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Purification and Properties of L(+)-Lactate Dehydrogenase from Potato Tubers

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1. A purification of L(+)-lactate dehydrogenase is described. 2. The final preparation is active with NADH and NADPH and with a number of keto acids, but evidence is presented to support the view that a single enzyme is involved. 3. NAD^+ showed product inhibition, but at slightly acid pH values there was evidence of co-operative binding. 4. At acid pH values ATP was a potent inhibitor and appears to be an allosteric effector. At neutral or alkaline pH values ATP behaved as a weak competitive inhibitor. 5. The physiological significance of inhibition by ATP is discussed.

Alcohol is the characteristic product of anaerobic fermentation in plants, but many plants produce lactic acid. The tuber of *Solanum tuberosum* (potato) produces L(+)-lactic acid under anaerobic conditions (Barker & El Saifi, 1963) and contains a NAD-linked dehydrogenase (Barron *et al.*, 1950). The enzyme has been little studied although the stereospecificity of NAD^+ reduction has been established as A side (Loewus & Stafford, 1960). The limited amount of work on the plant enzyme stands in sharp contrast to the extensive work on animal, fungal and bacterial lactate dehydrogenases.

The control of lactate dehydrogenase in relation to fermentation remains uncertain. ATP has been proposed as a controlling ligand for bacterial D(−)-lactate dehydrogenases (Tarmy & Kaplan, 1968; Wittinberger, 1968) as well as for the L(+)-lactate dehydrogenase of *Hevea latex* (Jacob & D'Anzac, 1967, 1968). GTP has been proposed as an allosteric inhibitor of D(−)-lactate dehydrogenases in fungi (Le John, 1971), and NADH appears to play a regulatory role in the lobster tail L(+)-lactate dehydrogenase (Kaloustian & Kaplan, 1969).

The present work is an attempt to characterize the potato enzyme and, in particular, to examine possible control mechanisms.

Experimental

Materials

Calcium phosphate gel. $CaCl_2$ (29.4g) was dissolved in 250ml of water and mixed with 250ml of a solution containing K_2HPO_4 (68.4g). The gelatinous precipitate was filtered at the pump and washed with 500ml of water. The gel was sucked dry and stored at 0°C.

Buffers. All buffers were prepared with glass-distilled water. 2-(*N*-Morpholino)ethanesulphonic acid (MES) and *N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid (TES) (Good *et al.*, 1966) were obtained from Calbiochem, Los Angeles, Calif., U.S.A. The buffers were dissolved in water at 25°C and adjusted to the required pH with freshly prepared KOH. Tris was obtained under the trade name Trizma from Sigma Chemical Co., St. Louis, Mo., U.S.A. It was dissolved in water at 25°C and adjusted to the required pH with HCl (A.R. grade).

Other chemicals. NAD^+, NADH, NADPH, ATP, ADP and sodium pyruvate were obtained from Sigma Chemical Co. Lithium lactate was from Calbiochem, sodium mercaptobenzothiazole from British Drug Houses Ltd., Poole, Dorset, U.K., DE-52 cellulose from W. and R. Balstron Ltd., Maidstone, Kent, U.K. and Sephadex G-200 from Pharmacia, Uppsala, Sweden.

Methods

Protein measurement. Protein was determined by the spectrophotometric method of Warburg & Christian (1942).

Enzyme assay. The standard assay was carried out at pH6.0 by measuring the decrease in E_{340} associated with NADH oxidation. The assay mixture contained MES buffer (pH6.0, 33mM), NADH (0.4mg) and sodium pyruvate (0.33mM) or sodium hydroxypyruvate (3.3mM) or sodium glyoxylate (3.3mM) or sodium oxobutyrate (22mM) in a volume of 3ml. The reaction was started by the addition of 0.1ml of enzyme suitably diluted to give an activity of less than 50units/ml (the unit of activity is defined below). Under these conditions, the relation between reaction rate and enzyme concentration was linear. Assays were carried out at 25°C with either a Cary spectrophotometer or a Unicam SP. 500 spectrophotometer.

Unit of enzyme activity. A unit of enzyme activity is defined as the removal of 1.0μmol of NADH/min, which is equivalent to a decrease in E_{340} of 2.07min.

Specific activity is defined as the units of enzyme activity/mg of protein.

Enzyme purification. Potato tubers were peeled, cut into segments and rinsed in ice-cold water. Subsequently all steps were carried out at 2°C. Protein was extracted from the tissue by passing potato segments (750g) and 750ml of a solution containing tris–HCl buffer (pH7.4, 0.05M), saturated with sodium mercaptobenzothiazole, through a domestic Kenwood centrifuge juice extractor. The centrifuge basket was lined with Miracloth (Calbiochem) to retain cell debris and starch grains.

