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Determination of some biochemical and structural features of alcohol dehydrogenases from *Drosophila simulans* and *Drosophila virilis*

Comparison of their properties with the *Drosophila melanogaster* Adh^s enzyme

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The biochemical properties of the enzyme alcohol dehydrogenase of two different *Drosophila* species, *Drosophila simulans* and *Drosophila virilis*, were studied and compared with those of *Drosophila melanogaster* Adh^s enzyme. All of them consist of two identical subunits of molecular weight 27800 and share significant similarities in function. The substrate specificities of these enzymes were characterized and $K_{m(app.)}$ and $V_{max.(app.)}$ values were calculated. All these alcohol dehydrogenases show greater affinity for secondary rather than for primary alcohols. The amino acid compositions of the three enzymes were determined, and there is a close similarity between the *D. simulans* and the *D. melanogaster* enzymes, but there are significant differences from the alcohol dehydrogenase of *D. virilis*. The *N*-terminal amino acid is blocked and the *C*-terminal amino acid is the same for all three alcohol dehydrogenases. The enzymes from the three species were carboxymethylated and digested with trypsin. The peptide 'maps' reveal, as expected, more homologies between the enzymes of *D. simulans* and *D. melanogaster* than with the enzyme of *D. virilis*.

The alleloenzymes of alcohol dehydrogenase from *Drosophila melanogaster* have been intensely studied in population genetics and biochemistry. They have been purified to homogeneity (Thatcher, 1977; Leigh Brown & Lee, 1979; Juan & González-Duarte, 1980), and not only are their biochemical features now known but the complete amino acid sequences of the different alleloenzymes have been determined (Thatcher, 1980). In contrast with all the information we have of the enzyme from *D. melanogaster*, we lack almost any biochemical and structural information concerning other species of *Drosophila*. The purpose of the present investigation is to start a comparative study of different alcohol dehydrogenases from species close to and distant from *D. melanogaster*. We wish not only to shed some light on the structural evolution of this molecule in *Drosophila* species but to try to relate the subsequent modifications with the function of this enzyme.

Experimental

Materials

The methods used to obtain the strains of *Drosophila melanogaster* Adh^s, *Drosophila simulans* and *Drosophila virilis* and the pure alcohol dehydrogenases from these species have already been described (Juan & González-Duarte, 1980).

NAD^+, guanidinium chloride, carboxypeptidase A (di-isopropyl phosphorofluoridate-treated), carbonic anhydrase from bovine erythrocytes, trypsin (diphenylcarbamoyl chloride-treated), tryptamine and toluene-*p*-sulphonic acid were purchased from Sigma (London) Chemical Co. (Poole, Dorset, U.K.). *N*-Ethylmorpholine was from Pierce Chemical Co., Rockford, IL, U.S.A. Sephadex G-75 (superfine grade) was from Pharmacia Fine Chemicals (Uppsala, Sweden). Dansyl chloride was from Fluka A.G., Buchs, Switzerland. Polyamide plates were bought from BDH Chemicals, Poole, Dorset, U.K. Bovine serum albumin, ovalbumin and horse myoglobin were purchased from Serva Feinbiochemica (Heidelberg, Germany). Propan-2-ol, propan-1-ol, 2-methylpropan-1-ol and butan-1-ol were from Doesder (Barcelona, Spain). All other laboratory reagents and biochemicals were from Merck (Darmstadt, Germany).

pH optima

The effect of pH on the rate of reaction catalysed by purified alcohol dehydrogenases was determined

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with 50mM-Tris/HCl. The activity was determined at pH 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5 and 10.0. The pH values were attained by adjustment with 1.0M-HCl. Alcohol dehydrogenase activity was quantified as described under 'Spectrophotometric measurements' below.

Spectrophotometric measurements

All spectrophotometric measurements were made in a Beckman DB-G recording spectrophotometer.

Alcohol dehydrogenase activity was quantified by measuring the rate of increase in absorbance at 340nm. One unit of activity reduces 1μmol of NAD^+/min at 25°C. In the standard assay 0.15ml of propan-2-ol (6.5%, v/v, in buffer) and 0.15ml of NAD^+ (10mg/ml in buffer) were added to 0.75ml of 10mM-sodium phosphate buffer, pH 8.0. The reaction was initiated by the addition of 0.15ml of enzyme solution, and the initial velocity was recorded. Kinetic constants were defined by using the direct linear plot described by Eisenthal & Cornish-Bowden (1974). Michaelis parameters were determined by varying the alcohol concentrations in the presence of a saturating concentration of NAD^+. The $K_{m(app.)}^{NAD^+}$ was determined by varying the NAD^+ concentrations in the presence of a saturating concentration of propan-2-ol.

