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AN NAD$^+$-INDEPENDENT L-LACTATE DEHYDROGENASE FROM *RHIZOPUS ORYZAE*

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SUMMARY

Two distinct lactate dehydrogenases are present in cultures of a lactic acid-producing strain of *Rhizopus oryzae*. During rapid vegetative growth, when lactic acid is being produced, the mycelium contains an NAD$^+$-dependent lactate dehydrogenase (L-lactate:NAD$^+$ oxidoreductase, EC 1.1.1.27) which catalyses the reduction of pyruvate to lactate but not, apparently, the reverse reaction. Following exhaustion of the glucose from the medium and the onset of sporulation the activity of this enzyme decreases rapidly to an undetectable level and it is replaced by an NAD$^+$-independent lactate dehydrogenase (L-lactate:(acceptor) oxidoreductase) catalysing the oxidation of L-lactic acid to pyruvic acid. This enzyme shows no activity with D-lactic acid or glycollate, is competitively inhibited by oxalate and can use dichlorophenol indophenol, ferricyanide or cytochrome *c* but not oxygen as electron acceptors.

INTRODUCTION

The physiology of lactic acid production by fungi of the genus *Rhizopus* has been studied extensively[1,2], but the enzymes involved in lactic acid production and utilisation have not been thoroughly investigated. The production of ethanol and lactic acid in approximately equimolar quantities[1] has suggested an analogy with the heterofermentative lactic acid bacteria[3]. However, isotopic studies[4] have shown that lactic acid formation in *Rhizopus* occurs solely by the Embden, Meyerhof, Parnas pathway. To account for the apparent stoichiometry between lactic acid and ethanol formation a pyruvate dismutation reaction has been suggested[2]. Recently OBAYASHI *et al.*[5] have reported the presence of an NAD$^+$-dependent lactate dehydrogenase (L-lactate:NAD$^+$ oxidoreductase, EC 1.1.1.27) catalysing the reversible reduction of pyruvate to lactate.

In the course of a study of the relationship between enzyme activity and product formation and utilisation in various species of *Rhizopus* it became apparent that the NAD$^+$-dependent enzyme in *Rhizopus oryzae* which catalyses lactic acid formation was not involved in the oxidation of lactic acid in the later stages of growth. A study of sporulating mycelia revealed the existence of a NAD$^+$-independent lactate

dehydrogenase (L-lactate:(acceptor) oxidoreductase) similar to that found in *Lactobacillus plantarum*[6]. A partial purification and some properties of this enzyme are described.

MATERIALS AND METHODS

Growth of cultures

Rhizopus oryzae (IMI 40564) was obtained from the Commonwealth Mycological Institute, Kew, England. Cultures were grown in a glucose–glutamate–mineral salts medium described previously[7]. For studies on changes in enzyme activity during growth the fungus was grown as surface cultures in 40 ml liquid medium in 250-ml erlenmeyer flasks at 25°. These were inoculated with 1 ml of spore suspension prepared by washing spores from surface cultures grown on glucose–glutamate agar with sterile water. For enzyme purification the fungus was grown in aerated submerged culture in a New Brunswick 40-l Fermacell fermentor. A heavy spore inoculum from 6 petri dishes suspended in 100 ml of water was added to 20 l of glucose-glutamate medium aerated at 12 l/min and stirred at 200 rev./min. Although these growth conditions are very different from those in surface cultures, the time course of changes in lactate dehydrogenase activity appear to be similar in the two methods of culture.

Enzyme assays

Mycelia were harvested by filtration, washed thoroughly with distilled water, dried between filter paper, weighed and frozen to −15°. The frozen mycelium was ground to a fine flour in a mortar maintained at −10° and then ground in the frozen state with ice-cold 0.1 M Tris–HCl buffer (pH 7.5) using 1.5 ml of buffer per g of frozen mycelium. The homogenate was rapidly thawed and centrifuged at 12 500 × *g* for 10 min. The debris was washed with cold buffer using 1 ml/g of mycelium. The pooled supernatants represented the crude cell-free extract. Reproducible extraction was obtained by this procedure and further treatment of the residue by passage through a French press, while yielding a considerable amount of additional protein, yielded very little additional lactate dehydrogenase activity.

