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Influence of Carboxylic Acids on the Stereospecific Nicotinamide Adenine Dinucleotide-Dependent and Nicotinamide Adenine Dinucleotide-Independent Lactate Dehydrogenases of *Leuconostoc mesenteroides*

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Leuconostoc mesenteroides increased its lactic acid production from glucose threefold when malic acid was added to the culture. This increase resulted also in a reduction of the ratio of D-lactic acid to L-lactic acid (31.5 to 1.23). Addition of malic acid increased 6.5-fold the specific activity of nicotinamide adenine dinucleotide (NAD)-linked L-lactate dehydrogenase and increased 3.2-fold that of NAD-linked D-lactate dehydrogenase. The Michaelis constant (K_m) for NAD of the NAD-linked L-lactate dehydrogenase increased with the addition of malate, but no change was observed in the K_m values for the respective D-enzyme. The effect of carboxylic acids on the NAD-linked L-lactate dehydrogenase activities was tested by using partially purified enzyme preparations from cells grown with glucose alone and from cells grown with glucose plus malate. Malate stimulated the L-enzyme and inhibited the D-lactate dehydrogenase. The NAD-linked L-lactate dehydrogenase exhibited the same activity bands on polyacrylamide gel electrophoresis whether the cell-free preparation originated from cells grown on glucose plus malate or on glucose as the sole carbon source. The NAD-linked D-lactate dehydrogenase, however, exhibited a different pattern of electrophoretic mobility, depending upon the source of origin of the cell-free preparation. The results suggest that malate has a stimulatory effect on the synthesis of both enzymes and may result in rearrangement of the protein structure of the D-lactate dehydrogenase. This rearrangement apparently makes the D-enzyme more susceptible to inhibition of catalytic activity. The L-lactate dehydrogenase, however, is stimulated not only in its synthesis but also in its activity. It is proposed that these effects are responsible for the regulation of lactic acid production.

Investigations of the nicotinamide adenine dinucleotide (NAD)-linked lactate dehydrogenases of the reverse reaction (lactate to pyruvate) in homo- and heterofermentative lactic acid bacteria revealed (4) that the *p*H optima of the heterofermentative group are very high (*p*H 9.0 to 10.0) compared with the homofermentative (*p*H 7.8 to 8.2). This raised the question as to whether changes in these enzymes regulate not only the total lactic acid production but also influence the type of optical isomer being produced. It has been observed that the addition of malate to a glucose-containing medium increases the yield of lactic acid from glucose (5, 6) and that the cessation of exponential growth in batch culture coincides with the formation of undissociated lactic acid in the medium (N. Gillespie, Honors thesis, Univ. of Queensland, Brisbane, Australia, 1969). Such results suggest a regulatory function for the lactate dehydrogenases. In *L. mesenteroides*, the addition of malic acid to a glucose-containing culture causes a switch in the lactic acid isomer production from predominantly D-lactic acid with glucose alone to L-lactic acid (13, 14). Thermodynamic and biochemical considerations, however, reveal that utilization of malate alone cannot produce the energy necessary for growth (see reference 10), and thus glucose is required as the main energy source.

This investigation is concerned with the influence of malic acid and other carboxylic acids on

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the stereospecific NAD-dependent and NAD-independent lactate dehydrogenases. It will be shown that the NAD-dependent lactate dehydrogenases regulate the amount of each isomer of lactic acid.

MATERIALS AND METHODS

Growth of the culture and preparation of cell-free extracts. The microorganisms used in this investigation were *L. mesenteroides* 39 (4) and *L. mesenteroides* 99 from the culture collection of the Department of Microbiology at the University of Queensland, Brisbane. Both microorganisms were cultivated as described earlier (4), with 2.2% glucose or 2.2% glucose plus 2.0% DL-malic acid as carbon source. The preparation of the cell-free extract was carried out as described earlier (4)

Lactate dehydrogenase assays. The assays of NAD-dependent and NAD-independent lactate dehydrogenases were made as described earlier (4), with a 0.2 M glycine-NaOH buffer (*p*H 9.8) used for the NAD-dependent and *p*H 6.4 for the NAD-independent lactate dehydrogenases.