N-Termini

A 10nmol portion of protein was dried down in a 6mm × 30mm Durham tube. Dansylation was performed by the method of Gray (1967). To identify the last residue of a protein the sample was dissolved in 5μl of 50% (v/v) pyridine and spotted on to both sides of a polyamide sheet. A standard mixture was added to one side. The plates were run by ascending chromatography with the following solvents: 1, 6% (v/v) formic acid; 2, toluene/acetic acid (9:1, v/v); 3, butyl acetate/methanol/acetic acid (30:20:1, by vol.). Solvents 2 and 3 were run at right-angles to solvent 1.

Dansyl-amino acids were detected under u.v. light.

Carboxypeptidase A digestion

A 2mg/ml solution of carboxypeptidase A was prepared as described by Ambler (1972). Portions (5, 10, 25 and 50μl) of this solution were added to 20nmol samples of each alcohol dehydrogenase, each dissolved in 50μl of 0.2M-*N*-ethylmorpholine/acetate buffer, pH 8.5 (Thatcher, 1977). After incubation for 1h at 37°C the reaction was stopped by lowering the pH to 3.0 with acetic acid. Any protein that was precipitated was removed by centrifugation, and the supernatant was dried in a desiccator. Each sample was then suspended in 500μl of 0.2M-sodium citrate buffer, pH 2.2, and applied directly to a Beckman 119C amino acid analyser.

Amino acid analysis

The amino acid composition of the protein was determined by analysis of five samples. Three samples of native enzyme were hydrolysed in 6M-HCl at 105°C for 24, 48 and 72h. A sample of oxidized alcohol dehydrogenase was hydrolysed in the same way for 24h, and another sample of native enzyme was hydrolysed in the presence of 3M-toluene-*p*-sulphonic acid containing 0.2% 3-(2-aminoethyl)indole in evacuated sealed tubes at 110°C for 24h (Liu & Chang, 1971). The same procedure was repeated three times with native alcohol dehydrogenases from *D. melanogaster* and *D. virilis* from three different purifications and twice with the native enzyme from *D. simulans* from two different purifications. All protein hydrolysates were analysed on a Beckman 119C automatic amino acid analyser.

Molecular-weight determination

The molecular weights of the crude enzyme extracts were determined by gel-permeation chromatography on a column (90cm × 1cm diam.) of Sephadex G-75 (superfine grade). Bovine serum albumin (mol.wt. 68750), ovalbumin (mol.wt. 45000), carbonic anhydrase (mol.wt. 31000) and myoglobin (mol.wt. 17800) were used as standards dissolved at 1mg/ml in 20mM-Tris/HCl buffer, pH 7.5. The samples were prepared as follows. Frozen *Drosophila* adults (1.5g) were blended with 2ml of 20mM-Tris/HCl buffer, pH 8.6, containing 0.2% (v/v) 2-mercaptoethanol and 1.1% (v/v) propan-2-ol for three 1min periods with 1min intervals. The slurry was then centrifuged at 23000*g* for 30min at 4°C. Next the supernatant was treated with 0.2ml of 2% (w/v) salmine sulphate, and the precipitate formed was discarded. A 1ml sample of the supernatant was directly applied to the top of the column.

The subunit molecular weight of alcohol dehydrogenase was determined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis on a thin-layer system as described by Juan & González-Duarte (1980).

Carboxymethylation and trypsin digestion

Pure alcohol dehydrogenase was carboxymethylated by the method of Crestfield *et al.* (1963). After the Sephadex G-25 step, which removes the salts and iodoacetic acid, the samples from *D. simulans* and *D. virilis*, in order to make the tryptic digest more efficient, were freeze-dried to 2ml, and then 2ml of 100mM-NH_4HCO_3, pH 8.6, was added and the whole was dried completely. After carboxymethylation the samples were digested with 2%

(w/v) trypsin in 100mM-NH_4HCO_3, pH 8.6, for 6.5h at 37°C. The enzyme solution was freeze-dried and dissolved in pyridine/acetic acid/water (1:10:289, by vol.), pH 3.6.

Peptide 'mapping'

The analytical peptide 'maps' were obtained by paper chromatography and electrophoresis by the method of Bennett (1967). A 1.5–2.0mg portion of digested sample was analysed by descending chromatography with butan-1-ol/acetic acid/water/pyridine (15:3:12:10, by vol.) and then electrophoresed at pH 3.6 in pyridine/acetic acid/water (1:10:89, by vol.). The following stains were used for the identification of the components: ninhydrin for peptides, the Ehrlich reaction for tryptophan, Pauli reaction for tyrosine and histidine and the Sagakuchi reaction for arginine.