Alcohol dehydrogenase (alcohol:NAD^+ oxidoreductase, EC 1.1.1.1) was assayed by following the reduction of NAD^+ at 340 nm in the reaction mixture: 250 μmoles Tris–HCl buffer (pH 7.7), 250 μmoles ethanol, 4 μmoles NAD^+, 0.2 ml of mycelial extract in a total volume of 3.15 ml.

NAD^+-dependent lactate dehydrogenase was assayed by following the oxidation of NADH at 340 nm in the reaction mixture: 250 μmoles phosphate buffer (pH 6.5), 25 μmoles sodium pyruvate (adjusted to pH 6.5), 1 μmole NADH, 0.2 ml of mycelial extract in a total volume of 3.15 ml. The reaction was started by addition of pyruvate after measuring any NADH oxidase activity in the extract. For confirmation of the specificity of this assay a radiochemical assay based on the conversion of [1-^{14}C]-pyruvate to [^{14}C]lactate was used. The complete reaction mixture contained: 12.5 μmoles of phosphate buffer (pH 6.5), 1 μmole NADH, 1 μmole of [1-^{14}C]pyruvate containing 2 μC of ^{14}C, 0.1 ml of mycelial extract in a total volume of 0.55 ml. 0.1-ml samples taken at suitable intervals were dropped into 0.1 ml of 0.2% (w/v) 2,4-dinitrophenylhydrazine in 0.5 M H_2SO_4. This converted the unreacted pyruvate to the dinitrophenylhydrazone allowing it to be readily separated from the lactic acid formed

by paper chromatography. 20-μl samples spotted onto Whatman No. 1 paper were developed in chloroform–*tert.*-amyl alcohol–formic acid–water (80:80:30:80, by vol.) for 12 h. 1 μmole of lithium lactate was added to each spot as a carrier. The dried chromatograms were scanned using a Packard Model 7200 radiochromatogram scanner and radio activity in the lactate and pyruvate hydrazone peaks determined by planimetry.

The NAD^+-independent lactate dehydrogenase was determined by the method of SNOSWELL[6]. The reaction mixture contained: 250 μmoles phosphate buffer (pH 7.0), 50 μmoles lactate, 0.2 μmole 2,6-dichlorophenolindophenol, 0.2 ml of mycelial extract or purified enzyme in a total volume of 3.15 ml. The rate of dye reduction was determined at 600 nm. With crude extracts the reaction mixture was incubated for about 5 min in the absence of lactate after which time the endogenous dye reduction rate was virtually zero. For assays during purification of the enzyme, lithium DL-lactate (British Drug House) was used. For determination of properties of the purified enzyme sodium L-lactate (pH 6.5) prepared from Grade L-1 L(+)-lactic acid (Sigma) was used. No significant difference in rate was detectable using the less pure lactate.

Analytical methods

Glucose was estimated by alkaline ferricyanide oxidation[8]. Ethanol was measured in steam distillates of the medium by acid dichromate oxidation followed by iodometric estimation of the excess dichromate. Lactic acid was measured by the colorimetric method of BARKER AND SUMMERSON[9]. An independent estimation of lactic acid in certain samples was made by silica-gel column chromatography[10]. Protein was determined by the method of LOWRY *et al.*[11] using bovine serum albumin as a standard.

RESULTS

Relationship between product formation and enzyme activity

In a study of the relationship between ethanol and lactic acid formation in *R. oryzae*, changes in the activity of the corresponding NAD^+-dependent dehydrogenases were determined by harvesting surface cultures at various intervals over a 3-day period of growth (Fig. 1).

