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OXIDIZED NICOTINAMIDE–ADENINE DINUCLEOTIDE-INDEPENDENT LACTATE DEHYDROGENASES OF *LACTOBACILLUS ARABINOSUS* 17.5

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SUMMARY

1. The object of this work was to investigate the nature and properties of an L- and a D-lactate dehydrogenase of *Lactobacillus arabinosus*[1] and to establish the relationship of these two enzymes to the two lactate dehydrogenases purified from the same organism by DENNIS AND KAPLAN[2].

2. The D- and L-lactate dehydrogenases were partially purified from cell-free extracts by ammonium sulphate and protamine sulphate treatments followed by chromatography on DEAE-cellulose columns. Finally the two enzymes were completely separated on CM-cellulose columns.

3. The L-lactate dehydrogenase was further purified (a total of 1200-fold) by chromatography on TEAE- and Ecteola–cellulose columns. This enzyme contained 1 molecule of FMN per molecule of enzyme and approx. 20% of the flavin was reduced by the addition of sodium L-lactate.

4. Both enzymes were NAD^+-independent and only reduced oxidation–reduction dyes. The K_m and optimum pH values were quite different from those reported for the D- and L-lactate dehydrogenases isolated by DENNIS AND KAPLAN, also the former enzymes readily separated by electrophoresis in contrast to the latter enzymes.

5. Both the NAD^+-independent D- and L-lactate dehydrogenases were separated from the NAD^+-dependent enzymes on a TEAE-cellulose column. Thus *Lactobacillus arabinosus* appears to contain two pairs of lactate dehydrogenases, two of which are NAD^+-linked and another two which are NAD^+-independent. Of these latter two enzymes the L-lactate dehydrogenase is a flavoprotein while the D-lactate dehydrogenase is probably a flavoprotein also.

INTRODUCTION

The partial purification of a lactate dehydrogenase from *Lactobacillus arabinosus* which did not require NAD^+ was reported previously[3]. This enzyme was specific for L-lactic acid. It was subsequently found[1] that *L. arabinosus* contained two stereo-

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specific lactate dehydrogenases which were NAD^+-independent. One enzyme was specific for L-lactic acid and the other was specific for D-lactic acid. DENNIS AND KAPLAN[2] reported that *Lactobacillus plantarum (arabinosus)* contains two stereospecific lactate dehydrogenases which are NAD^+-dependent (*i.e.* L-lactate : NAD oxidoreductase, EC 1.1.1.27 and D-lactate : NAD oxido-reductase, EC 1.1.1.28).

The present paper describes the isolation and properties of the two NAD^+-independent lactate dehydrogenases. The results presented indicate that these two enzymes are quite distinct from those isolated by DENNIS AND KAPLAN and that the NAD^+-independent L-lactate dehydrogenase is a flavoprotein enzyme containing FMN as a prosthetic group. A preliminary report of this work has been presented elsewhere[4].

METHODS

Preparations and materials

Cultures of L. arabinosus 17.5 were maintained on glucose–nutrient agar (Bacto) medium supplemented with yeast extract (80 ml/l). The yeast extract was prepared as described previously[5]. Liquid subcultures of the organism were grown in medium containing 20 g glucose, 20 g peptone (Bacto), 1 g KH_2PO_4, 1 g $MgSO_4 \cdot 7H_2O$, 50 mg $MnCl_2$ and 80 ml yeast extract per l.

Large scale production of L. arabinosus. The medium used for the large preparations contained 20 g enzymically hydrolysed casein (Nutritional Biochemical Corporation), 20 g glucose, 15 g sodium citrate, 5 g sodium acetate, 1 g KH_2PO_4, 1 g $MgSO_4 \cdot 7H_2O$, 50 mg $MnCl_2$ and 80 ml yeast extract per l. The medium was dispensed into 8-l Florence flasks which were subsequently inoculated with 250-ml subcultures which had been grown for 7 h at 30°. The large flasks were then incubated for 18 h at 30°. The cells were then harvested by centrifuging in a Sharples centrifuge and washed twice with distilled water. The yield was approx. 2.8 g dry wt. of cells per l of medium.

Preparation of cell-free extract. The cells were suspended in 0.1 M phosphate buffer (pH 7.0) to give a concentration of approx. 100 mg dry wt. of cells per ml and 100-ml aliquots of this suspension were then placed in a 300-ml stainless-steel vessel of a Lourdes high-speed homogenizer together with 60 g No. 14 Ballotini glass beads. The whole vessel was immersed in crushed ice and the cells were disrupted by homogenizing for 3 periods of 5 min at 17 000 rev./min. The glass beads were removed by centrifugation at $100 \times g$. The cell debris and unbroken cells were subsequently removed by centrifuging at $15\,000 \times g$ for 20 min in a refrigerated Servall centrifuge. The supernatant is referred to as the cell-free extract and contained approx. 17 mg protein/ml. This method of preparation of cell-free extract yielded approx. 10% of the original dry weight of the cells as soluble protein.