Results

pH optima

The effects of pH on the activity of purified alcohol dehydrogenase of *D. simulans*, *D. virilis* and *D. melanogaster* were determined. The results are illustrated in Fig. 1. pH optima values are identical for the *D. melanogaster* and *D. virilis* enzymes (pH 8.0 and 9.5) and differ slightly for the *D. simulans* enzyme (pH 7.5 and 9.9).

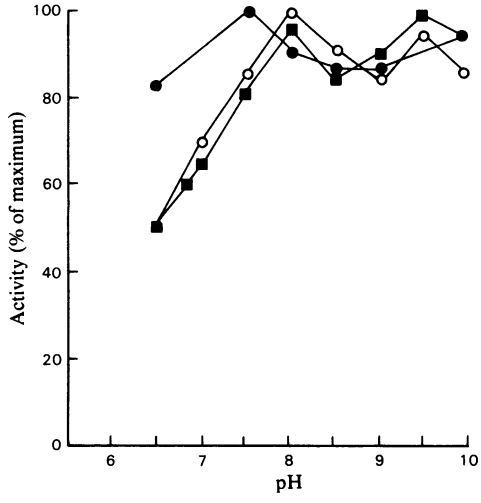

Fig. 1. *Activity of purified Drosophila alcohol dehydrogenases as a function of pH*

Experimental details are given in the text. The buffer used was 50mM-Tris/HCl. ■, *D. melanogaster* Adh^s enzyme; ●, *D. simulans* enzyme; ○, *D. virilis* enzyme.

Substrate specificities of alcohol dehydrogenases

Assays were performed with pure enzyme preparations to determine substrate specificities of the three alcohol dehydrogenases, and $K_{m(app.)}$ and $V_{max.(app.)}$ values are illustrated in Table 1. The alcohol dehydrogenases from *D. simulans* and *D. virilis* appear to have higher affinity for secondary rather than primary alcohols. The same effect was observed with the enzyme from *D. melanogaster*, as previously described by other authors (Day *et al.*, 1974; Thatcher, 1977; McDonald *et al.*, 1977).

It is difficult to assess the biological implications of these kinetic values because of the lack of knowledge of the real conditions in which the reaction takes place *in vivo*.

N-Termini

To characterize the *N*-terminal residues of alcohol dehydrogenases from *D. simulans* and *D. virilis* a dansylation reaction was performed as described in the Experimental section. It appears that, as in the *D. melanogaster* enzyme, these alcohol dehydrogenases have a blocked *N*-terminal amino acid that is unable to form any dansyl derivative.

Carboxypeptidase A digestion

The results of carboxypeptidase A digestion for the three alcohol dehydrogenases are presented in

Table 1. *Substrate specificity of alcohol dehydrogenases from D. melanogaster Adh^s, D. simulans and D. virilis*

For each substrate the values in the first row correspond to the enzyme from *D. melanogaster* Adh^s, the values in the second row to the enzyme from *D. simulans* and the values in the third row to the enzyme from *D. virilis*. For experimental details see the text.

	$K_{max.(app.)}$ (mM) $\bar{x} \pm e_s$	$V_{max.(app.)}$ (mol/min per mol of subunit) $10^2 \times \bar{x} \pm e_s$
NAD^+	0.26 ± 0.05	
	0.19 ± 0.02	
	0.20 ± 0.02	
Propan-2-ol	1.87 ± 0.03	9.78 ± 1.01
	0.93 ± 0.05	6.67 ± 1.07
	1.99 ± 0.16	8.15 ± 1.66
Butan-2-ol	0.86 ± 0.06	9.34 ± 0.85
	0.41 ± 0.12	6.02 ± 0.95
	1.60 ± 0.28	7.75 ± 1.66
Ethanol	8.92 ± 0.35	4.78 ± 0.5
	5.22 ± 0.86	2.08 ± 0.23
	6.44 ± 0.99	4.01 ± 0.17
Propan-1-ol	3.42 ± 0.45	4.60 ± 0.94
	3.33 ± 0.18	2.15 ± 0.15
	4.16 ± 0.46	4.99 ± 0.36
Butan-1-ol	3.31 ± 0.35	5.75 ± 0.91
	3.01 ± 0.27	6.13 ± 0.19

Fig. 2. The *C*-terminal residue is isoleucine for the three enzymes, and the results obtained are consistent with the *C*-terminal sequences given in Fig. 3. We have to remark that an unusual facet of the carboxypeptidase A experiment is the rapid release of glycine and other residues adjacent to glycine in the interior sequence of the molecule. This is in apparent contradiction with the carboxypeptidase A behaviour described by Ambler (1972), and there is no evident explanation that could account for that result.