Ethanol was the dominant product of glucose catabolism up to 34 h during which time the mycelium existed mainly as submerged hyphae. The appearance of lactic acid as a major product coincided with the development of an extensive aerial mycelium. A similar relationship was shown by the corresponding dehydrogenases. Lactate dehydrogenase was barely detectable in the 24-h mycelium in contrast to the high activity of alcohol dehydrogenase. However, a 34-h-old mycelium showed high lactate dehydrogenase activity as assayed by the pyruvate-dependent oxidation of NADH. Attempts to assay the enzyme by lactic acid-dependent reduction of NAD^+ showed no activity.

Both alcohol and lactate dehydrogenases decreased in activity after 34 h. The products, ethanol and lactic acid continued to accumulate until the glucose in the medium had been exhausted (after about 45 h). Thereafter both products declined although lactic acid later showed a further increase between 55 and 72 h. This pattern

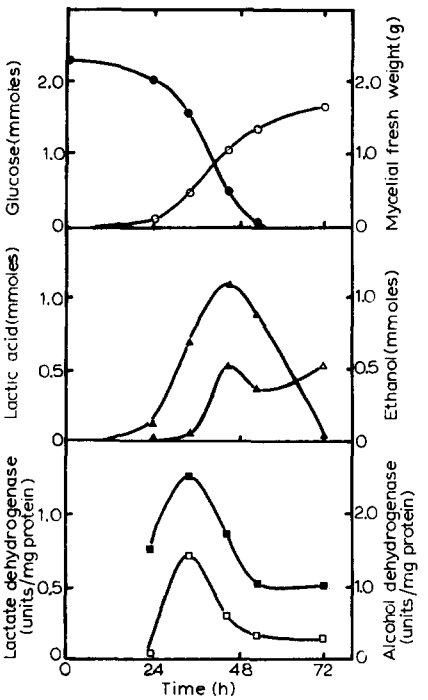

Fig. 1. Product formation, glucose utilisation and alcohol and lactate NAD^+-dependent dehydrogenase activity during growth of cultures of *R. oryzae*. ○—○, mycelial fresh weight; ●—●, glucose content of medium; ▲—▲, ethanol content of medium; △—△, lactic acid content of medium; ■—■, alcohol dehydrogenase activity (μmoles NAD^+ reduced per min per mg protein); □—□, lactate dehydrogenase activity (μmoles NADH oxidised per min per mg protein).

of changes in enzyme activity and product concentration has been confirmed in two further time-course experiments.

Confirmation of NAD^+-dependent lactate dehydrogenase activity and existence of a NAD^+-independent lactate dehydrogenase

In view of the inability to demonstrate lactate dehydrogenase activity by lactic acid-dependent reduction of NAD^+, the possibility arose that the pyruvate-dependent oxidation of NADH was due, not to the activity of lactate dehydrogenase, but to the combined activity of pyruvate decarboxylase and alcohol dehydrogenase. This could account for the similar time-course of changes in activity of the two dehydrogenases although it would not explain the absence of apparent pyruvate reductase activity in the 24-h-old mycelium which was actively forming ethanol. The previous demonstration of an NAD^+-dependent lactate dehydrogenase in *R. oryzae*[5] also utilised the spectrophotometric assay of NADH oxidation by pyruvate. Accordingly an independent assay based on the NADH-dependent conversion of [^{14}C]pyruvate to lactate was used.

Incubation of 1 μmole of [1-^{14}C]pyruvate with an extract from a 48-h-old mycelium in the presence of NADH resulted in progressive conversion of the pyruvate to [^{14}C]lactate which was complete after 10 min incubation. In the absence of NADH no conversion occurred.