The clear extract (1 litre) was stirred while $(NH_4)_2SO_4$ (150g) was added. After stirring for 10min, the extract was centrifuged at 10000*g* for 15min. The precipitate, which contained little or no lactate dehydrogenase, was discarded and the supernatant treated with a further 150g of $(NH_4)_2SO_4$. The second precipitate, collected by centrifuging at 10000*g* for 15min, was dissolved in 60ml of tris–HCl buffer (pH7.4, 0.05M) and dialysed for 3h against 5 litres of tris–HCl buffer (pH7.4, 0.02M).

The dialysed solution (approx. 100ml) was stirred while calcium phosphate gel (5g) was added. After 10min the gel was collected by centrifuging at 7000*g* for 5min and discarded. The supernatant was treated with a further 5g of calcium phosphate gel, which was similarly collected and discarded. The supernatant was then treated with 10g of calcium phosphate gel, which was collected as described above, but this time was retained. The gel was resuspended in 40ml of potassium phosphate buffer (pH7.8, 0.02M) and after standing for 10min the gel was collected by centrifuging at 7000*g* for 5min and the supernatant was discarded. The gel was treated with 40ml of potassium phosphate buffer (pH7.8, 0.1M) and after standing for 10min the calcium phosphate was removed by centrifuging at 7000*g* for 5min and the supernatant, which contained the lactate dehydrogenase, was collected.

DE-52 cellulose (1g) was added and stirred for 5min. The cellulose was then removed by centrifuging at 7000*g* for 5min.

The supernatant was rapidly stirred and acetic acid (2%) slowly added until the pH fell to 5.1. After standing for 10min the precipitate was collected by centrifuging at 10000*g* for 5min and dissolved in 10ml of MES buffer (pH6.5, 0.05M). After standing for 10min the fraction was clarified by centrifuging at 37000*g* for 15min.

A portion (5ml) of the extract was applied to a column (2.5cm×35cm) of Sephadex G-200. The enzyme was eluted with MES buffer (pH6.5, 0.02M) and 2ml fractions were collected. The enzyme emerged in a volume of eluate corresponding to a molecular weight range of 155000–200000. The active fractions were pooled and stored at −20°C. Little or no loss of activity occurred on storage for several months.

Results

Specificity of lactate dehydrogenase

Crude and purified preparations of potato lactate dehydrogenase catalyse the reduction of pyruvate and a number of α-oxo acids by NADH and NADPH.

Table 2. *Kinetic constants of four substrates of potato lactate dehydrogenase*

The assay system contained MES buffer (pH6.0, 0.033M), NADH (0.125mM), the oxo acid and 0.1ml of enzyme (30μg/ml) in a final vol. of 3ml. For further details see the Experimental section.

Substrate	K_m (mM)	$V_{max.}$ (units/mg of enzyme)
Pyruvate	0.2	6.5
Hydroxypyruvate	8.0	14.1
Glyoxylate	25.0	9.7
α-Oxobutyrate	77.0	5.1

Table 1. *Summary of purification of lactate dehydrogenase from potato tubers*

The fractionation and assay procedures are described in the Experimental section.

Fraction	Vol. (ml)	Total activity (units)	Specific activity (units/mg)	Ratio of activities: Pyruvate / Hydroxypyruvate	Pyruvate / Glyoxylate	Pyruvate / α-Oxobutyrate
Extract	1000	325	—	1.0	3.8	3.6
$(NH_4)_2SO_4$	100	205	0.3	1.1	3.7	3.7
Calcium phosphate eluate	40	120	2.2	0.95	3.8	3.4
DE-52 cellulose eluate	38	100	4.2	1.0	3.5	3.6
pH5.1 precipitate	10	80	5.0	0.98	3.7	3.6
Sephadex peak	30	65	18.5	0.98	3.7	3.6

Plants and animals contain an NAD-linked D-glycerate dehydrogenase which catalyses the reduction of hydroxypyruvate, glyoxylate and pyruvate. In addition, plants contain an NADPH-specific glyoxylate reductase, which is much more active with glyoxylate than with either hydroxypyruvate or pyruvate (Zelitch & Gotto, 1962; Tolbert *et al.*, 1970). It is thus of importance to establish the substrate specificity of enzymes acting on α-oxo acids.

Evidence that a single protein catalyses the reduction of α-oxobutyrate, pyruvate, hydroxypyruvate and glyoxylate was obtained by comparing the activity ratios during purification and by a kinetic study of the interaction of substrates with the enzyme.

Activity ratios

Table 1 shows that the activity ratios with different substrates were, within the limits of experimental error, constant throughout purification. Purification on a column of Sephadex G-200 gave a single symmetrical peak with activity towards pyruvate, glyoxylate, oxobutyrate and hydroxypyruvate.