Other preparations. NADH and NAD^+ (99%) were obtained from Sigma Chemical Co. D- and L-lactic acids were obtained from Calbiochem as the calcium salts which were subsequently converted to the sodium salts by treatment with Zeo-Carb 225 ion-exchange resin (Permutite Co.). Lithium DL-lactate was a laboratory-grade reagent (British Drug House) and was recrystallised from distilled water and ethanol.

Cellulose ion-exchange resins. The anion-exchange resin DEAE-cellulose was prepared from Solka-Floc cellulose by Dr. E. S. HOLDSWORTH according to the method of PETERSON AND SOBER[6]. The exchange capacity of this resin was 0.61 mequiv/g.

CM-cellulose was prepared from Whatman powdered cellulose according to the method of PETERSON AND SOBER[6] and the ion-exchange capacity of this resin was 0.42 mequiv/g. Ecteola–cellulose (*epi*chlorotriethanolamine–cellulose) was a commercial product (Cellex E) of the Bio-Rad Laboratories and TEAE-cellulose was a product of the Serva Co. (Germany). The ion-exchange capacity of the former resin was 0.64 mequiv/g and of the latter 0.56 mequiv/g.

Estimations

Protein was estimated by the biuret method[7] or by measurement of the absorbancy at 280 mμ according to the method of WARBURG AND CHRISTIAN[8].

Pyruvate was estimated by measuring the oxidation of NADH spectrophotometrically in the presence of rabbit-muscle lactate dehydrogenase and 0.02 M Tris–maleate buffer (pH 6.0).

The NAD^+-dependent lactate dehydrogenases were estimated by the method of DENNIS AND KAPLAN[2] by following the reduction of NAD^+ spectrophotometrically in the presence of sodium D- or L-lactate.

The NAD^+-independent lactate dehydrogenases were estimated by measuring the reduction of 2,6-dichlorophenolindophenol at 600 mμ with either sodium D- or L-lactate as a substrate. The combined lactate dehydrogenases activity was measured with lithium DL-lactate as substrate. A Beckman spectrophotometer Model G2400 fitted with double thermospacers was used for the assays. The temperature in the cell compartment was maintained at 28°. In the assay procedure used, the following reagents were added to a cuvette of 1-cm light path; 0.3 ml 0.2 M Tris–maleate buffer (pH 6.0 for the L-lactate dehydrogenase and pH 6.7 for the D-enzyme), 0.1 ml 0.001 M 2,6-dichlorophenolindophenol, 0.5 ml 0.2 M sodium D- or L-lactate and distilled water to give a final volume of 2.9 ml. The reaction was started by the addition of 0.2 ml enzyme solution and the absorbancy at 600 mμ was measured at intervals of 30 sec for 3 min. One unit of enzyme activity was defined as that amount catalysing the oxidation of 1 μmole of lactate per min and specific activity as units/mg protein.

Electrophoresis of the NAD^+-independent D- and L-lactate dehydrogenases

Protein fractions containing the enzymes were first dialysed against 0.005 M Tris–maleate buffer (pH 7.9) and then applied to a strip of No. 3 MM paper and subsequently placed in a L.K.B. 3276 paper electrophoresis apparatus. The paper was then subjected to a potential difference of 10 V/cm for 16 h using 0.05 M Tris–maleate (pH 7.9) as a buffer. The enzymes were located by spraying the damp paper strip with a mixture of 0.005 M 2,6-dichlorophenolindophenol, 0.5 M sodium DL-lactate and 0.25 M Tris–maleate buffer (pH 6.0). The enzymes appeared as white bands on a blue background. The stereospecificity of each enzyme was determined by the usual assay after elution from a corresponding strip.

RESULTS

Fractionation and separation of the two NAD^+-independent lactate dehydrogenases

All the procedures described below were carried out at 4°. The cell-free extract was first diluted with 0.1 M sodium phosphate buffer (pH 7.0) until the protein

concentration was reduced to 10 mg/ml. Solid ammonium sulphate was added to give 50% satn. and the precipitate was removed by centrifugation at 10 000 × *g* for 20 min. The supernatant was then brought to 70% satn. by the further addition of solid ammonium sulphate and the precipitate was collected by centrifugation, as above. This precipitate was dissolved in 0.005 M Tris–maleate (pH 7.6), containing 0.01 M sodium lactate, and dialysed for 16 h against 48 l (six changes of 8 l) of 0.005 M Tris–maleate buffer (pH 7.6), containing 0.002 M sodium lactate, in a rocking dialyser.