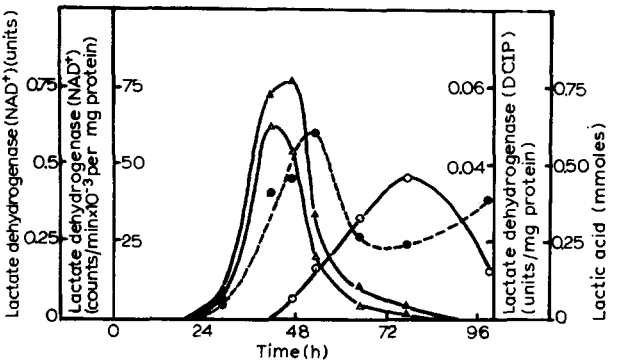

Fig. 2. Changes in activity of NAD+-dependent and NAD+-independent lactate dehydrogenases in relation to lactic acid formation and utilisation during growth of cultures of *R. oryzae*. ●—●, lactic acid content of medium; △—△, NAD+-dependent lactate dehydrogenase activity assayed by following pyruvate-dependent NADH oxidation spectrophotometrically at 340 nm; ▲—▲, NAD+-dependent lactate dehydrogenase activity assayed by rate of conversion of [^{14}C]pyruvate to [^{14}C]lactate; ○—○, NAD+-independent lactate dehydrogenase assayed by following lactic acid-dependent reduction of dichlorophenol-indophenol (DCIP) at 600 nm.

Changes in the activity of the NAD+-dependent lactate dehydrogenase during growth were compared using the two different assay methods. It is clear (Fig. 2) that the activity of lactate dehydrogenase measured spectrophotometrically closely follows the much more specific radiochemical assay and that the spectrophotometric method is a valid measure of lactate dehydrogenase activity. It was confirmed that the extracts, active in catalysing NADH-dependent reduction of pyruvate to lactate, did not catalyse reduction of NAD+ by either L(+)-lactate or D(−)-lactate over a wide range of pH values. Thus the NAD+-dependent lactate dehydrogenase appears to catalyse only pyruvate reduction to lactate and not lactate oxidation by NAD+.

However, lactic acid determinations on the medium showed that as in the previous experiment, lactic acid in the medium decreased after 48 h growth and subsequently increased again. If lactic acid utilisation is not catalysed by the NAD+-dependent enzyme an alternative enzyme must fulfil this role. An extract made from a four day old culture, which was inactive in catalysing pyruvate-dependent oxidation of NADH, possessed an enzyme catalysing the reduction of dichlorophenolindophenol by lactate. The rate of reduction was unaffected by addition of NAD+. Changes in the activity of this enzyme during the course of growth are shown in Fig. 2. No activity was detectable until near the end of the period of lactate synthesis and then activity increased during the sporulation phase reaching a maximum after about 3–3.5 days growth. It is thus active during the period of lactate decline and subsequent increase. Although the specific activity of this enzyme is much lower than that of the NAD+-dependent enzyme, it is adequate to account for the rate of lactate disappearance.

In a confirmatory experiment the relationship between glucose utilisation, lactic acid content and enzyme activity was determined. The peak activity of the NAD+-dependent lactate dehydrogenase coincided with the period of rapid glucose utilisation whereas peak activity of the NAD+-independent enzyme was reached after glucose had disappeared from the medium. As in both previous experiments a second rise in the lactate content of the medium occurred in the sporulation phase after exhaustion of glucose. To examine the possibility that this apparent rise in lactic acid

content was due to the accumulation of some other product giving the same colour reaction as lactic acid in the BARKER AND SUMMERSON[9] procedure the organic acid content of the medium from a culture harvested at 84 h was determined by silica gel partition column chromatography[10]. Lactic acid was the only organic acid peak found and the amount of lactic acid agreed closely with that estimated by the BARKER AND SUMMERSON colorimetric method on the same medium.

Purification of the NAD$^+$-independent lactate dehydrogenase

A partial purification of the NAD$^+$-independent lactate dehydrogenase from a sporulating culture of *R. oryzae* was carried out based on the procedure used for purification of an apparently similar enzyme in cultures of *L. plantarum*[6]. The fungus was harvested after 3 days growth in submerged aerated culture in 20 l of glucose–glutamate medium. The mycelium was frozen and disrupted by grinding in 0.1 M Tris–HCl buffer (pH 7.5) and thawing as described previously. All subsequent operations were carried out at 4°.