Mixed substrates

Thorn (1949) and, independently, Whittaker & Adams (1949) have shown that the rate of reaction with two substrates is given by the equation:

$$V_t = \frac{V_{max.1}[S_1]}{[S_1]+K_{m1}\left(1+\frac{[S_2]}{K_{m2}}\right)} + \frac{V_{max.2}[S_2]}{[S_2]+K_{m2}\left(1+\frac{[S_1]}{K_{m1}}\right)}$$

The rate of the reaction with two substrates if separate enzymes are involved is given by:

$$V_t = \frac{V_{max.1}[S_1]}{[S_1]+K_{m1}} + \frac{V_{max.2}[S_2]}{[S_2]+K_{m2}}$$

Table 3. *Activity of lactate dehydrogenase with mixed substrates*

The calculated velocities are based on the equations given in the text and the kinetic constants given in Table 2. For experimental details see the text.

Substrates	Values calculated by assuming two enzymes (units/mg of enzyme)	Values calculated by assuming one enzyme (units/mg of enzyme)	Values observed (units/mg of enzyme)
Pyruvate (200 μM) + hydroxypyruvate (670 μM)	4.40	3.70	3.70
Pyruvate (120 μM) + hydroxypyruvate (670 μM)	3.52	2.99	2.99
Pyruvate (80 μM) + hydroxypyruvate (670 μM)	2.99	2.46	2.46
Pyruvate (40 μM) + hydroxypyruvate (670 μM)	2.11	1.94	1.94
Pyruvate (120 μM) + glyoxylate (11 mM)	5.46	3.52	3.87
Pyruvate (80 μM) + glyoxylate (5.6 mM)	4.22	2.82	2.82
Hydroxypyruvate (830 μM) + glyoxylate (11 mM)	4.22	3.52	3.52

Table 4. *Michaelis constants for lactate dehydrogenase with various substrates*

For experimental details see the text.

pH	Varied substrate	Constant substrate	K_m apparent for varied substrate
6.1	Pyruvate	NADH (0.125 mM)	0.2 mM
6.5	Pyruvate	NADH (0.125 mM)	0.3 mM
7.1	Pyruvate	NADH (0.125 mM)	0.55 mM
7.8	Pyruvate	NADH (0.125 mM)	1.25 mM
6.1	NADH	Pyruvate (2 mM)	25 μM
6.5	NADH	Pyruvate (2 mM)	11 μM
7.1	NADH	Pyruvate (2 mM)	8 μM
7.8	NADH	Pyruvate (6 mM)	5 μM
6.5	Pyruvate	NADPH (0.15 mM)	5 mM
6.5	NADPH	Pyruvate (6 mM)	16 μM
6.5	NADPH	Hydroxypyruvate (33 mM)	20 μM
7.8	Lactate	NAD^+ (2.1 mM)	8.5 mM
7.8	NAD^+	Lactate (33 mM)	0.4 mM

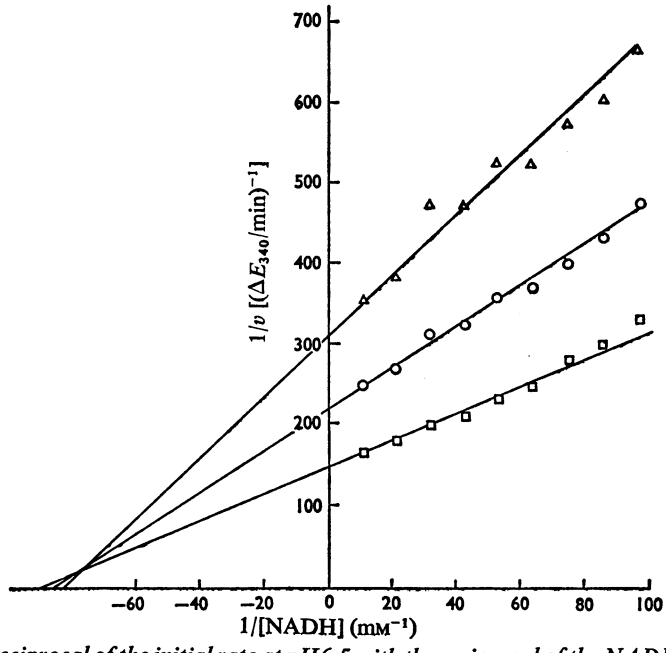

Fig. 1. *Variation of the reciprocal of the initial rate at pH 6.5 with the reciprocal of the NADH concentration for three different concentrations of pyruvate*

Experimental details are given in the text. Additions were: △, pyruvate (0.025 mM); ○, pyruvate (0.05 mM); □, pyruvate (0.2 mM).

