

## Purification and Properties of NAD-dependent Lactic Dehydrogenases of Different Species of *Lactobacillus*

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### SUMMARY

Six nicotinamide adenine dinucleotide (NAD)-dependent lactic dehydrogenases, four specific for D(−) lactate and two specific for L(+) lactate have been purified from different *Lactobacillus* species. Four of these enzymes appeared to be pure by several criteria. Some of the physical and catalytic properties of these enzymes are described and compared with those previously reported for the D- and L-lactic dehydrogenases of *Lactobacillus plantarum*.

### INTRODUCTION

The NAD-dependent lactic dehydrogenases of different species and subspecific groups of lactobacilli have been shown to differ not only in their specificity towards D(−) or L(+) lactate but also in their electrophoretic migration (Gasser, 1970). The D- and L-lactic dehydrogenases of *Lactobacillus plantarum* have been purified and studied by Dennis & Kaplan (1960). In the present studies, the purification of several D- and L-lactic dehydrogenases from selected *Lactobacillus* strains is described and their properties are compared. The most highly purified preparations of these enzymes were used for the preparation of antisera. The immunological cross-reactions among the purified enzymes and crude enzyme preparations of other strains will be described in a subsequent paper.

### METHODS

**Bacterial strains and cultivation.** The bacterial strains used in the present studies were chosen from among those previously examined for their complement of lactic dehydrogenases and the electrophoretic properties of these enzymes (Gasser, 1970). They included: *Lactobacillus leichmannii* NCDO 302 (ATCC 7830), *L. fermenti* CNRZ 236, *L. jensenii* 62 G, *L. acidophilus* (group III) A18 and *L. acidophilus* (group I) 65 K. The cultures were grown anaerobically at 37° in 10 l. fermenters purged with a constant stream of gas (95 % N<sub>2</sub> and 5 % CO<sub>2</sub>). The medium used was that previously described by Gasser (1970), but supplemented with sodium acetate (0.05 %, w/v) and dibasic ammonium citrate (0.02 %, w/v) for the growth of all strains except *L. leichmannii* NCDO 302.

**Preparation of crude extracts.** Bacteria were harvested at the end of the exponential phase of growth, washed with 0.05 M-phosphate (KH<sub>2</sub>PO<sub>4</sub>–Na<sub>2</sub>HPO<sub>4</sub>) buffer, pH 7, resuspended in the same buffer (100 mg./ml.) and disintegrated in 50 ml. batches for

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30 min. with a Raytheon 11 KC sonic oscillator and then centrifuged at 4° for 30 min. at 15,000 g. The protein contents of the crude extracts ranged from 5 to 18 mg./ml., depending on the bacterial strain used.

*Assays of lactic dehydrogenases.* During the course of purification, assays of lactic dehydrogenase activity were made with D(−) or L(+) lactate as substrate and NAD as coenzyme. The activity was measured at 25° with a Gilford model 2000 recording spectrophotometer by determining the initial rate of NAD reduction at 340 nm. in a mixture (1 ml.) containing: tris-HCl buffer, pH 8, 200  $\mu$ moles; the lithium salt of either D(−) or L(+) lactate, 100  $\mu$ moles; NAD, 2  $\mu$ moles. The reaction was initiated by the addition of sufficient enzyme to give an increase in extinction (*E*) of approximately 0.05 optical density unit during the first minute. The reaction was linear for at least 3 to 4 min. at all steps of the purifications. In crude extracts, NAD oxidase activity inflected the progress curve after the first minute of the reaction. In all assays the initial rate of NAD reduction was measured during the first minute of the reaction.

After purification, enzyme activity was also measured with pyruvate as substrate by measuring the initial rate of oxidation of the reduced form of nicotinamide adenine dinucleotide (NADH) at 340 nm. in the following mixture (1 ml.): tris-HCl, pH 8, 200  $\mu$ moles; sodium pyruvate, 20  $\mu$ moles; NADH, 0.2  $\mu$ moles. The reaction was initiated by the addition of enzyme.

One unit of enzyme was defined as that amount which reduced 1  $\mu$ mole of NAD/min./ml. or which oxidized 1  $\mu$ mole of NADH/min./ml. Specific activity is expressed as units/mg. protein.