(1) $(NH_4)_2SO_4$ fractionation. To 1 l of crude cell-free extract derived from the disruption of 350 g (wet weight) of mycelium, solid $(NH_4)_2SO_4$ was added to bring the solution to 70% saturation. The activity was quantitatively precipitated and could be stored in this form for 2 to 3 weeks with less than 5% loss of activity. The precipitate was collected by centrifugation and redissolved in 120 ml of cold 0.1 M Tris–HCl buffer (pH 7.5). $(NH_4)_2SO_4$ was added to bring the solution to 30% saturation and the suspension stirred for 1 h before centrifuging. The precipitate was discarded. Further $(NH_4)_2SO_4$ was added to bring the supernatant to 50% saturation. After 1 h the suspension was centrifuged, and the precipitate containing 75–85% of the activity, was redissolved in 25 ml cold 5 mM Tris–HCl (pH 7.5) containing 2 mM lithium lactate (hereafter referred to as Tris–lactate buffer). The solution was dialysed against 2 l of Tris–lactate buffer for 2 h and then 5 l of Tris–lactate overnight.

(2) DEAE-cellulose chromatography. DEAE-cellulose, equilibrated with Tris–lactate buffer (pH 7.5), was packed into a 20 cm × 2 cm column. The dialysed solution containing the enzyme was stirred for 10 min with a further 3 g of equilibrated DEAE-cellulose and the suspension then packed on top of the column. The enzyme was removed by gradient elution with 5 mM Tris buffer (pH 7.5) containing an increasing concentration of lithium lactate from 2 mM to 0.4 M. 5-ml fractions were collected and the most active fractions of the eluate (between 220 and 250 ml) were pooled and dialysed against two changes of Tris–lactate buffer.

After dialysis the solution was applied to a second DEAE-cellulose column (12 cm × 2 cm) and eluted with 5 mM Tris buffer (pH 7.5) containing an increasing concentration of lactate between 2 mM and 0.3 M. The activity was eluted between 140 and 210 ml with the most active fractions between 185 and 200 ml.

(3) Sephadex G-200 treatment. The fractions between 185 and 200 ml from the second DEAE-cellulose column were passed through a 30 cm × 2 cm column of Sephadex G-200 and eluted with 5 mM Tris–HCl buffer (pH 7.5). The activity was eluted in the earliest fractions containing protein between 28 and 60 ml. The highest specific activity fractions (between 28 and 46 ml) were pooled and used to study some properties of the enzyme. They represent a 30-fold purification relative to the specific activity in the crude cell-free extract.

The results of this purification procedure are shown in Table I.

TABLE I

PURIFICATION OF NAD$^+$-INDEPENDENT LACTATE DEHYDROGENASE FROM *R. oryzae*

Steps	*Vol. (ml)*	*Total enzyme units*	*Total protein (mg)*	*Specific activity (units/mg protein)*	*Yield (%)*
Crude cell-free extract	1000	63	4570	0.0137	100
30–50% $(NH_4)_2SO_4$ fraction (after dialysis)	45	46	1260	0.0364	73
Peak fractions from first DEAE-cellulose column	35	15	87	0.171	24
Peak fractions from second DEAE-cellulose column	20	2.8	7.7	0.368	5
Peak fractions from Sephadex G-200 column	20	1.2	2.7	0.415	2

Properties of the partially purified NAD$^+$-independent lactate dehydrogenase

(1) Reaction product. The product was identified as pyruvate by preparation of the 2,4-dinitrophenylhydrazone derivative. The two isomeric dinitrophenylhydrazones had the same R_F value as those of authentic pyruvate in *tert.*-amyl alcohol–ethanol–water (50:5:40, by vol.) and the absorption spectra in 0.2 M $NaHCO_3$ and in 0.1 M NaOH were identical with those of pyruvate dinitrophenyl hydrazone[12]. Oxidation of 3 μmoles of lactic acid yielded 2.58 μmoles of pyruvate estimated by colorimetric determination of the dinitrophenylhydrazone.

(2) Substrate specificity. The enzyme is specific for L(+)-lactic acid and shows no activity with D(−)-lactate, glycollate or L-malate. The presence of D-lactate has no effect on the rate of oxidation of L-lactate.