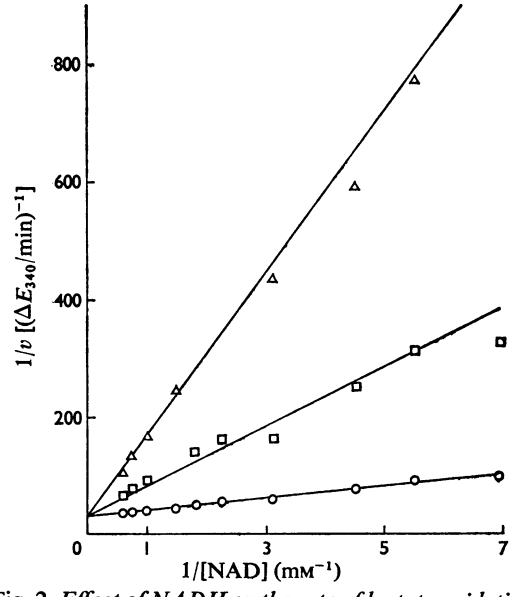

Fig. 2. *Effect of NADH on the rate of lactate oxidation by NAD at pH 7.8*

Experimental details are given in the text. TES buffer (pH 7.8, 33 mM) was used. Additions were: △, lactate (33 mM) and NADH (55 μM); □, lactate (33 mM) and NADH (22 μM); ○, lactate (33 mM) and no NADH.

where $V_{max.}$ represents the velocity in the presence of a saturating concentration of substrate S and K_m is the Michaelis constant. The kinetic constants for four oxo acids with NADH as hydrogen donor have been measured (Table 2). The velocities calculated for mixed substrates are compared with observed velocities in Table 3 and the results are in good agreement with the hypothesis of a single enzyme.

Kinetics

The pH optimum for reduction of oxo acids by NADH or NADPH was pH 6.5.

The enzyme shows normal Michaelis–Menten kinetics and the Michaelis constants for substrates and coenzymes at various pH values are shown in Table 4. In the terminology of Cleland (1963), the reaction is Bi Bi, i.e. the enzyme binds both substrates simultaneously. When the NADH concentration is varied and the pyruvate concentration kept constant, a Lineweaver–Burk plot gives a family of lines intersecting to the left of the $1/v$ axis (Fig. 1). Similar families of curves were obtained with other pairs of reactants, although the precise order of substrate addition cannot be deduced from these plots. In principle, the sequence can be determined by the method of product inhibition described by Cleland (1963); however, as shown in the next

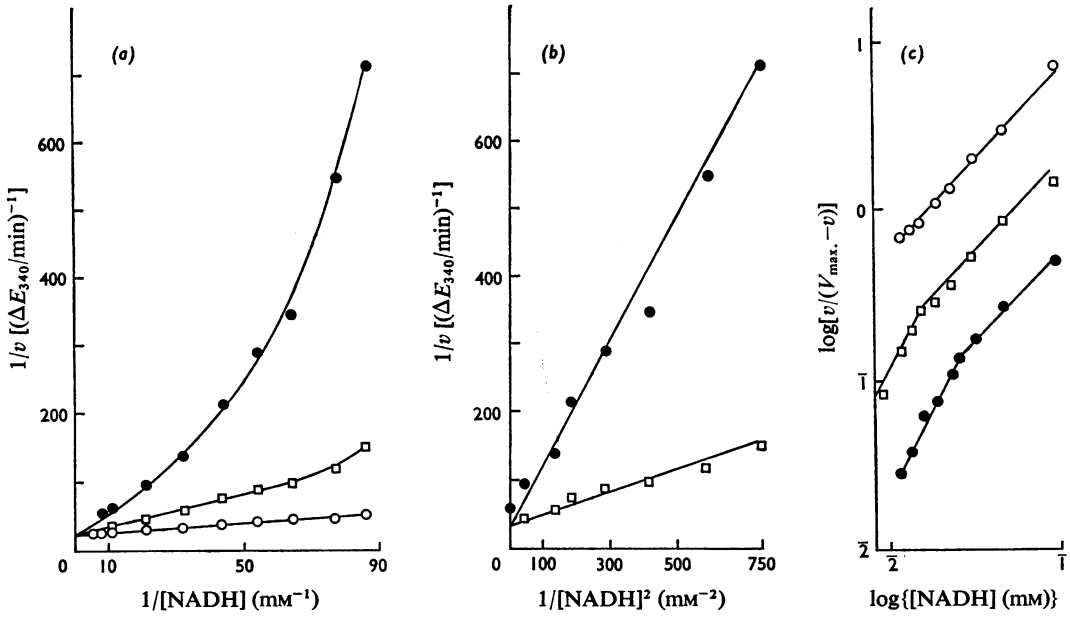

Fig. 3. *Effect of NAD⁺ on the reduction of pyruvate by NADH at pH* 6.1

Experimental details are given in the text. MES buffer (pH 6.1, 33 mM) and pyruvate (0.2 mM) were used. Additions were: ●, NAD^+ (3 mM); □, NAD^+ (1 mM); ○, control, no NAD^+.

