	–	–
R-(−)-Borneol	28	–	–	–
R/S-Menthol	27	–	–	–
R-(−)-Menthol	21	–	–	–
Benzyl alcohol	37	–	–	–
2-Methoxybenzyl alcohol	24	–	–	–
3-Methoxybenzyl alcohol	37	–	–	–
4-Methoxybenzyl alcohol	27	–	–	–
4-Nitrobenzyl alcohol	29	–	–	–
2-Phenylethanol	7	–	–	–
2-(3-Methoxy-4-hydroxy-phenyl)ethanol	0	–	–	–
3-Phenyl-2-propen-1-ol	30	–	–	–
R/S-1-Phenylethanol	196	1.1	0.95	1.2
R-(+)-1-Phenylethanol	350	5.2	3.4	1.5
S-(−)-1-Phenylethanol	169	2.5	3.9	0.64
2-Amino-1-phenylethanol	15	–	–	–
Diphenylmethanol	23	–	–	–
bis-(4-Hydroxy-3-methoxy-phenylglycol)	5	–	–	–
α,β,γ-Hydroxybutyrate	0	–	–	–
DL-Lactate	0	–	–	–
Glycerol	0	–	–	–
Rhamnose	0	–	–	–
Sorbitol	0	–	–	–

Table 4. Kinetic isotope effects on the kinetic coefficients for *Drosophila lebanonensis* Adh

Substrate	V_m^H/V_m^D	$(V_m/K_m)^H/(V_mK_m)^D$
Ethanol/[$^2H_6$]ethanol	2.9	4.6
Propan-2-ol/[$^2H_8$]propan-2-ol	1.0	1.9
Cyclohexanol/[$^2H_{12}$]cycohexanol	1.0	2.6

The absorption spectrum of *D. lebanonensis* Adh (10 μM) and NADH (10 μM) was the sum of the individual spectra. Addition of isobutyramide (33 mM) to this mixture caused no spectral change.

A mixture of 10 μM-Adh and 10 μM-NADH produced large changes in the fluorescence emission spectrum of NADH (Fig. 6). The $\lambda_{max.}$ of NADH changed from 460 nm to 424 nm in the mixture and a 20-fold increase

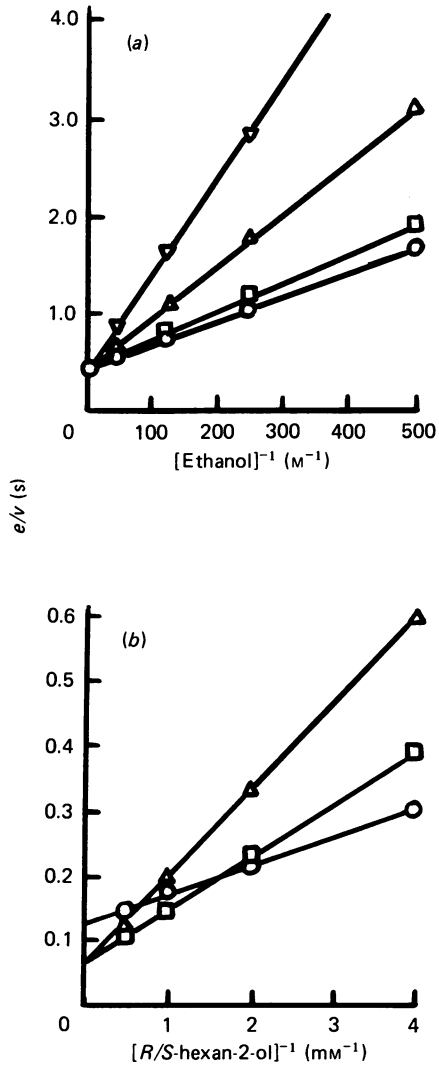

Fig. 4. Double-reciprocal plots of v/e against concentration of ethanol (*a*) and hexan-2-ol (*b*)

(*a*) Imidazole (mM): ○, 0; □, 200; △, 400; ▽, 500. (*b*) Imidazole (mM): ○, 0; □, 400; △, 500.

in the fluorescence intensity occurred at 424 nm. Addition of isobutyramide (5–33 mM) to this mixture caused no further change in the spectrum.