*pH Optima.* The effect of pH value on enzyme activity was determined in tris-maleic acid-sodium maleate buffers, 0.05 M with respect to both tris and the total maleic acid + maleate content, and adjusted to the desired pH value with NaOH.

*Kinetic studies.* The *K<sub>m</sub>* values for lactate, pyruvate and NADH were estimated by the graphical method of Lineweaver & Burk (1934). Reaction rates were determined at 25° in tris-maleic buffer as described above at the optimum pH for each reaction and enzyme. NAD was used at  $2 \times 10^{-3}$  M for assays with lactate, and pyruvate at  $2 \times 10^{-2}$  M for assays with NADH. For assays with pyruvate  $1.7 \times 10^{-4}$  M-NADH was used for the D- and L-lactic dehydrogenases of *Lactobacillus acidophilus* 65K and the L-lactic dehydrogenase of *L. acidophilus* A18. For other enzymes,  $1 \times 10^{-4}$  M was employed.

*Thermal stability.* The relative stability of the enzymes to heat inactivation was tested by immersing small test-tubes containing 0.8 ml. of  $6.6 \times 10^{-2}$  M-tris-HCl buffer, pH 8, and appropriate amounts of enzyme in water baths at various temperatures, cooling them rapidly to 0° after 5 min. of incubation, rewarming to room temperature and assaying the enzyme activity by the usual procedure using D(−) or L(+) lactate as substrate and NAD as coenzyme.

*Protein assays.* Protein was assayed by the method of Lowry, Rosebrough, Farr & Randall (1951) in the early and final steps of purification. During the course of purification (after removal of the nucleic acids) the concentration of protein was estimated spectrophotometrically using the formula:

$$\text{Protein (mg.)} = (E_{280 \text{ nm.}} \times 1.5) - (E_{260 \text{ nm.}} \times 0.75).$$

*Electrophoresis.* Polyacrylamide gel electrophoresis was performed according to the method of Davis (1964), using samples containing 60 to 120  $\mu$ g. of protein.

*Ultracentrifugation.* The Beckman-Spinco Model E analytical ultracentrifuge was used at 59,780 rev./min. with schlieren optics for the determination of sedimentation coefficients and at 20,410 rev./min. with Rayleigh interference optics for sedimentation equilibrium studies. All centrifugations were carried out at 4° in 0.01 M-phosphate buffer.

*Immunology.* Details of the preparation of antisera and of the procedure for the agar double diffusion technique will be reported in a subsequent paper. Immuno-electrophoresis experiments were conducted according to the method of Grabar & Williams (1955), using an agar gel containing barbital buffer, pH 8 ( $\mu\text{m.} = 0.1$ ).

*Purification of the D-lactic dehydrogenases of Lactobacillus  
leichmannii, L. jensenii and L. fermenti*

In this and in all other enzyme purifications, the procedures were carried out at 0°.

*Step 1.* After acidification of the crude extract to pH 5.5 with acetic acid, a protamine sulphate solution (2 %, w/v) was added slowly with stirring to a final concentration of 0.3 % (w/v). After 15 min. the mixture was centrifuged and the pellet discarded. The ratio of extinction ( $E$ ) at 280 nm. to that at 260 nm. of the supernatant liquid increased from c. 0.55 to 0.8–0.9 after this treatment.

*Step 2.* After adjustment of the pH to 7, solid ammonium sulphate was added slowly to 50 % saturation. After 2 h. the mixture was centrifuged and the precipitate discarded. The ammonium sulphate concentration was increased to 85 % saturation by the addition of solid ammonium sulphate and the mixture centrifuged after 12 h. The pellet was redissolved in a minimum volume of 0.05 M-phosphate buffer, pH 7, and dialysed against the same buffer.

*Step 3.* The enzyme preparation was applied to a DEAE Sephadex A50 column equilibrated with 0.05 M-phosphate buffer, pH 7. The column was washed with 0.05 M-NaCl in the same phosphate buffer (2 void vol.) and the enzyme was eluted with a linear gradient concentration of NaCl. The peak of eluted enzyme was obtained with 0.16 M-NaCl for *Lactobacillus leichmannii*, 0.17 M for *L. jensenii* and 0.28 M for *L. fermenti*.