Molecular weight

The molecular weights of the three native enzymes were determined by gel-permeation chromatography as described in the Experimental section. The regression line of the plot of log(mol.wt.) of standards versus elution volume is $y = 2.62 - 0.03x$ and the correlation coefficient is $r = 0.99$. According to the elution volumes the following molecular weights were obtained: 55600 ± 4560 for the *D. melanogaster* Adh^s enzyme, 57600 ± 4690 for the *D. simulans* enzyme and 54600 ± 1960 for the *D. virilis* enzyme. For the enzyme from each species the value of the elution volume was taken as the average of three different experiments. The differences are not significant for the three enzymes. The molecular weight of the native enzyme in each species is in accordance with the subunit molecular weight of these alcohol dehydrogenases (27800) determined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis in a thin-layer system (Juan & González-Duarte, 1980), and also with the value that can be deduced (27400) from the amino acid sequence data obtained by Thatcher (1980) for the *D. melanogaster* enzyme.

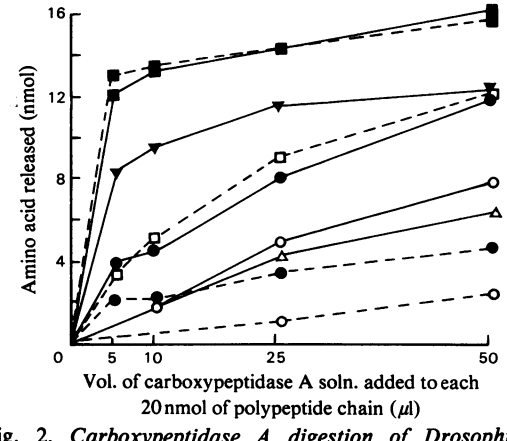

Fig. 2. *Carboxypeptidase A digestion of Drosophila alcohol dehydrogenases*

Experimental details are given in the text. The amino acids were quantified by amino acid analysis. Initial concentration of polypeptide chains was 20nmol. ——, *D. virilis*; – – – –, *D. simulans*. ■, Isoleucine; □, glycine; ●, serine; ▼, histidine; △, threonine; ○, aspartic acid.

Amino acid composition

The amino acid compositions of the three purified alcohol dehydrogenases are given in Table 3. The results obtained for *D. melanogaster* are in close agreement with the complete amino acid sequence determined by Thatcher (1980). The details of the amino acid analysis for the *D. virilis* are given in Table 2.

Peptide 'mapping'

The peptide 'maps' were obtained to show the structural homologies of the three alcohol dehydrogenases. Composite 'maps' obtained by using a general stain (ninhydrin), and specific stains are shown in Fig. 4. When repeating these 'maps' we observe that there is a remarkable constancy in the number and position of the peptides located outside the square marked with a dotted line on the 'maps'. This region corresponds to the most soluble part of the molecule and shows close homology in the three species. The other peptides show a much more complex pattern, implying that they correspond to a region of the molecule to which trypsin has a difficult access. They often vary in number and position in different hydrolysates, and this makes the comparison difficult and unreliable.

There are always more peptides obtained after trypsin digestion than would be expected from the results of the amino acid analyses. These are probably due to incomplete tryptic cleavage.

By using ninhydrin as a general stain we detect 33 peptides with the *D. melanogaster* enzyme, 45 peptides with the *D. simulans* enzyme and 43 peptides with the *D. virilis* enzyme. The expected number would be 23, 25 and 24 peptides respec-

D. simulans	-Asp-Ser-Gly-Ile
D. virilis	-Asp-Thr-Ser-His-Ile
D. melanogaster Adh^s	-(Thr / Leu, Ala)-Ser-Gly-Ile
D. melanogaster Adh$^{UF(1)}$	-(Gly / Ala, Leu-Thr)-Ile
D. melanogaster Adh$^{s(2)}$	-Asp-Ser-Gly-Ile

Fig. 3. *C-Terminal sequences of Drosophila alcohol dehydrogenases on the basis of carboxypeptidase A digestion*

(1) Thatcher (1977); (2) Thatcher (1980).