The protein concentration of the solution was then reduced to 5 mg/ml by dilution with 0.005 M Tris–maleate (pH 7.6) containing 0.002 M sodium lactate, and the pH was lowered to 5.9 by the cautious addition of 0.25 M acetic acid. Protamine sulphate (2%, pH 4.0) was added to give a final concentration of approx. 0.18 mg/mg protein or sufficient to raise the $A_{280}:A_{260}$ ratio from 0.5–0.6 to greater than 1.0 thus indicating the extensive removal of nucleic acids. The precipitate was removed by centrifugation at 1000 × *g* for 10 min. The pH of the supernatant was readjusted to 7.6 by the addition of 1 M Tris solution and the protein concentration reduced to 1.75 mg/ml by the addition of 0.005 M Tris–maleate buffer (pH 7.6), containing 0.002 M sodium lactate. A suspension of DEAE-cellulose (75 mg/ml) was added to the dilute protein solution and stirred with a magnetic stirrer for 15 min. The DEAE-cellulose was removed, by centrifugation, washed twice with 0.005 M Tris–maleate buffer (pH 7.6), containing 0.002 M sodium lactate, and the protein was eluted with 0.18 M sodium lactate. The eluate contained some 90% of the total lactate dehydrogenase activity but only 15% of the original protein.

This eluate was dialysed for 4 h against 0.005 M Tris–maleate buffer (pH 7.6), containing 0.002 M sodium lactate, and then freeze-dried. The freeze-dried material was dissolved in a small volume of distilled water and again dialysed against the above buffer. This concentrated fraction was then applied to a DEAE-cellulose column

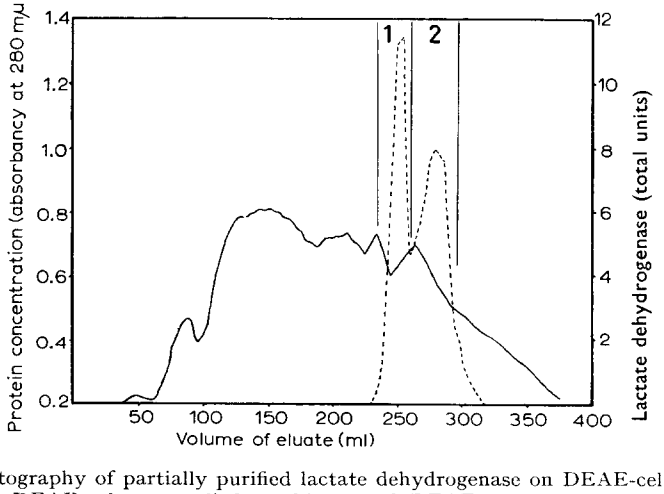

Fig. 1. Chromatography of partially purified lactate dehydrogenase on DEAE-cellulose. 130 mg protein from a DEAE-column applied to this second DEAE-cellulose column (21 × 2.5 cm) equilibrated with 0.005 M Tris–maleate (pH 7.5), 0.002 M sodium lactate. Elution with sodium lactate (pH 7.5) using a linear gradient to 0.3 M in a volume of 400 ml. Solid line, protein; dotted line, lactate dehydrogenase. Peak 1 contained predominantly D-lactate dehydrogenase; Peak 2 contained predominantly L-lactate dehydrogenase.

(23 × 4 cm, containing 35 g cellulose) which had previously been equilibrated overnight against the dilute Tris–maleate buffer described above. The protein was then eluted with sodium lactate (pH 7.6), the concentration of the sodium lactate being increased in a linear gradient from 0.002 to 0.3 M in a volume of 400 ml. The fractions containing lactate dehydrogenase activity were pooled, freeze-dried and dialysed as before. The concentrated fraction was then applied to a second DEAE-cellulose column (21 × 2.0 cm, containing approx. 15 g cellulose) and the process repeated. Fig. 1 shows the elution pattern of protein and lactate dehydrogenase from this second DEAE-cellulose column. The figure also shows the division of the lactate dehydrogenase into two peaks. The activity of each peak was subsequently re-examined using sodium L- and D-lactates as substrates in separate assays. It was found that the first peak (marked 1 in Fig. 1) contained predominantly D-lactate dehydrogenase whereas the second peak (marked 2 in Fig. 1) contained predominantly L-lactate dehydrogenase. The fractions contained in each peak were pooled separately and freeze-dried.

The freeze-dried material of the second peak was dissolved in distilled water and dialysed for 4 h against 8 l of 0.005 M Tris–maleate buffer (pH 6.0), containing 0.002 M sodium lactate. This fraction was then applied to a CM-cellulose column (24 × 1 cm, containing 5 g cellulose) which had been equilibrated overnight against 0.005 M Tris–maleate buffer (pH 6.0), containing 0.002 M sodium lactate. The protein was subsequently eluted with Tris–maleate buffer by increasing the concentration and pH in a linear gradient to 0.05 M and pH 7.5 in a volume of 50 ml. Fig. 2a shows the elution pattern of protein and lactate dehydrogenase from this column. The L-lactate dehydrogenase was eluted as a sharp symmetrical peak and was completely separated from the small amount of contaminating D-lactate dehydrogenase. The L-lactate dehy-