Table 5. *Effect of various compounds on the activity of lactate dehydrogenase*

The assay system contained MES buffer (pH 6.5, 0.1 M), sodium pyruvate (0.2 mM), NADH (0.1 mM) and enzyme (0.1 ml of 30 μg/ml solution) in a total vol. of 3 ml. The effector concentration was varied as indicated.

Concn. of effector	Inhibition (−) or activation (+) (%)
3-Phosphoglycerate (2 mM)	+15
α-Glycerophosphate (1 mM)	+7
α-Glycerophosphate (2 mM)	+15
α-Glycerophosphate (10 mM)	+45
α-Glycerophosphate (20 mM)	+22
Phosphoenolpyruvate (3.3 mM)	−33
Fructose diphosphate (0.66 mM)	−5
TES buffer (50 mM)	−10
Oxalate (1 mM)	−40
Oxalate (2 mM)	−56
UTP (0.1 mM)	−4
NAD^+ (0.1 mM)	−12
ADP (0.1 mM)	−50
GTP (0.1 mM)	−64
ATP (0.1 mM)	−86

section, anomalous results were obtained in studies of product inhibition.

Product inhibition

NADH was found to be competitive with respect to NAD^+ in the oxidation of lactate at pH 7.8 (Fig. 2). At pH values above 7.4, NAD^+ was competitive with respect to NADH in the reduction of pyruvate, but as the pH was lowered below 7, linearity in Lineweaver–Burk plots disappeared. A plot of $1/v$ against $1/[NADH]^2$ gave a reasonably good fit to a straight line and a slope of 2 was found when $\log v/(V_{max.}-v)$ was plotted against log[NADH]. It should, however, be noted that the Hill number, nH, defined as the slope of a tangent to the curve at half-maximal velocity (Wyman, 1963), is not 2. A slope of 2 is only observed when $v/V_{max.}-v$ is small (Fig. 3).

Effectors of lactate dehydrogenase

A number of compounds affected the rate of pyruvate reduction (Table 5). The action of ATP on the enzyme has been investigated in more detail. Inhibition by ATP is most pronounced at lower pH values (Fig. 4) and is non-competitive with respect to pyruvate (Fig. 5). When the inhibition by ATP was

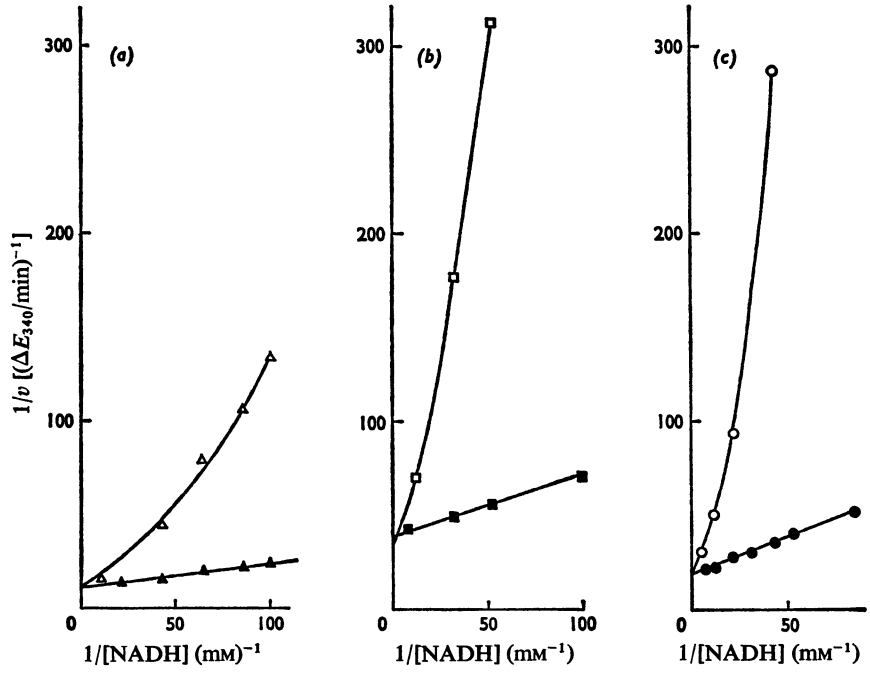

Fig. 4. *Effect of ATP on pyruvate reduction by NADH at three pH values*

Experimental details are given in the text. The concentration of pyruvate used was 0.2mM. Conditions were: △, pH7.1, TES buffer (33mM) and ATP (0.2mM); ▲, pH7.1, control, no ATP; □, pH6.5, TES buffer (33mM) and ATP (0.2mM); ■, pH6.5, control, no ATP; ○, pH6.1, MES buffer (33mM) and ATP (0.1mM); ●, pH6.1, control, no ATP.