*Step 4.* Peak tubes of enzyme were pooled and their contents submitted to the following treatments for further purification. (a) For *Lactobacillus leichmannii* and *L. fermenti* the pool was precipitated with ammonium sulphate at 85 % saturation and the amorphous precipitate was submitted to a back extraction procedure with decreasing concentrations of ammonium sulphate. The resulting enzyme solutions were allowed to stand at 0° until what were presumed to be crystalline precipitates appeared. Those precipitates with the highest specific activity were pooled and repeatedly reprecipitated in the same manner to a constant specific activity. (b) Because the enzyme of *L. jensenii* was unstable to repeated ammonium sulphate fractionation, it was treated differently. The pool of the peak tubes of enzymic activity from the DEAE Sephadex column was applied to a second DEAE Sephadex column and again eluted with a linear NaCl gradient. The tubes containing the enzyme peak from this second DEAE Sephadex column were pooled, the solution concentrated in a mechanical membrane concentrator (Diaflo, Amicon Corporation, Lexington, Massachusetts, U.S.A.) and then applied to a G-100 Sephadex column equilibrated at pH 7 with 0.05 M-phosphate buffer containing 0.1 M-NaCl. The enzyme was eluted with the same

solution. Peak fractions with equivalent specific activity were pooled. Results using this procedure are summarized in Table 1.

*Separation and purification of the D- and L-lactic dehydrogenases of  
Lactobacillus acidophilus 65 K*

The L-lactic dehydrogenase of *Lactobacillus acidophilus* 65 K was very unstable both to the acidification involved in protamine sulphate treatment and to ammonium sulphate precipitation. The D-lactic dehydrogenase also proved to be unstable to repeated ammonium sulphate fractionation in the later stages of purification. Hence a procedure was devised by which the two enzymes were separated and partially purified with minimum use of these reagents.

Table 1. *Purification of D-lactic dehydrogenases of  
Lactobacillus leichmannii, L. fermenti and L. jensenii*

Preparations	<i>L. leichmannii</i> NCDO 302			<i>L. fermenti</i> CNRZ 236			<i>L. jensenii</i> 62 G		
	Total activity* (units)	Specific activity (units/mg. protein)	Yield (%)	Total activity* (units)	Specific activity (units/mg. protein)	Yield (%)	Total activity* (units)	Specific activity (units/mg. protein)	Yield (%)
Crude extract	84,700	5.5	100	68,800	7.5	100	35,600	2.9	100
Supernatant after protamine sulphate ppt. (step 1)	68,800	9.7	81	65,400	15.6	95	34,900	3.24	98
Ammonium sulphate ppt. (50 to 85 % saturation) (step 2)	28,800	34	34	60,220	22.9	87.5	25,500	6.4	71.6
Peak eluted from DEAE Sephadex column (step 3)	20,000	190	24.6	40,900	248	59.4	12,900	51.4	36.2
Repeated precipitation from ammonium sulphate (step 4(a))	11,700	260	13.8	18,000	330	26.2	—	—	—
Peak eluted from DEAE Sephadex column (step 4(b))	—	—	—	—	—	—	2,740	82.2	7.7
Peak eluted from Sephadex G-100 (step 4(b))	—	—	—	—	—	—	1,070	123.5	3

\* Per 100 g. initial wet weight of bacteria.

*Step 1.* The crude extract was dialysed against several changes of  $1.6 \times 10^{-3}$  M-phosphate buffer, pH 7.0, and treated with streptomycin as described by Oxenburgh & Snoswell (1965). The ratio of absorbance at 280 nm. of the centrifuged supernatant liquid increased from c. 0.55 to 1.4.

*Step 2.* The supernatant liquid was dialysed against 0.05 tris-HCl buffer, pH 8, and applied to a DEAE 50 Sephadex column equilibrated with the same buffer. The D-lactic dehydrogenase was eluted with this same buffer containing 0.2 M-NaCl, and the L-lactic dehydrogenase was subsequently eluted with a linear gradient of NaCl (between 0.2 and 0.35 M-NaCl) in the same buffer.