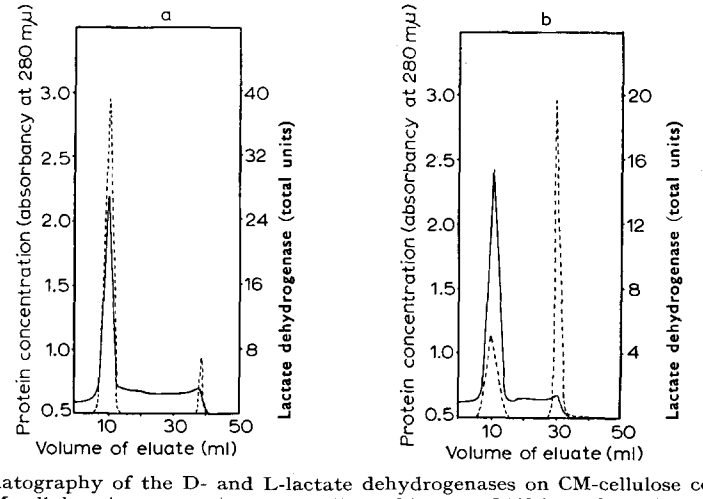

Fig. 2. Chromatography of the D- and L-lactate dehydrogenases on CM-cellulose columns. The columns of CM-cellulose (24 × 1 cm) were equilibrated in 0.005 M Tris–maleate (pH 6.0), 0.002 M sodium lactate. Elution with Tris–maleate buffer using a linear gradient to 0.05 M and pH 7.5 in a volume of 50 ml. Solid line, protein; dotted line, lactate dehydrogenase. (a) 13 mg protein (Peak 2, Fig. 1) applied to the column. L-Lactate dehydrogenase eluted at approx. 10 ml and D-lactate dehydrogenase at 37 ml. (b) 9 mg protein (Peak 1, Fig. 1) applied to the column. L-Lactate dehydrogenase eluted at approx. 11 ml and D-lactate dehydrogenase at 31 ml.

TABLE I

THE FRACTIONATION OF TWO STEREOSPECIFIC LACTATE DEHYDROGENASES OF *L. arabinosus*

The lactate dehydrogenase assay systems contained 100 μmoles sodium L- or D-lactate, 75 μmoles Tris–maleate buffer at pH 6.0 (for D-enzyme) and 6.7 (for L-enzyme), 0.12 μmole 2,6-dichlorophenolindophenol and enzyme in a total volume of 3 ml. Decrease in absorbancy at 660 mμ measured every 30 sec for 3 min at 28°. Protein was estimated by the biuret method[7] in the preliminary stages and by the 280-mμ absorbancy method[8] in the later stages. Peaks 1 and 2 refer to those identified in Fig. 1.

	L-*Lactate dehydrogenase*						D-*Lactate dehydrogenase*					
	Total volume (ml)	*Units/ml*	*Total units*	*Protein (mg/ml)*	*Specific activity (units/mg protein)*	*Recovery (%)*	*Total volume (ml)*	*Units/ml*	*Total units*	*Protein (mg/ml)*	*Specific activity (units/mg protein)*	*Recovery (%)*
Cell-free extract	555	0.530	294	10.2	0.052		555	0.540	300	10.2	0.053	
50–70% $(NH_4)_2SO_4$ fraction	205	0.933	191	18.6	0.050	65	205	0.938	192	18.6	0.050	64
Protamine sulphate supernatant	1760	0.166	292	1.85	0.063	99	1760	0.016	292	1.85	0.089	97
DEAE-cellulose eluate eluted with 0.18 M lactate	22.4	10.8	242	29.4	0.377	90	22.4	11.7	263	29.4	0.40	88
Eluate from 1st DEAE-column	20.7	7.60	151	6.4	1.19	54	20.7	8.10	168	6.4	1.27	56
Peak 1 eluted from 2nd DEAE-column							3.2	17.2	54.9	2.8	6.50	18
Peak 2 eluted from 2nd DEAE-column	3.1	19.9	61.8	4.2	4.74	21						
Peak 1 after chromatography on CM-cellulose							1.7	12.0	21.3	0.4	30	7
Peak 2 after chromatography on CM-cellulose	2.2	24.7	54.3	4.3	5.74	18						

drogenase eluted from this column had a specific activity of 5.74 and contained 18% of the original activity and represented a 110-fold purification when compared with the activity of the original cell-free extract (Table I).

The freeze-dried material of the first peak (marked 1 in Fig. 1) was treated in a similar manner to that of the second peak and also applied to a CM-cellulose column. The protein was eluted with Tris–maleate buffer as described for the first CM-cellulose column and Fig. 2b shows the complete separation of the D-lactate dehydrogenase from the small amount of contaminating L-lactate dehydrogenase. The D-lactate dehydrogenase had a specific activity of 30 and represented a 560-fold purification as compared with the original cell-free extract (Table I). The yield was 7%.