Electrophoresis of NAD-dependent lactate dehydrogenases (7). Electrophoresis was run on polyacrylamide gel at 4 C. The gel mixture consisted of one part 0.05 M tris(hydroxymethyl)aminomethane-maleate buffer (*p*H 8.5), two parts acrylamide-*N*,*N*'-methylene bis-acrylamide (28 g of acrylamide and 735 mg of bis-acrylamide in 100 ml of distilled water), one part 0.84% (v/v) *N*,*N*,*N*',*N*'-tetramethyl-ethylene-diamine, and four parts 0.175% (w/v) ammonium persulfate. The gel set in 10 min. After setting of the gel, the sucrose-saturated enzyme preparation, to which bromophenol blue had been added, was transferred onto the gel, and a current of 4 mA per gel was applied until the dye had migrated close to the opposite side of the gel. The enzyme was located by direct staining. The gels were first equilibrated in 0.2 M glycine-NaOH buffer (*p*H 9.8) for 10 min and then immersed in a solution consisting of 50 mg of NAD, 10 mg of nitroblue tetrazolium, 2 mg of phenazine methosulfate, and 1.4 mmole of sodium L-lactate or calcium D-lactate in 100 ml of 0.2 M glycine-NaOH buffer (*p*H 9.8). The gels were left in the staining solution in the dark until fully developed.

Lactic acid. Lactic acid was determined enzymatically with the Boehringer Kit (2) as described by Hohorst (8), with L-lactate dehydrogenase from rabbit muscle and D-lactate dehydrogenase from *L. leichmanii*.

Protein. Protein was determined with the Folin phenol reagent (11).

RESULTS AND DISCUSSION

Influence of malic acid on lactic acid production. *L. mesenteroides* is a heterofermentative lactic acid bacterium which converts less than 80% of its glucose carbon to lactic acid (P. A. Hansen, *Type Strains of Lactobacillus Species*, American Type Culture Collection, Rockville, Md.); the remainder is converted to other end products such as ethanol, acetate, and CO_2. With glucose as the sole carbon source, 18.6% of the glucose carbon was converted to lactic acid by strain 39 and 14.7% by strain 99 (Table 1). Since the latter strain does not contain NAD-dependent L-lactate dehydrogenase, only D-lactate was expected. However, strain 39 also produced both isomers, predominantly the D-isomer of the acid. This ratio of lactic acid isomer production would seem to be caused by the different specific activities of the NAD-dependent lactate dehydrogenases (as reflected in the D/L activity ratio of 12.01) and the NAD-independent lactate dehydrogenases (D/L ratio of 3.1) and confirms the data previously published (4). The addition of 75 mmoles of L-malic acid caused an increase in the total lactic acid production by 488.8% to a final 222.4 mmoles. This increase meant that 69.7% of the total carbon source added was converted to lactic acid by strain 39. If one assumes that the 75 mmoles L-malic acid is converted completely to lactic acid and subtracts this amount from the total lactic acid value, the remaining 147.4 mmoles would represent the glucose product and a 324% increase in lactic acid production from glucose equivalent to 60% of the glucose carbon. If one assumes that the 75 mmoles of L-malate is completely converted to 75 mmoles of L-lactic acid, this increase was evenly divided between the L- (50.8 mmoles) and D- (51.1 mmoles) isomer of lactic acid. Thus the ratio of D/L lactic acid resulting from the dissimilation of glucose changes from 31.3 with glucose alone to 1.82 with glucose plus malate as carbon source.

The specific activities of the NAD-linked enzymes increased 6.5-fold in the case of L- and only 3.2-fold in the case of the D-lactate dehydrogenase. In the light of the earlier established correlation between the stereospecific lactate dehydrogenases and their end products (4), a higher yield of L-lactic acid would have been expected.