examined with respect to various concentrations of NADH, sigmoid kinetics were observed (Fig. 6). Plotting $\log v/(V_{max.}-v)$ against log[NADH] gave a slope of 2 at low concentrations of NADH, but, as noted for NAD^+, the Hill number, nH, as defined by Wyman (1963) is 1. Similarly, a Dixon plot of $1/v$ against [ATP] is curved upwards but a plot of $1/v$ against $[ATP]^2$ is approximately linear (Fig. 7). Assuming that these plots establish competitive inhibition they gave the following K_i values for ATP: at pH7.1, $K_i = 0.06$mM; at pH6.5, $K_i = 0.03$mM; at pH6.1, $K_i = 0.018$mM.

Discussion

Under anaerobic conditions potato tubers first accumulate lactate, but after some days lactate disappears and ethanol accumulates. This situation requires that potato lactate dehydrogenase resembles most animal lactate dehydrogenases in being readily reversible, rather than those bacterial lactate dehydrogenases which are not so readily reversed (Wittenberger, 1968; Tarmy & Kaplan, 1968). The kinetic properties of the preparation reported in the present paper are consistent with its designation as L(+)-lactate dehydrogenase; the preparation is active with L(+)-lactate, but not with D(−)-lactate and at pH7.8 the ratio $V_{max.}$ (lactate oxidation)/$V_{max.}$ (pyruvate reduction) is 0.18. On the other hand, at substrate saturation the preparation shows greater activity with hydroxypyruvate or glyoxylate than with pyruvate [$V_{max.}$ (hydroxypyruvate)/$V_{max.}$ (pyruvate) = 2 and $V_{max.}$ (glyoxylate)/$V_{max.}$ (pyruvate) = 1.5] and it could be argued that hydroxypyruvate is the 'best' substrate. The potato pathogen *Rhizoctonia solani* contains an oxo acid decarboxylase which at substrate saturation decarboxylates pyruvate at 91% of the rate observed with α-oxobutyrate. Quinn & Strobel (1971) suggest that α-oxobutyrate is the 'best' substrate for this decarboxylase, which they designate α-oxobutyrate decarboxylase. Against this viewpoint it should be noted that the K_m for pyruvate is much lower than the K_m for hydroxypyruvate or glyoxylate (Table 2), and if each oxo acid was at a concentration of 0.1mM, the rate of pyruvate reduc-