Further purification of the L-*lactate dehydrogenase*

Although the L-lactate dehydrogenase eluted from the CM-cellulose column sedimented as a single peak in the ultracentrifuge ($s_{20,w} = 1.01$) it was possible to separate a protein fraction containing the enzyme activity from the bulk of the protein in this eluate by electrophoresis. It was found subsequently that this separation could also be achieved by the use of anion-exchange cellulose resins. Thus L-lactate dehydrogenase eluted from the CM-cellulose column (as shown in Fig. 2a) was dialysed against the buffer described previously for the DEAE-cellulose column and applied to a TEAE-cellulose column (21 × 2 cm). The protein was eluted with sodium lactate (pH 7.6) by increasing the salt concentration in a linear gradient to 0.5 M in a volume of 500 ml. The L-lactate dehydrogenase from this column was then rechromatographed on an Ecteola–cellulose column which had also been equilibrated in the same buffer system used for the DEAE-column and again the enzyme was eluted with sodium lactate as above. The L-lactate dehydrogenase eluted from this column had a specific activity of 53.6 which represented a 1200-fold purification of the enzyme when compared with the activity of the original cell-free extract. The elution pattern from this column also showed a flavin peak (as measured by the absorbancy at 450 mμ) in the same position as the lactate dehydrogenase.

Separation of the NAD$^+$-independent lactate dehydrogenases from the NAD$^+$-dependent lactate dehydrogenases

Although the protein fraction applied to the TEAE-cellulose column, described in the previous section, contained only about 3% of the original NAD$^+$-dependent lactate dehydrogenase activity it was found that both the NAD$^+$-dependent D- and L-lactate dehydrogenases were eluted as separate peaks after the NAD$^+$-independent L-lactate dehydrogenase as shown in Fig. 3.

Properties of the NAD$^+$-independent D- *and* L-*lactate dehydrogenases*

Stability: The D-lactate dehydrogenase was found to be considerably less stable than the L-lactate dehydrogenase during the fractionation procedure (Table I). This table indicates that there were considerable losses of D-lactate dehydrogenase in the final stages of fractionation and these losses occurred mainly during the freeze-drying steps. The final preparation of L-lactate dehydrogenase retained 30% of its activity

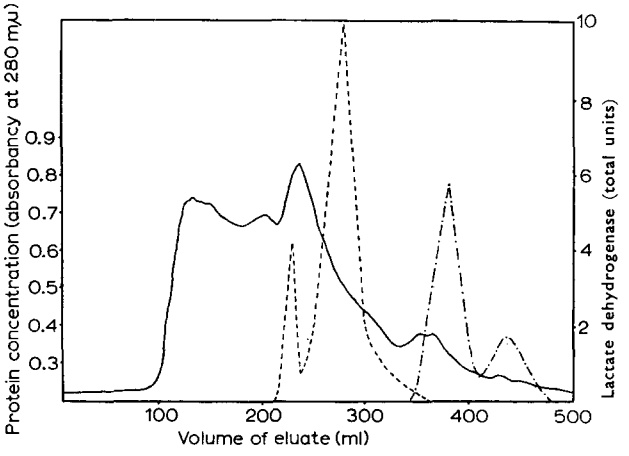

Fig. 3. Chromatography of lactate dehydrogenases on TEAE-cellulose. 90 mg of partially purified lactate dehydrogenase applied to a TEAE-cellulose column (20 × 2 cm) equilibrated in 0.005 M Tris–maleate (pH 7.5), 0.002 M sodium lactate. Elution with sodium lactate (pH 7.5) using a linear gradient to 0.5 M in a volume of 500 ml. Solid line, protein: dotted line, NAD^+-independent lactate dehydrogenase; dash-dotted line, NAD^+-dependent lactate dehydrogenase. The peak eluted at 220 ml contained NAD^+-independent D-lactate dehydrogenase, at 280 ml NAD^+-independent L-lactate dehydrogenase, at 380 ml NAD^+-dependent D-lactate dehydrogenase and at 440 ml NAD^+-dependent L-lactate dehydrogenase.

after storage at −15° for 2 months while the D-lactate dehydrogenase retained less than 10% of its activity after 2 weeks.

Electrophoretic mobility: Paper electrophoresis of the two enzymes revealed that they possessed quite different electrophoretic mobilities. After 16 h electrophoresis at 10 V/cm in 0.05 M Tris–maleate buffer (pH 7.9) the D-enzyme moved approx. 4 cm from the origin towards the anode while the L-enzyme moved approx. 12 cm. Unfortunately this method was not suitable as a preparative procedure for separating the two enzymes as it only gave 10–15% yields.

Optimum pH: Optimum activity of the L-lactate dehydrogenase was observed at pH 6.0 compared with 6.7 for the D-lactate dehydrogenase.