Leuconostoc mesenteroides 99 was employed to establish whether or not the marked increases in the specific activity of NAD-linked L-lactate dehydrogenase was due to the L-malate utilization, which has been reported to be metabolized via pyruvate to lactic acid utilizing this enzyme (6). However, after the addition of 75 mmoles of L-malate, *L. mesenteroides* 99, which has no NAD-linked L-lactate dehydrogenase, produced 50 mmoles of L-lactic acid. This formation of L-lactic acid was observed despite the fact that no L-lactate dehydrogenase was detectable. If one assumes that L-malate was converted only to L-lactic acid (9, 14) and that this conversion does not involve the conversion of pyruvate to lactate via the NAD-dependent L-lactate dehydrogenase (16), it would mean that 66.6% of the malate carbon was converted to L-lactic acid.

The similarities between the two strains in regard to the stimulation of the NAD-dependent

TABLE 1. *Formation of* L- *and* D-*lactic acid from glucose and glucose plus malate in comparison to the specific activities of the respective NAD-dependent and NAD-independent lactate dehydrogenase (LDH)*[a]

Substrate	Production of		Total lactic acid production (mmoles)	Specific activity of NAD-dependent		D/L Ratio	Specific activity of NAD-independent		D/L Ratio
	L-Lactic acid (mmoles)	D-Lactic acid (mmoles)		L-LDH	D-LDH		L-LDH	D-LDH	
L. mesenteroides 39									
Glucose (122 mmoles) + malate (75 mmoles) ...	127.2	95.2	222.4	1.68	10.29	6.1	192.5	700.0	3.6
Glucose (122 mmoles) ...	1.41	44.1	45.5	0.26	3.14	12.01	109.5	336.8	3.1
Increase due to malate addition (calculated).....	125.8	51.1	176.9	1.42	7.15		83.0	363.2	
L. mesenteroides 99									
Glucose (122 mmoles) + malate (75 mmoles) ...	50.0	92.3	142.3	None	1.49		Absent	Present	
Glucose (122 mmoles)	0.0	35.8	35.8	None	0.48		Absent	Present	
Increase due to malate addition (calculated).....	50.0	56.5	106.5		1.01				

[a] The enzyme activities were obtained with crude cell-free extracts of the respective microorganisms under optimal assay conditions.

D-lactate dehydrogenase (3.1-fold) and D-lactic acid production (2.5- and 2.1-fold) suggested that the glucose and the glucose-plus-malate utilization were similar, although the malate utilization was completely separated from the glucose utilization. Thus, a 66.6% conversion of L-malate would mean a 3.79-fold increase in total lactic acid by *L. mesenteroides* 39. If one subtracts 50 mmoles from the 127.2 mmoles of L-lactic acid, a 54.8-fold increase is observed with the addition of L-malic acid. In the presence of malate, 70% of the glucose carbon was converted to lactic acid (172.4 mmole), compared with 18.6% with glucose as the sole carbon source. Despite the large increase in L-lactic acid production (to 77.2 mmoles) assumed to be from glucose alone, the D-isomer (95.2 mmoles) was still the predominant lactic acid isomer produced, although the D/L ratio shifted markedly toward the L-isomer (31.3 to 1.23).

With these calculations and the observation that L-malate utilization does not involve NAD-dependent L-lactate dehydrogenase (16), the D/L ratios of the specific activities of the NAD-dependent and NAD-independent lactate dehydrogenases exhibit the correct correlation to account for the sterospecificity of the isomer reported previously (4). These D/L ratios reflected both the predominance of D-lactic acid formation and the strong shift toward L-lactic acid production resulting from the addition of L-malate to the culture medium. Thus, L-malic acid had a stimulatory effect on both NAD-dependent enzymes, with the L-lactate dehydrogenase being more strongly affected. It was also observed that the specific activity of the NAD-independent D-lactate dehydrogenase of *L. mesenteroides* 99 was in the same order of magnitude (1.49 and 0.48) as the NAD-dependent L-lactate dehydrogenase (1.68 and 0.26) and not as the D-lactate dehydrogenase (10.29 and 3.14) of *L. mesenteroides* 39.