Table 2. *Amino acid composition of alcohol dehydrogenase from D. virilis*

Results from three different samples of three different purifications are given. For each amino acid the first row corresponds to the first sample, the second row to the second sample and the third row to the third sample. The best value was obtained by averaging the values for the 24h, 48h and 72h samples hydrolysed with HCl, except for serine and threonine (extrapolated to zero hydrolysis time) and for valine and isoleucine (extrapolated to infinite hydrolysis time). To determine the number of residues per molecule of alcohol dehydrogenase, in the first row the best-value results were divided by 4.45, in the second row by 7.03 and in the third row by 5.32. The first two factors were derived by summing the values for aspartic acid, glutamic acid, proline, glycine, leucine and phenylalanine and dividing by 158. The third factor was derived by dividing the value for tryptophan by 4.

	Amino acids recovered after acid hydrolysis (nmol)							
Amino acid	24h	48h	72h	Performic acid-oxidized	Toluene-*p*-sulphonic acid-hydrolysed	Best value	Content (residues/molecule)	
Lys	85.2	77.3	76.4	83.4	68.4	79.6	17.8	
	104.2	104.5	101.2	126.0	94.5	102.6	14.6	15.9 ± 1.7
	80.8	85.2	82.5	83.7	79.3	82.8	15.5	
His	21.6	20.2	22.8	18.0	21.5	21.5	4.8	
	31.0	27.8	28.7	22.9	25.2	29.1	4.1	4.6 ± 0.4
	24.9	25.7	28.1	25.1	20.1	26.1	4.9	
Arg	37.0	36.6	36.5	31.8	36.9	36.7	8.2	
	55.0	53.5	53.4	60.7	47.7	53.9	7.7	8.0 ± 0.2
	42.6	44.0	43.2	44.4	39.7	43.2	8.1	
Asx	110.6	115.0	115.4	114.9	114.1	113.6	25.5	
	182.2	185.9	189.5	184.7	178.2	185.8	26.4	26.1 ± 0.6
	104.7	139.9	141.7	141.8	146.2	140.7	26.4	
Thr	96.7	98.2	96.0	100.6	106.1	97.7	21.9	
	162.2	162.5	157.6	164.5	158.4	164.0	23.3	23.0 ± 1.1
	125.3	124.1	120.7	125.7	126.8	128.0	24.0	
Ser	54.6	44.8	45.1	52.3	53.1	62.0	13.9	
	104.4	100.4	100.9	78.0	88.2	107.8	15.3	14.3 ± 0.7
	69.4	66.6	61.5	64.1	68.9	73.5	13.8	
Glx	80.8	84.2	82.9	82.2	85.3	82.6	18.5	
	124.7	129.5	125.7	135.0	123.3	126.6	18.0	18.7 ± 0.9
	104.8	105.1	104.2	104.3	101.0	104.7	19.7	
Pro	44.1	49.4	40.8	49.3	53.2	44.7	10.0	
	89.4	88.1	87.4	81.6	86.4	88.3	12.5	11.4 ± 1.3
	65.1	61.3	61.1	61.2	69.8	62.5	11.7	
Gly	78.5	78.5	76.1	67.1	80.1	77.7	17.4	
	135.4	133.2	134.1	144.7	120.6	134.2	19.0	18.4 ± 0.4
	98.7	101.3	102.2	100.3	98.9	100.7	18.9	
Ala	94.5	88.9	97.1	94.9	96.1	93.5	21.0	
	165.9	165.3	162.1	158.4	150.3	163.7	23.2	22.1 ± 1.1
	118.5	118.4	119.3	120.1	117.1	118.7	22.3	
Val	78.3	78.2	81.2	74.0	69.1	83.0	18.6	
	128.3	133.2	135.6	128.5	113.0	139.0	19.7	19.4 ± 0.7
	91.0	98.1	101.0	93.4	79.5	107.0	20.1	
Met	5.0	5.0	6.7	7.0	—	7.0	1.5	
	—	5.9	6.1	15.0	4.0	15.0	2.1	1.5 ± 0.5
	4.7	4.3	4.2	6.6	2.4	4.9	0.9	
Ileu	103.7	103.2	109.6	97.8	90.2	112.5	25.2	
	146.0	156.1	150.6	164.0	137.0	165.0	23.4	24.1 ± 0.9
	108.0	116.5	122.2	107.3	89.3	127.7	23.9	
Leu	112.6	108.5	110.7	116.3	104.5	110.6	24.8	
	177.4	171.6	164.7	181.0	165.0	171.2	24.3	24.0 ± 0.9
	122.6	122.6	123.0	122.3	117.1	122.7	23.0	
Tyr	25.1	22.2	22.8	13.7	22.4	23.3	5.2	
	—	34.3	32.5	22.9	32.4	33.4	4.7	4.9 ± 0.2
	25.8	26.4	26.1	16.6	26.8	26.1	4.9	
Phe	44.5	42.0	42.4	41.1	39.5	42.9	9.6	
	—	63.3	62.2	71.3	64.0	62.7	8.9	9.2 ± 0.5
	48.2	49.4	48.6	47.6	47.3	48.7	9.1	
Cys				9.7		9.7	2.1	
				15.4		15.4	2.2	2.1 ± 0.0
				11.8		11.8	2.2	
Trp					20.1	20.1	4.5	
					28.8	28.8	4.0	4.1 ± 0.2
					21.3	21.3	4.0	