(3) Electron acceptors. 2,6-Dichlorophenolindophenol and ferricyanide are equally effective electron acceptors. The enzyme also catalyses the reduction of cytochrome *c* by L-lactate, but the rate of reduction is much slower than that with ferricyanide or dichlorophenolindophenol at a comparable concentration of acceptor. Using 67 μM dichlorophenolindophenol as electron acceptor, a purified enzyme preparation gave a reduction rate of 0.72 μmole/min per mg of protein whereas, using 82 μM cytochrome *c* the same enzyme preparation gave a rate of reduction of only 0.055 μmole/min per mg of protein. The enzyme did not catalyse the reduction of either NAD$^+$ or NADP$^+$ by L-lactate. When L-lactate was shaken with the enzyme alone in air for 30 min no pyruvate was detectable with the sensitive dinitrophenylhydrazone assay nor was any H_2O_2 formation detected by a benzidine–peroxidase assay. Therefore oxygen does not appear to act as an electron acceptor.

(4) Effect of pH on activity. The effect of pH on activity in 0.05 M Tris–maleate buffer between pH 5.2 and 8.4 is shown in Fig. 3. Ferricyanide was used as electron acceptor because of the pH dependence of the extinction coefficient of dichlorophenolindophenol. The enzyme shows highest activity between pH 7.2 and 8.4 and is almost inactive at pH 5.2.

(5) Effect of substrate concentration and inhibition by oxalate (Fig. 4). The K_m for L-lactate at pH 7.0 using dichlorophenolindophenol as electron acceptor is 3.85 mM. The enzyme is competitively inhibited by oxalate. K_i values determined at two

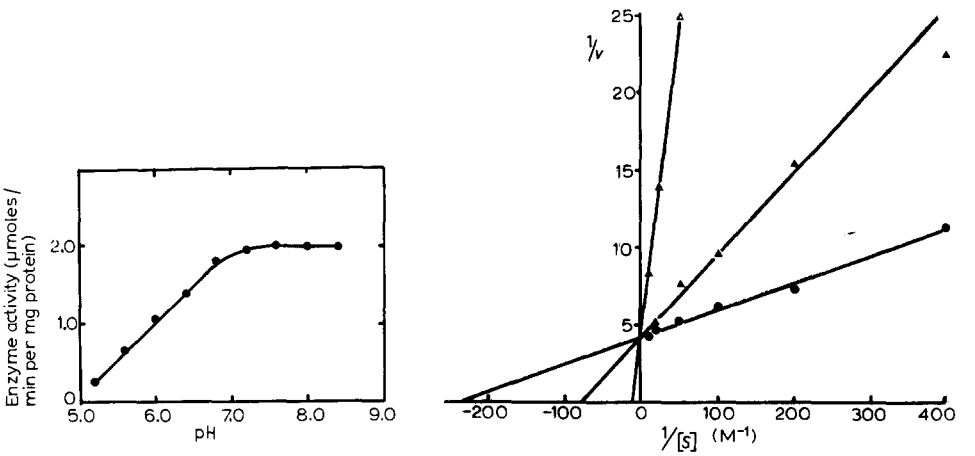

Fig. 3. Effect of pH (0.05 M Tris–maleate buffers) on the activity of the NAD^+-independent lactate dehydrogenase. Reaction mixture contained 100 μmoles Tris–maleate buffer, 40 μmoles L-lactate, 3 μmoles potassium ferricyanide and 0.5 ml of enzyme in a total volume of 3 ml. Ferricyanide reduction was followed at 420 nm.

Fig. 4. Effect of lactate concentration and oxalate on the activity of the NAD^+-independent lactate dehydrogenase. Lineweaver–Burk plot of $1/v$ against 1/lactate concentration. Enzyme assayed in 0.1 M phosphate buffer (pH 7.0) at 27° using standard assay conditions. ●—●, sodium L-lactate; △—△, lactate *plus* 10 mM oxalate; ▲—▲, lactate *plus* 1 mM oxalate.

different concentrations of oxalate (10 mM and 1 mM) were 0.40 mM and 0.445 mM, respectively.