*Step 3.* The pool of the most active fractions of the L-lactic dehydrogenase was concentrated on a mechanical membrane concentrator, applied to a Sephadex G-200 column equilibrated with 0.05 M-tris-HCl buffer, pH 8, containing 0.1 M-NaCl and eluted with the same solution. The peak fractions were pooled, concentrated using the membrane concentrator, and examined for purity and properties of the enzyme.

*Step 4.* The D-lactic dehydrogenase eluted from the DEAE Sephadex was precipitated with ammonium sulphate at 90% concentration and dialysed against 0.05 M-phosphate buffer, pH 7.

*Step 5.* The preparation from step 4 was applied to a DEAE Sephadex column equilibrated with 0.05 M-phosphate buffer, pH 7, and eluted with a linear gradient of NaCl concentration in the same buffer. The peak of eluted enzyme was obtained with 0.12 M-NaCl.

Table 2. *Purification of D- and L-lactic dehydrogenases of Lactobacillus acidophilus 65 K*

Preparations	D-Lactic dehydrogenase			L-Lactic dehydrogenase		
	Total activity* (units)	Specific activity (units/mg. protein)	Yield (%)	Total activity* (units)	Specific activity (units/mg. protein)	Yield (%)
Crude extract	3560	0.75	100	1450	0.305	100
Supernatant after streptomycin treatment (step 1)	3310	1.64	93	1200	0.590	83
DEAE Sephadex at pH 8 (step 2):						
Peak eluted with 0.2 M-NaCl	2785	3.5	78	0	—	—
Peak eluted with NaCl gradient 0.20 to 0.35 M	0	—	—	512	9.75	35
Peak eluted from Sephadex G-200 (step 3)	—	—	—	285	11.60	19.8
Ammonium sulphate ppt. (60 to 90 % saturation: step 4)	2165	7.5	61	—	—	—
Peak eluted from DEAE Sephadex at pH 7 (step 5)	1160	23.6	32.6	—	—	—
Peak eluted from Sephadex G-100	580	38.3	16.3	—	—	—

\* Per 100 g. initial wet weight of bacteria.

Table 3. *Purification of L-lactic dehydrogenase of Lactobacillus acidophilus A18*

Preparations	Total activity* (units)	Specific activity (units/mg. protein)	Yield (%)
Crude extract	11,120	0.64	100
Ammonium sulphate ppt. (50 to 70 % saturation: step 1)	9,340	0.95	84
Supernatant after streptomycin treatment (step 2)	8,600	2.25	77.3
Peak eluted from DEAE Sephadex column (step 3)	2,845	3.3	25.6
Repeated precipitation from ammonium sulphate (step 4)	2,770	7.65	24.9
Peak eluted from Sephadex G-100 (step 5)	1,645	12.2	14.9

\* Per 100 g. initial wet weight of bacteria.

*Step 6.* The pool of the most active fractions was concentrated with a membrane concentrator, applied to a G-100 Sephadex column, and eluted with the same buffer containing 0.1 M-NaCl. The fractions with the highest activities were concentrated by membrane ultrafiltration and examined for the purity and properties of the enzyme. Results with this procedure are summarized in Table 2.

*Purification of the L-lactic dehydrogenase of Lactobacillus acidophilus A18*

*Step 1.* Solid ammonium sulphate was slowly added to the crude extract to 50% saturation. After 2 h. the mixture was centrifuged and the precipitate discarded. The concentration of ammonium sulphate in the supernatant liquid was increased to 70% saturation by addition of solid ammonium sulphate. After 12 h., the mixture was centrifuged and the pellet redissolved in 0.05 M-phosphate buffer, pH 7, to a concentration of 10 mg. protein/ml.

*Step 2.* The enzyme preparation was dialysed and treated with streptomycin as described by Oxenburgh & Snoswell (1965).

*Step 3.* The enzyme preparation was dialysed against several changes of 0.05 M-phosphate buffer, pH 7, and applied to a DEAE Sephadex A 50 column. The column was washed with the same buffer and the enzyme was eluted with a linear gradient of NaCl. The peak tube of eluted enzyme was obtained with 0.12 M-NaCl.