Concentration of active sites: active site titration

As the difference spectrum of the enzyme–NAD^+–pyrazole complex minus the enzyme–NAD^+ complex showed a peak at 305 nm (Fig. 5*b*), spectrophotometric titrations were performed at this wavelength. Aliquots of 3 μl of 605 μM-NAD^+ solution were added to a 1 ml mixture of enzyme and 10 mM-pyrazole (Fig. 5*c*). A molar absorbance ϵ_{305} of 14.5×10^3 $M^{-1} \cdot cm^{-1}$ was obtained.

The protein concentration in these experiments was calculated from amino acid analysis and for the dimer resulted in a molar absorbance ϵ_{280} of 13.3×10^4 $M^{-1} \cdot cm^{-1}$.

The difference spectra titrations showed that one NAD^+ molecule was bound to each enzyme subunit and that there are two active sites per enzyme molecule.

Concentration of active sites: rate assay

Using the rate assay, the initial velocities were linearly related to the concentration of enzyme active sites determined by titration (Fig. 5*d*). Thus, enzyme concentrations can be accurately determined as from the slope ΔA_{340}/min per μM enzyme active site was 0.82.

DISCUSSION

Amino acid analysis of the *D. lebanonensis* Adh polypeptide chain accounts for 247 amino acids. This is similar to the 255 residues of the *D. melanogaster* subunit (Benyajati *et al.*, 1981). It also agrees with the M_r values determinated for the dimeric enzymes and their subunits of both *Drosophila* species. The amino acid composition of the *D. lebanonensis* enzyme compared with that of several other *Drosophila* species shows that the frequency of only two amino acids, cysteine and tryptophan, have been maintained (Vilageliu *et al.*, 1982). Phylogenetic relationships based on the amino acid composition also suggest that *D. lebanonensis* is not closely related to *D. melanogaster* (Vilageliu & Gonzalez-Duarte, 1984).

Table 3 shows that the substrate stereospecificity of *D. lebanonensis* Adh is similar to that of other *Drosophila* species Adh (Chambers *et al.*, 1981; Juan & Gonzalez-Duarte, 1981; Oakeshott *et al.*, 1982; Winberg *et al.*, 1982*a*; Hovik *et al.*, 1984), with secondary alcohols being better substrates than primary alcohols. This implies that all *Drosophila* alcohol dehydrogenases studied so far contain a substrate-binding region where both alkyl groups of a secondary alcohol are bound to the enzyme. The relatively constant V_m value of 7.4 s^{-1} for secondary alcohols (Table 3), the lack of a primary kinetic ^2H isotope effect (Table 4), substrate activation at high alcohol concentrations (Results section) and the inhibition/stimulation with imidazole (Fig. 4*b*) shows that these alcohols are oxidized in an ordered reaction pathway with rate-limiting enzyme–NADH dissociation (Winberg *et al.*, 1982*a*,*b*; Hovik *et al.*, 1984). With primary alcohols, V_m varies and the values are much lower than those for the secondary alcohols following a Theorell–Chance mechanism. With ethanol/[$^2H_6$]ethanol, a primary kinetic ^2H isotope effect of 2.9 on V_m^H/V_m^D and a isotope effect of 4.9 for $(V_m/K_m)^H/(V_m/K_m)^D$ was obtained. These values, along with the competitive inhibition of imidazole against varied ethanol (Fig. 4*b*) show that the interconversion of the ternary complexes has become rate-limiting for ethanol oxidiation. For butan-1-ol the maximum velocity, V_m, was 4.3 s^{-1}, which is between that of ethanol (2.3 s^{-1}) and the dissociation rate of the enzyme–NADH complex (7.4 s^{-1}). With this primary alcohol, substrate activation occurred at high alcohol concentrations and competitive inhibition with stimulation occurred with imidazole. Thus the rate-limiting step for butan-1-ol and alcohols with a similar V_m seems composite, comprising at least the interconversion of the ternary complexes and the dissociation of reduced coenzyme from the binary complex.