Table 3. *Amino acid compositions of alcohol dehydrogenases from D. melanogaster Adh^s, D. simulans and D. virilis*

	D. melanogaster Adh^s (Thatcher, 1980) (from sequence)	*D. melanogaster* Adh^s (Thatcher, 1977)	*D. melanogaster* Adh^s	*D. simulans*	*D. virilis*
Asx	28	25.3	27.2	27.4	26.1
Thr	27	22.2	24.6	25.3	23.0
Ser	9	9.7	10.7	10.1	14.3
Glx	17	18.0	17.7	16.9	18.7
Pro	11	9.7	10.0	10.2	11.4
Gly	19	16.5	18.7	19.4	18.4
Ala	23	19.9	21.5	21.7	22.1
Cys	2	2.8	1.7	1.9	2.1
Val	22	19.1	19.5	19.4	19.4
Met	—	—	—	—	1.5
Ile	23	18.6	22.7	21.4	24.1
Leu	27	24.1	28.7	27.6	24.0
Tyr	6	5.5	6.1	5.9	4.9
Phe	9	8.0	9.5	9.1	9.2
Lys	18	16.1	18.5	20.0	15.9
His	4	3.6	3.9	3.9	4.6
Arg	5	4.9	5.0	5.2	8.0
Trp	4	4.1	3.8	3.8	4.1
Total	254	228.0	249.8	249.2	251.8

tively. When using specific stains, we observe the same effect, i.e. six arginine peptides (five expected by amino acid analysis) with the *D. melanogaster* enzyme, seven arginine peptides (five expected) with the *D. simulans* enzyme and eleven arginine peptides (eight expected) with the *D. virilis* enzyme. The number of peptides appearing on the 'maps' is related to the solubility of the carboxymethylated enzyme. Carboxymethylation was always performed in identical conditions, and the number of carboxymethylcysteine residues determined by amino acid analysis was always in complete agreement with the number of cysteine residues present in the molecule. The alcohol dehydrogenase from *D. simulans* and *D. virilis* needed a special treatment, as described in the Experimental section, or previous denaturation with urea, to be partially hydrolysed by trypsin. Even so, the hydrolysis was more incomplete than with the *D. melanogaster* enzyme, as revealed by the greater number of peptides obtained. If we consider that, according to Fletcher *et al.* (1978), we obtain with the *D. melanogaster* enzyme peptides that represent approximately half of the molecule, we could say that the peptides obtained from the enzymes of *D. simulans* and *D. virilis* probably correspond to an even smaller and variable part of the molecule. When considering the number of histidine and tyrosine peptides on the 'maps' we observe the following: there are three histidine and tyrosine peptides with the *D. melanogaster* enzyme, five with the *D. simulans* enzyme and seven with the *D. virilis* enzyme. The number of histidine and tyrosine residues determined by amino acid analysis is ten in the enzyme from each of the three species. The trypsin used for the proteolytic cleavage of the protein was the same with the three enzymes. There is an apparent contradiction between the total number of peptides obtained after digestion and the number of histidine peptides and tyrosine peptides. The insolubility of the part of the molecule that is partially digested by trypsin could well explain the extra number of peptides observed on the 'maps'. Also, the lower number of histidine peptides and tyrosine peptides observed could be due to the fact that some of these residues remain either in the insoluble part of the molecule, not digested by trypsin, or in the peptides insoluble in the buffers used. We could also claim that the increase in the number of peptides is due to over-digestion producing a high degree of pseudo-tryptic cleavages and to the poor quality of the trypsin used. Then we would have to postulate that the histidine peptides and tyrosine peptides that do not appear on the 'maps' are located either in the insoluble part of the molecule (or in the insoluble peptides) or that more than one of these residues are found in the same peptide. This appears to be true when considering the complete amino acid sequence of *D. melanogaster* alcohol dehydrogenase (Thatcher, 1980).