K_m determinations. The apparent Michaelis-Menten constants (K_m) for NAD and for L- and D-lactic acid were estimated for the NAD-dependent enzymes of *L. mesenteroides* 39 (Table 2) by using linear plots of the initial rate data according to Lineweaver and Burk (11). Under the influence of malate, the K_m values for NAD of the L-lactate dehydrogenase increased approximately 2.3-fold, whereas no change was observed in the case of the D-lactate dehydrogenase. Malic acid also had no influence on the K_m values of either of the substrates. Thus it appears that the observed increase in total lactic acid production was reflected in an increase of the K_m for NAD for the NAD-dependent L-lactate dehydrogenase only. Although all these investigations were carried out on the back reaction (lactate to pyruvate), this change in K_m does not rule out the presence of an allosteric protein, whereby the product lactic acid could leave the enzyme faster and thus speed up the reaction. It was also interesting to note that the influence of L-malic acid was stronger in regard to the NAD-independent enzymes. Whereas the K_m of the L-lactate dehydrogenase was twofold higher, the corresponding value for the D-enzyme was nearly 50% lower. If these independent lactate dehydrogenases are only able to convert lactic acid to pyruvate (17), it appears that D-lactic acid is converted back to pyruvate faster than the L-lactic acid, which also would lead to an L-lactic acid accumulation.

Influence of carboxylic acids on lactate dehydrogenase activity. As the K_m values did not give a satisfactory explanation for the stimulation of lactic acid formation from glucose in the presence of malate, the influence of malic acid and other carboxylic acids on the enzyme activity were tested in partially purified cell-free extracts (Fig. 1). Malic acid and tartaric acid strongly stimulated the activity of the NAD-dependent L-lactate dehydrogenase, irrespective of whether the cells were grown on glucose or glucose plus malate. The addition of 200 μmoles of DL-malic acid (= 100 μmoles of L-malic acid) to the assay mixture doubled the L-lactate dehydrogenase activity. Further additions had no effect. Assuming that 250 μmoles of L-malate (= 500 μmoles of DL-malate) in the assay mixture of 3.0 ml corresponds to 83 mmoles in the 1-liter culture medium, the addition of 75 mmoles of L-malic acid would stimulate the L-lactate dehydrogenase about twofold. The effect of malate on the D-lactate dehydrogenase was only slight (approximately 20 to 30%). Cells which were grown on glucose plus malate, however, showed an earlier increase of D-lactate dehydrogenase activity, with malic acid concentrations up to 200 μmoles of DL-malic acid, which was approximately double (40 to 50%) the increase shown in extracts from cells grown on glucose. This increase was, however, followed by a linear decrease of the activity when the DL-malic acid concentration was increased to the range 200 to 400 μmoles. The presence of 500 μmoles resulted in a 90% inhibition of the original activity. In contrast to the D-lactate dehydrogenase from *L. mesenteroides* 39, the corresponding enzyme from *L. mesenteroides* 99 was strongly stimulated by the addition of DL-malate and did not lose any of its activity before the malate concentration in the assay mixture exceeded 500 μmoles (Fig. 2). These results suggest that, under the culture conditions used (122 mmoles of glucose plus 75 mmoles of L-malate in 1 liter), the D-lactate dehydrogenase of *L. mes-*

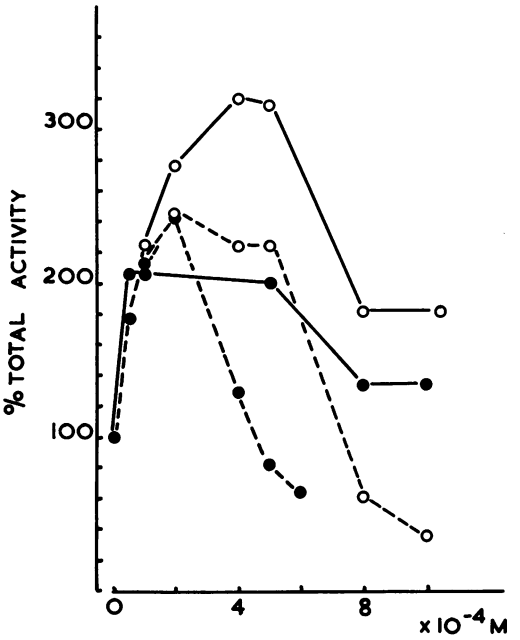

FIG. 2. *Influence of* D L-*malate (filled) and* L-*tartrate (open) on the activity of NAD-dependent* D-*lactate dehydrogenase with partially purified enzyme solutions from Leuconostoc mesenteroides 99 grown on glucose (unbroken line) and glucose plus malate (broken line).*