Kinetic studies: The K_m value of the L-lactate dehydrogenase was $1.6 \cdot 10^{-2}$ M, as determined from the Lineweaver–Burk plot (Fig. 4a), whereas the K_m value for the D-lactate dehydrogenase was $2.3 \cdot 10^{-3}$ M. Oxalate acted as a powerful competitive inhibitor of both enzymes (Fig. 4a and b), the K_i value for the L-enzyme was $2.0 \cdot 10^{-4}$ M ($K_{m,\ \text{L-lactate}}:K_{i,\text{oxalate}} = 80$) compared with $1.1 \cdot 10^{-5}$ M ($K_{m,\ \text{D-lactate}}:K_{i,\text{oxalate}} = 210$) for the D-enzyme. The latter value is similar to the value of $(5–10) \cdot 10^{-6}$ M ($K_{m,\ \text{D-lactate}}:K_{i,\text{oxalate}} = 350$) reported by TUBBS[9] for the D-2-hydroxy acid dehydrogenase (D-2-hydroxy acid:cytochrome *c* oxido-reductase, EC 1.1.2.4) of kidney. Oxamate acted as a non-competitive inhibitor of the L-enzyme and 50% inhibition was observed at a concentration of $2 \cdot 10^{-3}$ M. D-Lactate was found to be a weak competitive inhibitor of the D-enzyme, ($K_{m,\ \text{L-lactate}}:K_{i,\ \text{D-lactate}} = 0.27$) and L-lactate was a weak competitive inhibitor of the D-enzyme ($K_{m,\ \text{D-lactate}}:K_{i,\ \text{L-lactate}} = 0.06$). L-Lactate was also found to act as a competitive inhibitor of the D-2-hydroxy acid dehydrogenase of kidney[9] and yeast[10]. Neither the D- nor the L-lactate dehydrogenase oxidized any 2-hydroxy acid other than lactic acid to any significant

extent. This result is similar to that found with the D-lactate dehydrogenase of *Leconostoc mesenteroides*[11] but in contrast to D-2-hydroxy acid dehydrogenase of kidney[9] and yeast[10] which oxidize 2-hydroxy acids other than lactic acid at rapid rates.

Electron acceptors: Neither enzyme reduced NAD^+ (or $NADP^+$) in the presence of lactate nor oxidized NADH (or NADPH) in the presence of pyruvate. Several other lactate dehydrogenases from bacterial sources[11–14], are not NAD^+-linked

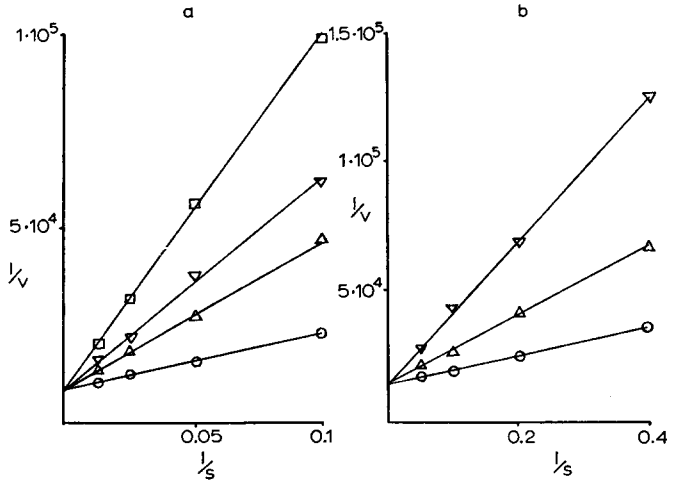

Fig. 4. The effect of lactate and oxalate on the activities of the L- and D-lactate dehydrogenases. (a) Lineweaver–Burk plot of $1/V$ against $1/S$ for the L-lactate dehydrogenase. Ordinate, $1/V$ where V = units of activity. Abscissa, $1/S$ where S = concentration of sodium L-lactate. ○—○, sodium L-lactate; △—△, lactate plus $1 \cdot 10^{-4}$ M oxalate; ▽—▽, lactate plus $2 \cdot 10^{-4}$ M oxalate; □—□, lactate plus $5 \cdot 10^{-4}$ M oxalate. (b) Lineweaver–Burk plot of $1/V$ against $1/S$ for the D-lactate dehydrogenase. Ordinate, $1/V$ where $1/V$ = units of activity. Abscissa, $1/S$ where S = concentration of sodium D-lactate. ○—○, sodium D-lactate; △—△, lactate plus $1 \cdot 10^{-5}$ M oxalate; ▽—▽, lactate plus $2 \cdot 10^{-5}$ M oxalate.

although none of these enzymes have been purified to any extent. Neither enzyme reduced ferricyanide or cytochrome *c* in the presence of lactate nor was capable of using molecular O_2 as an electron acceptor. Only oxidation–reduction dyes with an E_0' of +0.1 V or greater were utilized as electron acceptors and 2,6-dichlorophenol-indophenol ($E_0' = +0.217$ V at pH 7.0) was the most efficient of these acceptors. Table II shows the rate of the reaction of the L-enzyme with various oxidation–reduction dyes. Similar results were obtained with the D-enzyme.