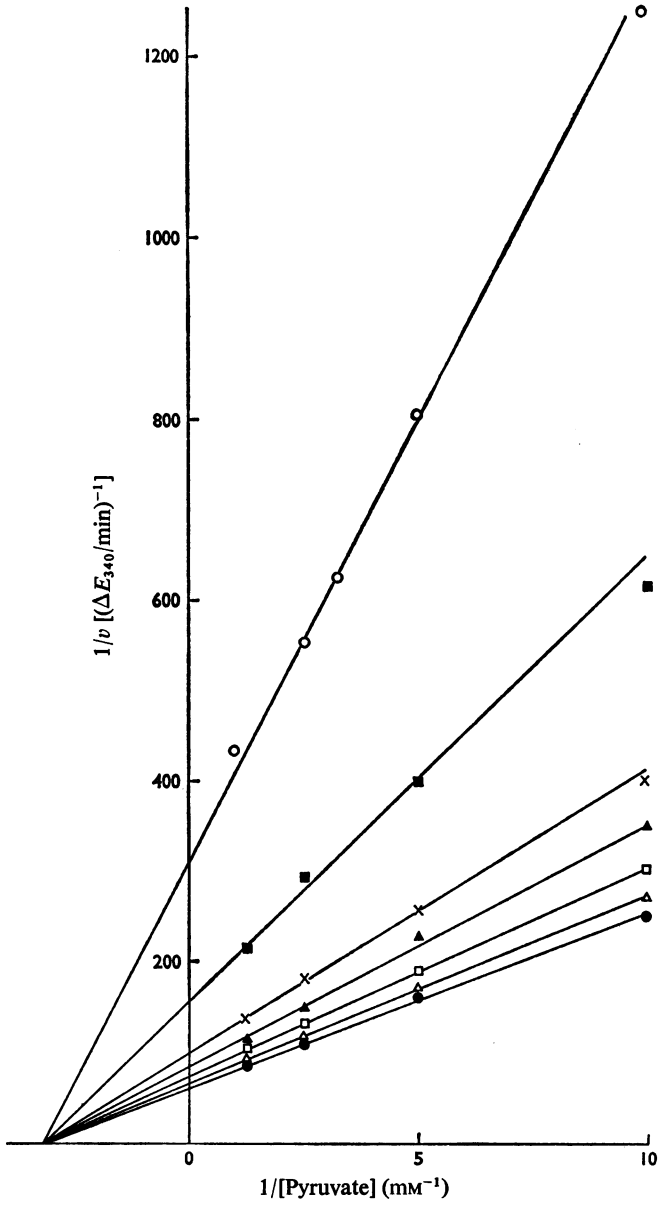

Fig. 5. *Effect of ATP on pyruvate reduction by NADH at pH6.5*

Experimental details are given in the text. TES buffer (33mM) and NADH (0.093mM) were used. Additions were: ○, ATP (0.66mM); ■, ATP (0.33mM); ×, ATP (0.166mM); ▲, ATP (0.11mM); □, ATP (0.055mM); △, ATP (0.22mM); ●, control, no ATP.

tion would greatly exceed the rate of reduction of either hydroxypyruvate or glyoxylate [v(pyruvate): v(hydroxypyruvate): v(glyoxylate) = 55.4:4.5:1]. It is therefore proposed that the physiological function of the α-hydroxy acid dehydrogenase is the interconversion of lactate and pyruvate and that the dehydrogenase is a lactate dehydrogenase (EC 1.1.1.27).

At alkaline pH values the inhibition of pyruvate

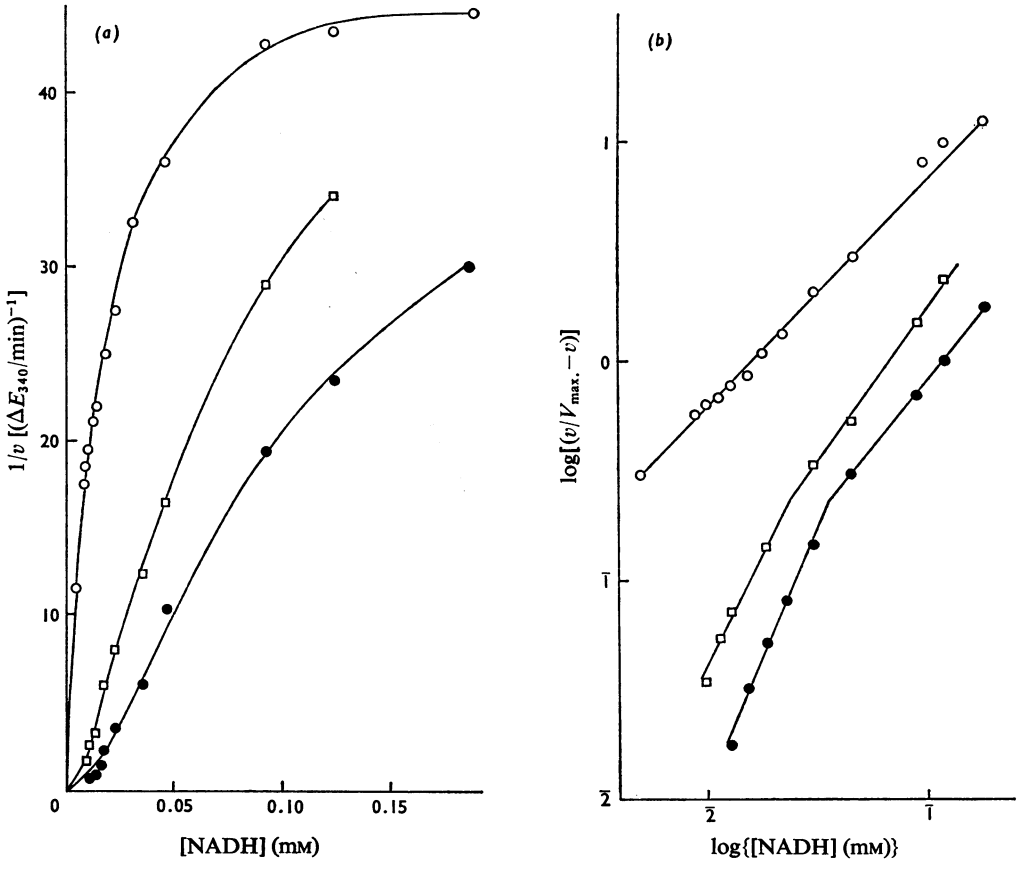

Fig. 6. *Effect of ATP on pyruvate reduction by NADH at pH* 6.1

Experimental details are given in the text. MES buffer (33 mM) and pyruvate (0.2 mM) were used. Additions were: o, control, no ATP; □, ATP (0.05 mM); ●, ATP (1.1 mM).

reduction by ATP and NAD^+ is slight and approximates to competitive inhibition. Under slightly acid conditions the inhibition is strong and co-operative. A possible explanation of the increased effect of ATP at low pH is that ATP^{3-} is effective rather than ATP^{4-}. One possible explanation of the co-operativity is that ATP and NAD^+ compete for the NADH substrate-binding site and in addition there is an allosteric site for ATP which can also be occupied by NAD^+. The affinity of the allosteric site for ATP is increased as the pH is lowered. Another possible explanation suggested to the authors is that the enzyme dissociates at pH values below 7 to an inactive form and that the inhibitors bind preferentially to the dissociated form. The physiological significance of the inhibition is difficult to evaluate, but the following proposal is advanced.