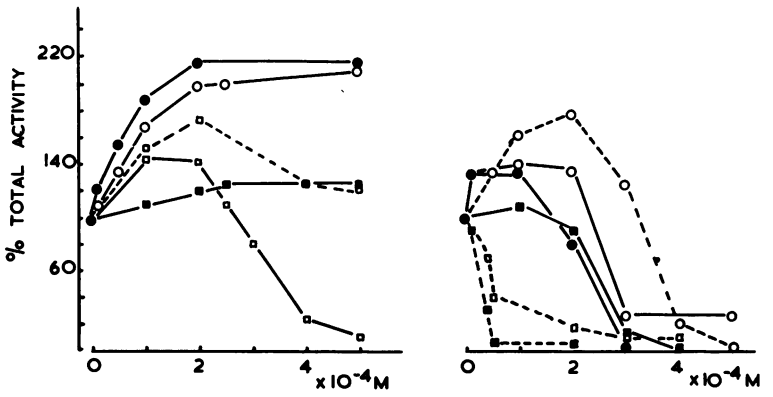

FIG. 1. *Influence of* D L-*malate,* L-*tartrate, succinate, and citrate on the activity of NAD-dependent* L-(○) *and* D-*lactate dehydrogenase* (□) *with partially purified enzyme solutions from Leuconostoc mesenteroides 39. Left: Glucose-grown cells and the effect of malate and tartrate* (○—○; ■—■)*; glucose plus malate-grown cells and the effect of malate* (●—●; □—□) *and tartrate* (○—○; □ - - - □)*. Right: Glucose-grown cells and the effect of succinate* (○—○; ■—■) *and citrate* (●—●; ■—■)*; glucose plus malate-grown cells and the effect of succinate* (○---○; □---□) *and citrate* (○—○; ■---■).

enteroides 39 is strongly inhibited, whereas the same enzyme of strain 99 is strongly activated. This activation on strain 99 was very similar to that observed with L-lactate dehydrogenase from strain 39 and could explain the earlier observations of similar magnitude of specific activity.

Tartaric acid in concentrations up to 500 μM weakly stimulated both stereospecific enzymes of strain 39 and the D-lactate dehydrogenase of strain 99. The details are shown in Fig. 1.

The pattern of influence of succinate and citrate on L-lactate dehydrogenase is similar to that observed with malate. However, when using cells grown on glucose plus malate, both succinate and citrate affected the D-lactate dehydrogenase more severely than did malate. These investigations indicated that the presence of malate in the cell culture solution renders the resultant D-lactate dehydrogenase more susceptible to inhibition of its activity. To determine whether this change is owing to changes in the protein structure or isozyme formation, or both, the partially purified cell-free extracts were studied on polyacrylamide gels.

Polyacrylamide gel electrophoresis. The partially purified cell-free extracts of *L. mesenteroides* 39 and 99 were transferred onto polyacrylamide gels; the resulting electrophoretic patterns are shown in Fig. 3. The NAD-linked L-lactate dehydrogenase (strain 39) exhibited two strong bands and one weak band, which were identical whether the cells were grown on glucose or on glucose plus malate, thus confirming the earlier results. The NAD-linked D-lactate dehydrogenases, however, exhibited entirely different patterns. In both strains, the bands for enzyme activity from cells grown on glucose were markedly different from those of the cells grown on glucose plus malate. There were also obvious differences between the band patterns of the two strains.