The soluble D-lactate dehydrogenase of *Acetobacter peroxydans*, which is not NAD^+-linked, only reduces dyes which have a positive oxidation–reduction potential and thionine ($E_0' = +0.062$ V at pH 7.0) is the most efficient acceptor[12].

Effects of chelating agents: Pre-incubation of a partially purified preparation of the L-lactate dehydrogenase (specific activity 5.5) with EDTA or 8-hydroxyquinoline for 6 min had no effect on the rate of the enzyme reaction. 1,10-Phenanthroline (3 mM) inhibited the reaction some 28% under similar conditions.

TABLE II

THE RATE OF THE L-LACTATE DEHYDROGENASE REACTION WITH VARIOUS ELECTRON ACCEPTORS

The standard assay procedure as indicated in Table I was used except the methylene blue reaction required a Thunberg cuvette. Reduction of the first dye was measured at 590 mμ, the third at 615 mμ, the fourth at 570 mμ and ferricyanide at 400 mμ. Corrections were made for the differing extinction coefficients. L-Lactate dehydrogenase with a specific activity of 4.2 was used.

Acceptor	E_0' *at pH 7.0*	*Rate of reaction**
Potassium indigo-tetrasulphonate	−0.046	nil
Methylene blue	+0.011	1
1-Naphthol-2-(sodium sulphonate)-indo-2,6-dibromophenol	+0.119	2
Guiacol-indo-2,6-bromophenol	+0.159	16
2,6-Dichlorophenolindophenol	+0.217	100
Ferricyanide	+0.36	nil

* The reaction rates are expressed as a percentage of the rate with 2,6-dichlorophenol-indophenol.

Reaction products: Pyruvate was positively identified as a product of the L-lactate dehydrogenase reaction by chromatography of the 2,4-dinitrophenylhydrazine derivative and comparison with the 2,4-dinitrophenylhydrazone of authentic pyruvic acid.

The nature of the prosthetic group: The highly purified preparation of the L-lactate dehydrogenase (specific activity 52) was used to identify the prosthetic group of this enzyme. The enzyme preparation was yellow-brown in colour and contained flavin in the form of FMN. Assuming a molecular weight of 10 000 for the enzyme (a previous

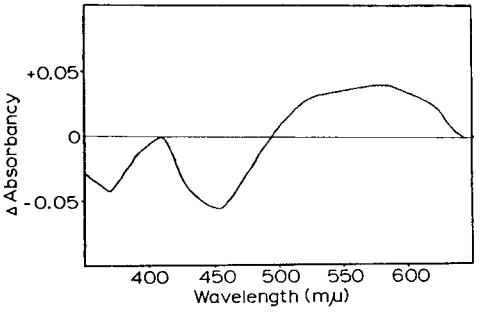

Fig. 5. Difference spectrum of the NAD^+-independent L-lactate dehydrogenase. 1.2 mg of enzyme (specific activity 52) in 0.1 ml Tris–maleate (pH 6.0) reduced with solid sodium L-lactate. The spectrum plotted is that of the reduced enzyme minus the oxidized enzyme.

estimation of the molecular weight of this enzyme indicated a figure near this value[15]) there was an equivalent of 1 molecule of FMN per molecule of enzyme. The absorption spectrum did not reveal any typical flavin absorption peaks although a shoulder in the region 360–460 mμ could be detected. Addition of sodium L-lactate resulted in an approximate 20% reduction in absorbancy in this region and the difference

spectrum (reduced minus oxidized enzyme) as shown in Fig. 5 revealed a maximum decrease in absorbancy at approx. 455 mμ. Also reduction of the enzyme with lactate resulted in the formation of a broad absorption band with a maximum at approx. 570 mμ (Fig. 5).

Preliminary experiments suggested that the D-lactate dehydrogenase is also a flavoprotein.

DISCUSSION

Originally[3] only an L-lactate dehydrogenase which was NAD^+-independent was detected in *L. arabinosus* but subsequently[1] a D- and L-lactate dehydrogenase were discovered in this organism and both were NAD^+-independent. Also evidence presented in the original paper[3] suggested that the L-lactate dehydrogenase was a flavoprotein yet no flavin could be detected in a subsequent preparation of much higher specific activity[1]. This latter enzyme preparation appeared to be virtually homogeneous, as judged by ultracentrifugal data, but the work presented in this paper indicates that while this preparation was approx. 95% pure protein all the lactate dehydrogenase activity was associated with the small amount of "impurity". Thus as the major fraction of protein examined was not in fact the lactate dehydrogenase it is not surprising that no flavin was detected in this enzyme preparation.