Peptide TV7, common to all three species studied, deserves comment. According to its mobility and also its specific stain for histidine it seems analogous to the T20 peptide (Thr-Thr-Leu-Val-His-Lys) described by Fletcher *et al.* (1978) for the enzyme from *D. melanogaster* Adh^s. This peptide has been consistently observed in these alcohol dehydro-

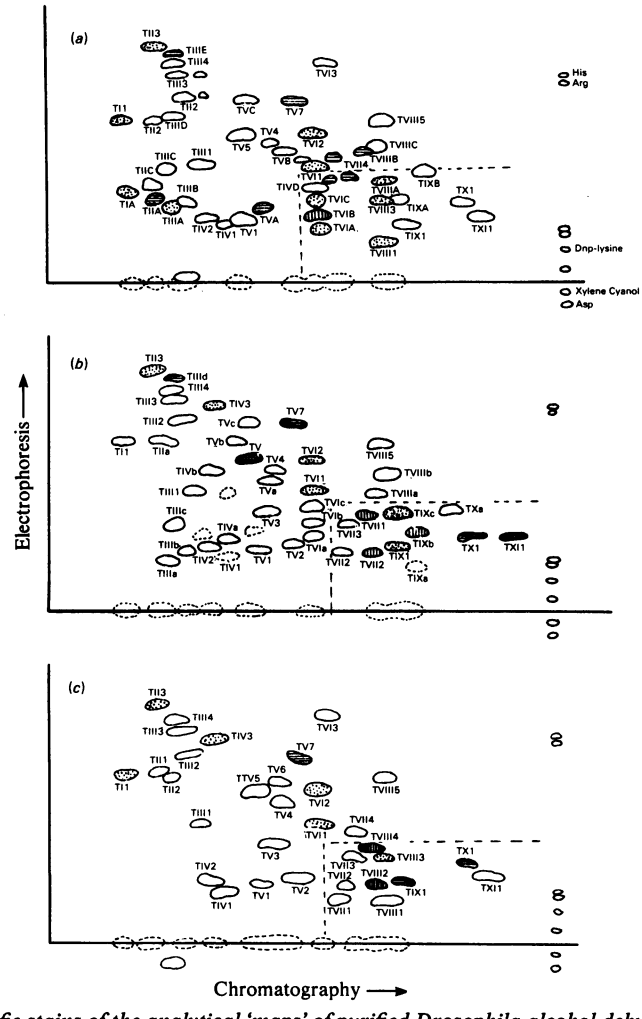

Fig. 4. *Specific stains of the analytical 'maps' of purified Drosophila alcohol dehydrogenases*
Experimental details are given in the text. First dimension: chromatography with butan-1-ol/acetic acid/water/pyridine (15:3:12:10, by vol.). Second dimension: electrophoresis at pH 3.6 with pyridine/acetic acid/water (1:10:89, by vol.). (*a*) *D. virilis* enzyme; (*b*) *D. simulans* enzyme; (*c*) *D. melanogaster* Adh^{s} enzyme. ▩, Arginine; ▤, histidine; ▥, tryptophan; ■, tyrosine.

genases, and seems to belong to a very conserved region of the enzyme in all the species studied. This would also support the hypothesis that Adh^{F} and Adh^{UF} enzymes originally come from the genes for the Adh^{s} enzyme, because there is an amino acid substitution (Lys→Thr) in this peptide in both allelic variants (Thatcher, 1980).

Peptides TII3 and TI1 specific for arginine are common to all three species, and peptide TIV3 only appears with the *D. simulans* and *D. melanogaster* enzymes, being absent with the *D. virilis* enzyme. The amino acid analysis of this peptide after elution shows that it only contains arginine. Looking at the full sequence of the Adh enzyme from *D. melanogaster* (Thatcher, 1980), we could tentatively explain the presence of this arginine residue. It would come from a trypsin digestion between residues

$$\overset{28}{\text{-Lys-}}\overset{29}{\text{Arg-}}\overset{30}{\text{Asp-}}$$

The substitution of lysine-28 by any amino acid other than arginine would produce a longer peptide, having arginine as the *C*-terminal. The original arginine peptide (TIV3) will not appear and instead we should get a larger one.