In air the pH of the cytoplasm is assumed to be slightly above pH 7.0. On transferring the potato to nitrogen, lactate is produced, and since ATP is a poor inhibitor the rate of lactate production is high. As fermentation proceeds lactate accumulates, the pH decreases and lactate dehydrogenase responds to the concentration of ATP to control the production of lactate, i.e. the enzyme functions as an ATP-controlled pH-stat. The initial rapid decrease in pH which occurs before lactate dehydrogenase functions as a control enzyme could also have physiological significance. Alcohol production requires the decarboxylation of pyruvate and pyruvate decarboxylase has extremely low activity at neutral or alkaline pH values. Thus pyruvate decarboxylation and alcohol production would be expected to occur after lactate production has occurred, a sequence of events

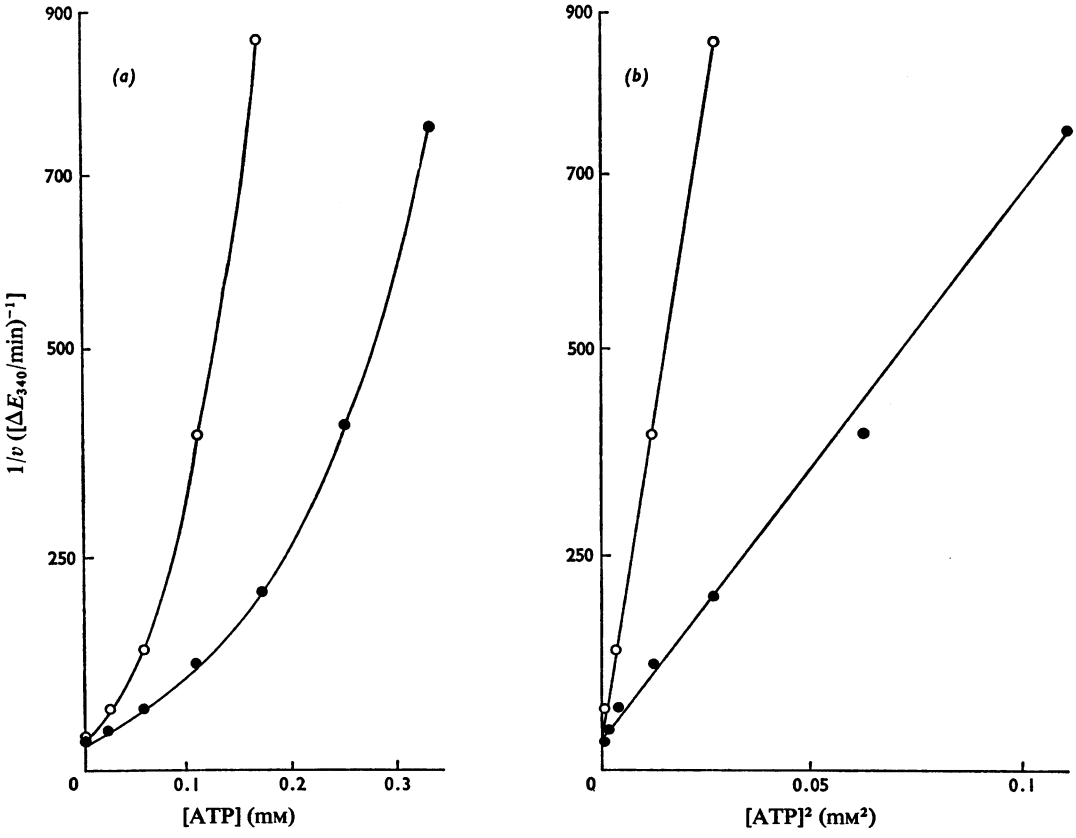

Fig. 7. *Dixon plot of effect of ATP on reduction of pyruvate by NADH at pH* 6.1

Experimental details are given in the text. Pyruvate (0.2mM) and MES buffer (33mM) were used. Additions were: o, NADH (0.07mM); •, NADH (0.014mM).

that has been confirmed by many workers (Forward, 1965).

Much of this work was completed while D. D. D. was a Visiting Professor in the Department of Plant Biochemistry and Biophysics, University of California at Los Angeles. We thank Professor George Laties for his help and hospitality.

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