The NAD^+-independent L-lactate dehydrogenase described in this paper contains 1 molecule of FMN per molecule of enzyme, based on a molecular weight of 10 000. The flavin was partially reduced by the addition of sodium lactate as revealed in the difference spectrum although the absorption due to the flavin group at 455 mμ was only reduced approx. 20%. In this respect the enzyme would appear to be similar to the bacterial flavoprotein enzymes 4,5-dihydroorotate:O_2 oxido-reductase (EC 1.3.3.1) of *Zymobacterium orotium*[16] and $NADH_2$:H_2O_2 oxido-reductase (EC 1.11.1.1) of *Streptococcus faecalis*[17]. The NAD^+-independent lactate dehydrogenase of *L. arabinosus* is thus a flavoprotein with FMN as a prosthetic group and in this respect is similar to flavoprotein enzymes oxidizing lactate found in other microorganisms[11,18–20].

The appearance of the broad absorption band with a maximum absorption at approx. 570 mμ on reduction of the L-lactate dehydrogenase is interesting as similar absorption bands have been attributed to flavin semiquinones[21]. Flavin semiquinones are involved in a number of flavoprotein enzyme reactions[22,23] while in other enzyme reactions involving flavoproteins the appearance of broad absorption bands in the 500–600-mμ region is due to the formation of enzyme–substrate complexes[24,25]. Further investigation of L-lactate dehydrogenase of *L. arabinosus* is required to determine whether the absorption band at 570 mμ which appears on reduction of this enzyme is due to a flavin semiquinone or an enzyme–substrate complex.

The inhibition of the NAD^+-independent lactate dehydrogenase by the metal-chelating agent 1,10-phenanthroline would possibly suggest that this enzyme may contain a metal. Extensive studies with chelating agents on the D-2-hydroxy acid dehydrogenase of kidney led TUBBS[9,26] to suggest that this enzyme contains a functional metal component. Similarly, it was considered that yeast D-2-hydroxy acid dehydrogenase contained a metal and this metal was Zn[10,27]. Subsequent work established that this enzyme does in fact contain Zn[28]. The D-lactate dehydrogenase of *Euglena gracilis*, which is not NAD^+-linked, also appears to contain Zn[29]. Once again

further work would be needed to establish whether or not the flavoprotein L-lactate dehydrogenase of *L. arabinosus* contains Zn (or some other metal).

A comparison of the properties of the D- and L-lactate dehydrogenases purified from the same organism by DENNIS AND KAPLAN[2] reveal the following differences:

1. The two enzymes described in this paper are NAD^+-independent whereas those described by DENNIS AND KAPLAN[2] are NAD^+-dependent. Further the results presented in this paper show that the NAD^+-independent L-lactate dehydrogenase is a flavoprotein and probably both NAD^+-independent lactate dehydrogenases are flavoproteins. There is no suggestion that the NAD^+-dependent dehydrogenases contain flavin[2].

2. The optimum pH values for the NAD^+-independent D- and L-lactate dehydrogenases are 6.7 and 6.0, respectively, whereas the values reported for the NAD^+-dependent enzymes are 8.5 and 7.5, respectively[2].

3. The NAD^+-independent D-lactate dehydrogenase has a greater affinity for its substrate than the L-enzyme as evidenced by the lower K_m values for the former enzyme, regardless of the nature of the hydrogen acceptor (*e.g.* $6.6 \cdot 10^{-3}$ M for the D-enzyme compared with $3.3 \cdot 10^{-2}$ M for the L-enzyme using 2,6-dichlorophenol-indophenol as the hydrogen acceptor). The opposite is reported for the NAD^+-dependent enzymes (*e.g.* $K_m = 2.9 \cdot 10^{-2}$ M for the D-enzyme compared with $7.1 \cdot 10^{-3}$ M for the L-enzyme using acetyl-NAD^+ as the hydrogen acceptor[2]).

4. The two NAD^+-independent lactate dehydrogenases are readily separated by electrophoresis whereas the NAD^+-dependent enzymes are not separated even after prolonged electrophoresis under similar conditions[2].

5. Finally the two NAD^+-dependent enzymes were completely separated from the NAD^+-independent enzymes on a TEAE-cellulose column by elution with sodium lactate (see Fig. 4).

Thus it is quite clear that *L. arabinosus* contains two pairs of lactate dehydrogenases, two which are NAD^+-dependent and two which are NAD^+-independent and are probably both flavoprotein enzymes.

It is interesting to speculate on the role of these two pairs of enzymes in the metabolism of the organism. The NAD^+-dependent enzymes are probably involved in the production of D- and L-lactate from pyruvate whereas the NAD^+-independent enzymes would appear to oxidize D- and L-lactate back to pyruvate. The metabolic significance of such a mechanism has been discussed previously[3].

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