*Step 4.* The enzyme preparation was submitted to the ammonium sulphate precipitation procedure described in step 4(a) for the purification of *Lactobacillus leichmannii* D-lactic dehydrogenase.

*Step 5.* The pool of fractions with the highest specific activity were dialysed against 0.05 M-phosphate buffer, pH 7, and applied to a G-100 Sephadex column equilibrated with the same buffer containing 0.1 M-NaCl. The enzyme was eluted with the same buffer-NaCl mixture and the peak fractions with equivalent specific activity were pooled. Results with this procedure are summarized in Table 3.

#### RESULTS AND DISCUSSION

The results of the purification of the various enzymes are shown in Tables 1 to 3.

*Criteria of purity.* The products of the procedures for the D-lactic dehydrogenases of *Lactobacillus leichmannii*, *L. jensenii* and *L. fermenti*, and for the L-lactic dehydrogenase of *L. acidophilus* A18 appeared to be pure, at least by the following three criteria: the constancy of their specific activity on repeated precipitation and on successive extraction with decreasing concentrations of ammonium sulphate; the appearance of a single protein band upon electrophoresis in polyacrylamide gel; and the formation of a single precipitation line with a homologous antiserum in double diffusion and immuno-electrophoresis experiments. The D-lactic dehydrogenase of *L. acidophilus* 65K did not appear to be pure and could not be further purified with ammonium sulphate because of its instability. The L-lactic dehydrogenase of the same organism originally showed a single protein band after electrophoresis on polyacrylamide gel. After precipitation with ammonium sulphate, two protein bands were detected, one containing most of the enzymic activity and another diffuse band with a lower migration rate exhibiting only slight activity. Immuno-electrophoresis of a sample of step 6 preparation (Table 2) showed three distinct zones of precipitation with the homologous antiserum.

Although all attempts to obtain well-formed crystals of the enzymes met with failure, the preparations of D-lactic dehydrogenases of *Lactobacillus leichmannii*, *L. jensenii* and *L. fermenti* and the L-lactic dehydrogenase of *L. acidophilus* A18 all showed bi-refringence of flow, characteristic of crystalline enzymes. The D-lactic dehydrogenase preparations consisted predominantly of minute rod-shaped elements from 2  $\mu$ m. to several  $\mu$ m. in length, sometimes apparently branched. The L-lactic dehydrogenase

preparation of *L. acidophilus* strain A 18 appeared to be more threadlike and somewhat beaded.

**Molecular weights.** The approximate molecular weights of the enzymes were estimated from their migration in Sephadex G-100 columns, using cytochrome *c*, adenosine deaminase, *Escherichia coli* alkaline phosphatase and ferritin as markers (Whitaker, 1963). All of the D-lactic dehydrogenase enzymes had an apparent molecular weight of *c.* 80,000. The L-lactic dehydrogenases from strains A 18 and 65 K of *Lactobacillus acidophilus* had an apparent molecular weight of 120,000 and 125,000 respectively. The molecular weights of the D- and L-lactic dehydrogenases of *L. plantarum* have been previously reported to be 60,000 to 70,000 and 155,000 respectively (Dennis, Reichlin & Kaplan, 1965).