Drosophila alcohol dehydrogenases forms strong ternary enzyme–NAD^+–pyrazole complexes which are characterized by an absorption peak at 305 nm (Fig. 5*b*; Place *et al.*, 1980; Winberg *et al.*, 1982*b*, 1985). In the metalloenzyme horse liver Adh, pyrazole forms a bridge between the active site zinc ion and C-4 of the positively charged pyridine ring of NAD^+ (Theorell & Yonetani,

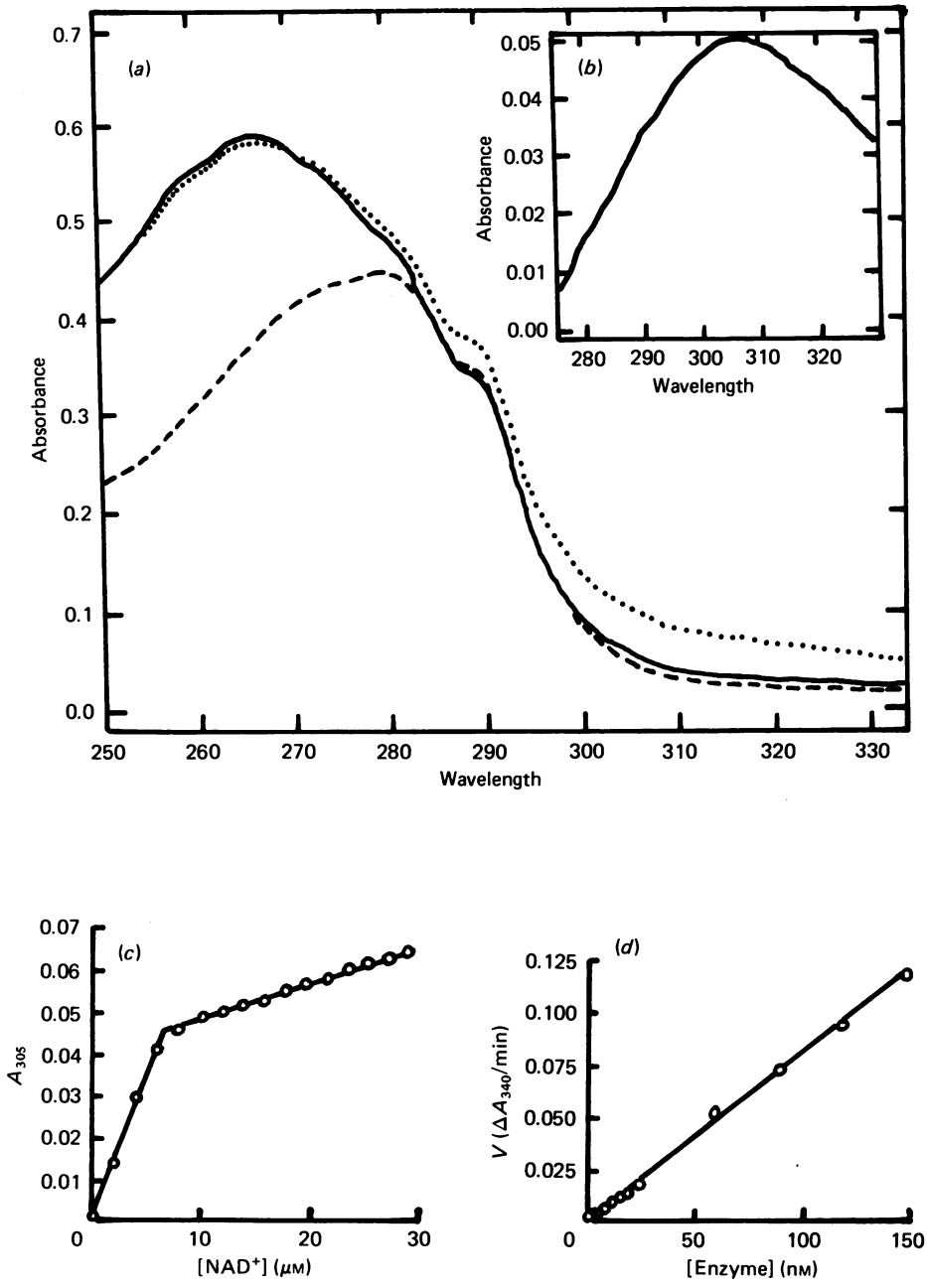

Fig. 5. *D. lebanonensis* Adh: absorption spectra, difference spectra, spectrophotometric titration and rate assay