DISCUSSION

The existence of an NAD^+-dependent lactate dehydrogenase in *R. oryzae* previously reported by OBAYASHI *et al.*[5] has been confirmed. However, the present study indicates that it functions solely as a pyruvate reductase during the period of lactate formation in the logarithmic phase of growth whereas OBAYASHI *et al.* state that the enzyme catalyses the oxidation of lactic acid as well as the reduction of pyruvic acid. In studying the oxidation of lactic acid, they did not, in fact, demonstrate reduction of NAD^+ by lactate but utilised electron acceptors such as dichlorophenolindophenol and ferricyanide and assumed that the enzyme preparation which they were using contained a diaphorase linking lactate and acceptor *via* NAD^+. In the light of the present study it seems that their enzyme preparation must have contained both the NAD^+-dependent and NAD^+-independent lactate dehydrogenases. In studying the properties of the NAD^+-dependent enzyme they used the $(NH_4)_2SO_4$ fraction precipitating between 30 and 50% saturation without further purification which, as the present study shows, contains most of the NAD^+-independent enzyme as well.

NAD^+-dependent L-lactate dehydrogenases catalysing only the reduction of pyruvate to lactate and not the reverse reaction have been reported from *Lactobacillus fermenti*, *Lactobacillus jugurti* and *Lactobacillus casei*[13].

Because of the low yields of the NAD^+-independent lactate dehydrogenase after passage through DEAE-cellulose columns it has not been possible to obtain sufficient

enzyme to characterise the prosthetic group. High activity fractions from the first DEAE-cellulose column, concentrated by $(NH_4)_2SO_4$ precipitation, are yellow in colour and show a difference spectrum following dithionite reduction fairly similar to that reported by SNOSWELL[6] for the flavoprotein L-lactate dehydrogenase from *L. plantarum* with a maximum decrease in absorbance at 455 nm. However, the *Lactobacillus* enzyme differs from the *R. oryzae* enzyme in several features:

(1) The *Lactobacillus* lactate dehydrogenase is unable to use ferricyanide or cytochrome *c* as electron acceptors whereas the *R. oryzae* enzyme catalyses reduction of both of these by L-lactate.

(2) The *Lactobacillus* L-lactate dehydrogenase is competitively inhibited by D-lactate whereas D-lactate has no effect on the rate of L-lactate oxidation by the *Rhizopus* enzyme.

(3) The *Lactobacillus* enzyme shows a pH optimum at 6.0 whereas the *Rhizopus* enzyme has very low activity at this pH and the activity is highest between pH 7.2 and 8.4.

The results of the time-course experiments and the properties of the two lactate dehydrogenases in *R. oryzae* are consistent with the suggestion that the NAD^+-dependent enzyme is involved in lactic acid biosynthesis whereas the NAD^+-independent enzyme serves to reoxidise the lactate after utilisation of glucose (*cf.* ref. 14). A somewhat comparable situation has been described in *Butyribacterium rettgeri*[15] where an NAD^+-dependent lactate dehydrogenase is responsible for lactic acid synthesis from glucose but a glucose-repressible NAD^+-independent dehydrogenase mediates lactic acid utilisation.

However, this simple interpretation is complicated by the fact that a second period of lactic acid synthesis occurs after the disappearance of glucose and of the NAD^+-dependent lactate dehydrogenase. The pyruvate required for the synthesis of lactate at this stage may originate from the oxidation of ethanol via the glyoxylate cycle, the enzymes for which are known to be synthesised in *Rhizopus* following disappearance of glucose from the growth medium[16], and subsequent decarboxylation of the malate formed. The glutamate added to the medium as a nitrogen source could also give rise to pyruvate by way of the tricarboxylic acid cycle and malic enzyme. However, the enzymic basis for reduction of pyruvate to lactate during the sporulation phase is not evident from the results of the present study and is in need of further investigation.

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