Table 4. *Some properties of D- and L-lactic dehydrogenases of lactobacilli*

	D-Lactic dehydrogenase					L-Lactic dehydrogenase		
	<i>L. leichmannii</i> NCDO 302	<i>L. acidophilus</i> 65 K	<i>L. jensenii</i> 62 G	<i>L. fermenti</i> CNRZ 236	<i>L. plantarum</i> * ATCC 8041	<i>L. acidophilus</i> 65 K	<i>L. acidophilus</i> A 18	<i>L. plantarum</i> ATCC 8041
(1) $\frac{\text{(Rate for pyruvate} \rightarrow \text{lactate)}}{\text{(Rate for lactate} \rightarrow \text{pyruvate)}}$ ; pH 8	7.6	7.3	9.6	4.1	—	7.9	9.6	—
(2) Specificity: relative rates with substituted substrates:†								
(a) $\alpha$ -Oxobutyrate	0.02	0	0	0.07	0.25	0.13	0.19	0.25
(b) Deamino-NAD	1.00	0	0.55	0.28	0.60	0	0.09	0
(c) 3-Acetylpyridine NAD	0.43	1.50	0.46	1.00	4.30	16.5	21.6	13.0
(d) Pyridine-3-aldehyde NAD	0	0	0	0	0.07	0.24	0	0.57
(3) pH Optimum in tris-maleate								
(a) Lactate $\rightarrow$ pyruvate	8.0	7.8	8.2	8.6	8.5	7.8	7.8	7.5
(b) Pyruvate $\rightarrow$ lactate	7.6	7.3	7.8	8.6	—	5.3	5.3	—
(4) $\frac{\text{(Rate in tris-maleate)}}{\text{(Rate in tris-HCl)}}$ ; pH 8								
(a) Lactate $\rightarrow$ pyruvate	0.65	1.07	1.04	0.76	—	0.94	1.84	—
(b) Pyruvate $\rightarrow$ lactate	0.58	2.30	1.31	0.77	—	1.73	2.40	—
(5) $K_m$ at optimal pH (tris-maleate)								
(a) Lactate ( $M \times 10^{-1}$ )	0.7	12.5	0.7	0.2	0.2	3.6	16.6	0.6
(b) Pyruvate ( $M \times 10^{-3}$ )	1.2	4.6	7.2	1.9	4.7	1.5	2.7	0.37
(c) NADH ( $M \times 10^{-5}$ )	7.1	16.0	16.6	10.0	—	6.9	7.4	—
(6) Heat inactivation: % activity destroyed after 5 min. at:								
(a) 45°	0	98	0	54	—	0	0	0
(b) 50°	50	100	25	98	100	0	0	0
(c) 80°	100	100	100	100	100	50	20	> 92

\* Previously reported data for *L. plantarum* enzymes are shown for comparison (Dennis & Kaplan, 1960; Dennis, Reichlin & Kaplan, 1965), but the conditions for some assays were not identical with those employed in the present studies.

† As compared with rates for pyruvate and NADH or lactate and NAD.

The D-lactic dehydrogenases of *Lactobacillus leichmannii* and *L. fermenti* appeared to be homogeneous in the ultracentrifuge and exhibited monodisperse peaks. Using conditions described in Methods, and 7.8 mg. protein/ml., the sedimentation co-

efficient ( $S_{20, w}$ ) of the *L. leichmannii* enzyme was calculated to be 3.9s. For the *L. fermenti* enzyme (10 mg./ml.), the sedimentation coefficient was 4.4s. Because of the unexpectedly low sedimentation coefficient of the *L. leichmannii* enzyme, its apparent molecular weight was determined by the short column sedimentation equilibrium technique (van Holde & Baldwin, 1958) using protein concentrations of 0.125, 0.25, 0.5, 1.0 and 1.5 mg./ml. The molecular weight, extrapolated to zero concentration, was found to be c. 68,000 ( $\bar{v}$  used for calculations, 0.74).

**Specificity.** The relative rate of NADH oxidation with pyruvate to that of NAD reduction with lactate was determined for each enzyme (Table 4 (1)) at pH 8 in tris-HCl buffer (see Methods). The specificity of the enzymes (Table 4 (2)) was tested under the usual assay conditions at pH 8.  $\alpha$ -Oxobutyrate was substituted for pyruvate with NADH as hydrogen donor (no reaction between NAD and either DL-hydroxybutyrate or DL- $\alpha$ -hydroxy- $\alpha$ -methylbutyrate was detected with any enzyme). NADH was oxidized with  $\alpha$ -oxoglutarate at one-tenth the rate observed with pyruvate by the D-lactic dehydrogenase of *Lactobacillus acidophilus* 65K; this might have been due to an impurity in this preparation.