On comparing both lactate dehydrogenases from cells grown on glucose, it appeared that two activity bands were characteristic for each enzyme. The L-lactate dehydrogenase exhibited two bands of nearly equal strength, whereas the D-enzyme produced one major band of low and a smaller band of high electrophoretic mobility. These two bands appeared to rearrange themselves under the influence of malate in the culture solution into two almost equal activity bands, which exhibited electrophoretic mobility similar to the two bands of the L-enzyme.

It appeared, therefore, that, in the case of the D-lactate dehydrogenase, the addition of malate to the culture solution had an effect on the synthesis of the enzyme which lead to a change in its protein structure. The data presented in Table 2 suggest that the amounts of both enzymes are increased threefold and that the activity of L-lactate dehydrogenase is increased twofold (Fig. 2) under the influence of malate.

These investigations suggest that the higher

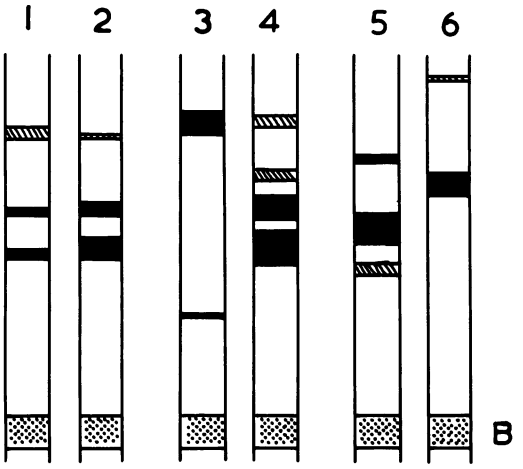

FIG. 3. *Electrophoretic pattern of the NAD-dependent L-LDH of Leuconostoc mesenteroides 39 grown on glucose (1); L-LDH of L. mesenteroides 39 on glucose plus malate (2); D-LDH of L. mesenteroides 39 grown on glucose (3); D-LDH of L. mesenteroides 39 grown on glucose plus malate (4); D-LDH of L. mesenteroides 99 grown on glucose (5) and D-LDH of L. mesenteroides 99 grown on glucose plus malate (6). Electrophoresis was carried out as described in Materials and Methods with partially purified enzyme solutions containing 200 μg of protein for 1, 2, 5, and 6, and 100 μg of protein for 3 and 4. B, Bromophenol blue.*

TABLE 2. *Comparison of K_m values for NAD and substrate of the NAD-dependent and the K_m values for substrate of the NAD-independent lactate dehydrogenases (LDH)*[a]

LDH	Glucose-grown	Glucose-malate-grown
NAD-dependent		
K_m-NAD for L-lactate dehydrogenase	3.57×10^{-3} M	8.3×10^{-3} M
K_m-NAD for D-lactate dehydrogenase	2.0×10^{-3} M	2.5×10^{-3} M
K_m for L-lactate ..	6.7×10^{-1} M	6.7×10^{-1} M
K_m for D-lactate ..	5.0×10^{-1} M	5.5×10^{-1} M
NAD-independent		
K_m for L-lactate ..	1.5×10^{-2} M	3.1×10^{-2} M
K_m for D-lactate ..	1.8×10^{-2} M	1.0×10^{-2} M

[a] From partially purified enzyme solutions from *Leuconostoc mesenteroides* 39 grown on glucose and on glucose plus malate.

lactic acid production in the presence of malic acid is due to the stimulatory effect of malic acid on the synthesis of both NAD-dependent lactate dehydrogenases. The shift in isomer production, however, appears to be related to the differential effect of malic acid on the catalytic activity of both enzymes. This differential effect of malate on the activity is almost certainly reflected in a change in the protein structure of the D enzyme. Whether the different activity bands on the polyacrylamide gel represent isoenzymes or not requires further quantitative investigation. It was also observed that the L-lactic acid production of *L. mesenteroides* 99 grown on glucose plus malate would make this strain an ideal microorganism for further investigation of malate utilization, as it does not possess any L-lactate dehydrogenase and the mechanism appears to be still obscure (see review 1; 14; M. Schütz, Diplom thesis, Univ. Mainz, Germany, 1969).

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