(*a*) Absorption spectra: ---, enzyme; ——, enzyme–NAD^+; . . ., enzyme–NAD^+–pyrazole. (*b*) Difference spectrum of enzyme–NAD^+–pyrazole minus enzyme–NAD^+. Enzyme, 10 μM; NAD^+, 10 μM; pyrazole, 10 mM. Measurements were carried out in 0.1 M-Tris/HCl buffer, pH 8.5. (*c*) Spectrophotometric titration with NAD^+ of enzyme and 10 mM-pyrazole in 1 ml of 0.1 M-Tris/HCl buffer, pH 8.5, enzyme: 6.6 μM. (*d*) Velocity versus concentration of enzyme active sites.

1963; Eklund *et al.*, 1982). The difference spectra of horse liver enzyme–NAD^+–pyrazole minus enzyme–NAD^+ complex gave an absorption peak at 290 nm, in contrast with the binary NAD^+–pyrazole adduct which had an absorption maximum at 305 nm. In addition, binding of NADH to the horse liver enzyme is accompanied by a 15 nm blue shift of the 340 nm absorption maximum of the coenzyme (Theorell & Bonnichsen, 1951). A non-polar environment (Shifrin & Kaplan, 1960) and a positive charge in the vicinity of the dihydropyridine ring (Kosower, 1962) has been evoked to explain the blue shift. No such shift is observed with the binary NADH complex of *Drosophila* Adh. The absence of blue shifts in the spectra of *Drosophila* Adh–coenzyme complexes may be explained by the lack of a metal ion in the active site of the enzyme.

Binding of NADH to *D. lebanonensis* Adh gave an increase in intensity and a shift to a shorter wavelength in the NADH fluorescence emission maximum. However, no enzyme–NADH–isobutyramide complex was formed as shown by the lack of further change in the binary complex fluorescence on addition of isobutyramide and by competitive inhibition between isobutyramide and butan-2-ol.

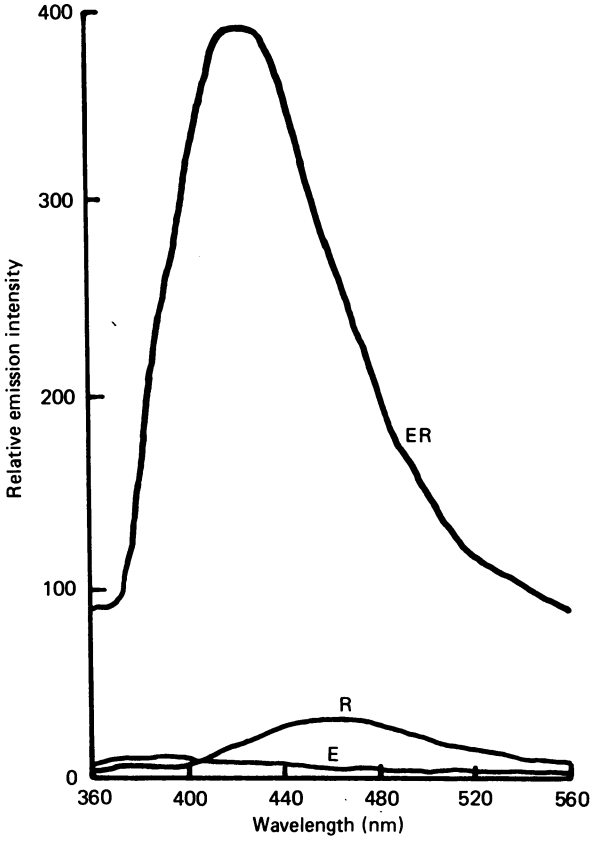

Fig. 6. Fluoroescence emission spectra of *D. lebanonensis* Adh

Fluorecence emission spectra (excitation 330 nm) of enzyme (E), NADH (R) and enzyme–NADH (ER); 10 μM-enzyme and/or 10 μM-NADH in 0.1 M-Tris/HCl, pH 8.5.

D. lebanonensis Adh, while differing markedly from mammalian, plant and other zinc alcohol dehydrogenases in amino acid composition, active site structure, substrate specificity etc., is however qualitatively similar to the alcohol dehydrogenase enzymes from other *Drosophila* species.

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