Analogues of NAD were assayed at  $2 \times 10^{-3}$ M concentration with D(–) or L(+) lactate as substrate. Nicotinamide adenine dinucleotide phosphate (NADP) was not reduced by any of the enzymes tested. The relative rates of reduction of other NAD analogues (Table 4 (2)) were computed by using the following wavelengths and molar extinction coefficients: deamino-NAD, 340 nm.,  $6.2 \times 10^3$ ; pyridine-3-aldehyde NAD, 355 nm.,  $9.3 \times 10^3$ ; 3-acetylpyridine NAD, 365 nm.,  $9.1 \times 10^3$ .

**pH Optima and kinetic properties.** The apparent optimal pH for the oxidation of lactate and the reduction of pyruvate was determined for each enzyme in tris-maleic acid-sodium maleate buffers (Table 4 (3)). As seen in Table 4 (4), the choice of buffer not only had different effects on different enzymes but also had different and sometimes opposite effects on the reaction rates of a given enzyme catalysing the same reaction in opposite directions. These observations have not been elucidated and the explanation may be complex, since the maleate buffer differs from the chloride buffer not only in the nature of its anion and the corresponding acid, but also in its sodium content and ionic strength.

The  $K_m$  values for lactate, pyruvate and NADH (see Methods) are shown in Table 4 (5). The determination of  $K_m$  value for NAD was impracticable. The D- and L-lactic dehydrogenases of *Lactobacillus plantarum* (Dennis & Kaplan, 1960) and those described in this article showed linear kinetic responses in double reciprocal plot. *Butyribacterium rettgeri* and *Escherichia coli* D-lactic dehydrogenases exhibited sigmoidal kinetics with respect to pyruvate (Wittenberger & Fulco, 1967; Tarmy & Kaplan, 1968). No such effect was found with lactic dehydrogenases of lactobacilli.

**Activators and inhibitors.** The effect of the different buffers on the rate of reaction has already been mentioned. Several compounds were tested for inhibitory or activating effects on the oxidation of pyruvate and reduction of lactate. Neither EDTA ( $10^{-3}$ M) nor fructose-1-6-diphosphate ( $10^{-2}$ M) had any effect. This last compound is required for the activity of the L-lactic dehydrogenases of *Streptococcus bovis* (Wolin, 1964), *Bifidobacterium bifidum* (de Vries, Gerbrandy & Stouthamer, 1967) and *Streptococcus faecalis* (C. L. Wittenberger, personal communication). Oxamate, which has been shown to inhibit strongly the D- and L-enzymes of *Lactobacillus plantarum* at concentrations below  $10^{-3}$ M (Dennis & Kaplan, 1960), had no effect on any of the enzymes



studied in the present work at  $5 \times 10^{-3}$  M. At  $10^{-2}$  M concentration, oxamate had a slight inhibitory effect (15 to 25 %) on the three enzymes obtained from the two strains of *L. acidophilus*.

Adenosine triphosphate (ATP) is an allosteric effector for the D-lactic dehydrogenase of *Butyrivibacterium rettgeri* (Wittenberger, 1968). In our study, neither ATP, ADP nor AMP ( $5 \times 10^{-3}$  M) had any effect on the activity of the lactic dehydrogenases, except for the L-lactic dehydrogenases of *Lactobacillus acidophilus*. For the enzyme of *L. acidophilus* strain A 18, all three compounds were somewhat inhibitory in the oxidation of lactate (AMP, 50 %; ADP, 21 %; ATP, 28 % inhibition) but increased the rate of pyruvate reduction by 5 to 10 %. With the enzyme of *L. acidophilus* 65K, AMP reduced the rate of enzyme action in both directions by c. 30 %; ADP increased only the rate of lactate oxidation by 22 %, while ATP was without any effect. The above results cannot be meaningfully interpreted in terms of specific control mechanisms and may be due to fortuitous side effects of the addition of relatively large quantities of the compounds in question.

The observations reported here show that, while the D-lactic dehydrogenases of different species of *Lactobacillus* have certain properties in common, they are readily distinguishable from each other on the basis of their physical and catalytic properties. The L-lactic dehydrogenases, which also show certain resemblances to each other, differ in several respects (e.g. their molecular weights and thermal stability) from the D-enzymes. As will be shown in a subsequent paper, the D-lactic dehydrogenases are also immunologically distinct from the L-enzymes and immunological cross-reactions are demonstrable among the enzymes of each type obtained from different species.

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