Discussion

The main aim of the present work has been to compare the biochemical and structural characteristics of three alcohol dehydrogenases. Two of them

belong to two morphologically almost identical and genetically very similar species, *D. melanogaster* and *D. simulans* (subgenus *Sophophora*). These sibling species differ in their alcohol tolerance; *D. melanogaster* is associated with alcohol-rich environments, whereas *D. simulans* is susceptible to the presence of alcohol and is seldom found in wine-cellars (McKenzie & Parsons, 1972). The enzyme present in the species *D. virilis*, although being able to utilize an alcohol substrate, is phylogenetically distant from *D. melanogaster*. The *virilis* group consists of 11 species, one of which, *D. virilis*, is currently cosmopolitan, and is an exception from the rest of the group in that it has been collected in breweries, markets and domestic situations (Spieth, 1979).

The results obtained clearly indicate that there are obvious homologies and differences among these three alcohol dehydrogenases. All of them in the native form consist of two identical subunits of mol.wt. 27800 and have a blocked *N*-terminal residue. Carboxypeptidase A digestion shows that the three residues in the *C*-terminal of the enzyme are identical with *D. melanogaster* and *D. simulans*, and identical with *D. virilis* except that in *D. virilis* the second residue glycine is replaced by histidine.

When the specificity of substrates is considered the results seem to indicate that there is closer similarity in function *in vitro* between the *D. melanogaster* and *D. virilis* enzymes than between the *D. simulans* enzyme and the other two enzymes. This is especially true when primary alcohols are used as substrates, and it is worth noticing that this situation is likely to be found *in vivo* because ethanol is probably the most frequent substrate found in their habitats.

The pH optima results support the similarity in function between the *D. melanogaster* and *D. virilis* enzymes, being identical in the same buffer system. The alcohol dehydrogenase from *D. simulans* shows higher activity at pH values closer to the physiological pH.

The amino acid compositions of alcohol dehydrogenases from *D. melanogaster* and *D. simulans* show a remarkable similarity, particularly when the least frequently found amino acids are considered. By using the Marchalonis parameter (Marchalonis, 1972) as a first approximation to the estimation of homology between these two proteins, the difference between them could not be distinguished. This parameter gives a higher value when the amino acid compositions of two identical enzymes (Adh^s) from *D. melanogaster* (Table 4) are compared.

The amino acid composition of alcohol dehydrogenase from *D. virilis* clearly differs from those of the other two enzymes. There are evident differences in the least frequently found amino acids; methionine appears for the first time, and there are three more arginine residues, one more histidine residue and one less tyrosine residue. The Marchalonis parameter gives a value of 9.3 between the *D. melanogaster* and *D. virilis* enzymes and a value of 13.3 between the *D. virilis* and *D. simulans* enzymes. To give an idea of the evolutionary divergence of these molecules, we could refer to the value obtained when comparing, on the basis of sequence data, the homology between two α-haemoglobin chains. The Marchalonis parameter between human α-haemoglobin and rhesus α-haemoglobin gives a value of 8 units and that between human α-haemoglobin and carp haemoglobin is 101 units (Marchalonis, 1972).

If we compare the chemical structure of the three enzymes, on the basis of the amino acid composition and peptide 'maps', we could conclude that the enzymes from *D. melanogaster* and *D. simulans* are very similar and that both differ from the *D. virilis* enzyme. However, the kinetic properties of these enzymes reveal a much closer similarity between the *D. melanogaster* and the *D. virilis* enzymes. We could explain this behaviour in terms of the ecology of these species. *D. melanogaster* appears to be a species specifically adapted to live in a rich alcoholic environment, whereas *D. simulans* is never found in wine-cellars and is generally collected in the wild away from the alcohol resources (Parsons, 1975). *D. virilis* seems also to be attracted by cellar environments. It has been collected in breweries (Spieth, 1979), and, in spite of being a rather rare species in Spain, it is probably in the process of introduction, since it has been found in two cellars (Monclús &

Table 4. *Comparison among alcohol dehydrogenases of Drosophila species in accordance with the Marchalonis parameter (Marchalonis, 1972)* $s\Delta Q = \Sigma_j (X_{ij} - X_{kj})^2$

i and *k* identify the particular proteins and x_j is the content (percentage of residues) of a given amino acid. Amino acid compositions were taken from Table 3.

	D. melanogaster Adh^s (Thatcher, 1980)	*D. melanogaster* Adh^s	*D. simulans*	*D. virilis*
D. melanogaster Adh^s (Thatcher, 1980)	0			
D. melanogaster Adh^s	3.1	0		
D. simulans	3.8	1.9	0	
D. virilis	12.5	9.3	13.3	0

Prevosti, 1979). So, although the chemical structure of the three alcohol dehydrogenases seems to be consistent with a pattern of divergent evolution, the kinetic properties reveal that this is a clear case of adaptation to the ecology of these species.

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