The Distribution of NAD-independent Lactic Dehydrogenases amongst Micro-organisms

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SUMMARY

Snoswell's (1959) postulate that NAD-independent lactic dehydrogenases are associated with anaerobiosis has been examined by searching for the enzymes in 20 heterotrophic bacteria. The NAD-independent dehydrogenases are widely distributed but are particularly associated with facultative anaerobes.

INTRODUCTION

Snoswell (1959) demonstrated that *Lactobacillus arabinosus* 17.5 possessed four enzymes which catalysed the oxidation of lactate to pyruvate. Two of the enzymes, which catalysed the oxidation of D(-) and L(+) lactate respectively, were NAD-dependent, while two were flavo-protein enzymes and did not require NAD. NAD-independent lactic dehydrogenase activities have now been detected in several micro-organisms including *Escherichia coli* (Kaback & Milner, 1970), *Aerobacter aerogenes* (Pascal & Pichnoty, 1963), *Propionibacterium pentosaceum* (Molinari & Lara, 1960) and *Leuconostoc mesenteroides* (Kauffmann & Dikstein, 1961), and there has been some speculation about their role in metabolism.

Following the observation that NAD-independent lactic dehydrogenases were more active in *Lactobacillus arabinosus* if the culture were grown without aeration, Snoswell (1959) suggested that the enzymes were associated with anaerobiosis. This paper reports a survey designed to test this theory. The distribution of D(-) and L(+) lactic dehydrogenases amongst micro-organisms grown in glucose containing media, some aerobically and some anaerobically, was examined, and the induction of lactic dehydrogenase by *Staphylococcus albus* investigated.

METHODS

Growth of micro-organisms. The organisms investigated (Table 1) were kept at the University of Adelaide, excepting *Lactobacillus casei* and *L. leichmannii* which were a gift from the Department of Animal Nutrition, C.S.I.R.O., Kintore Avenue, Adelaide, Australia. The organisms were grown in glucose nutrient media described in Table 2 and listed in Table 1 against the appropriate organism. Strict anaerobes were grown in deep cultures which contained 0.1% (v/v) thioglycollic acid, and facultative anaerobes were grown in media dispensed in 1-litre lots into 1-litre Florence Flasks. Aerobic bacteria were grown either on agar slopes in 40 oz flat bottles (medium 3, Table 2) or by shaking in 200 ml of liquid medium in 2 litre conical flasks. The bacteria were harvested when good growth had been obtained, usually after 16 h at 37 °C. The exceptions were *Bacteroides* species and

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Table 1. *A list of organisms used in this survey, their mode of growth, and their specific activities* for NAD-independent lactic dehydrogenases*

Organism	Type of growth	Medium	Specific activity with D-, L-lactate pH 6.0	Specific activity with L(+)-isomer, pH 6.0	Specific activity with D(-)-isomer, pH 6.6
<i>Escherichia coli</i>	Anaerobic	1	0.13	0.115	0.015
<i>Aerobacter aerogenes</i>	Anaerobic	1	0.019	0.004	0.011
<i>A. aerogenes</i>	Aerobic	3	0.038	0.006	0.019
<i>Salmonella</i> sp.	Aerobic	3	0.077	0.020	0.026
<i>Shigella</i> sp.	Anaerobic	1	0.019	†	†
<i>Proteus vulgaris</i>	Aerobic	4	0.065	0.035	0.036
<i>Pseudomonas fluorescens</i>	Aerobic	5	Nil	†	†
<i>Leuconostoc mesenteroides</i>	Anaerobic	1	0.039	†	†
<i>L. mesenteroides</i>	Aerobic	3	Nil	†	†
<i>Lactobacillus casei</i>	Anaerobic	1	0.122	0.043	0.030
<i>L. leichmannii</i>	Strict anaerobic	2	0.085	Nil	0.074
<i>Clostridium welchii</i>	Strict anaerobic	2	0.030	0.008	0.023
<i>C. sporogenes</i>	Strict anaerobic	2	Nil	†	†
<i>Bacillus subtilis</i>	Aerobic	1	Nil	†	†
<i>Bacillus</i> sp.	Aerobic	1	Nil	†	†
<i>Bacteroides</i> sp.	Strict anaerobic	2	Nil	†	†
<i>Streptomyces</i> sp.	Aerobic	1	Nil	†	†
<i>Staphylococcus albus</i>	Aerobic	4	0.535	0.510	0.110
<i>Staphylococcus</i> sp.	Aerobic	3	0.052	0.035	Nil
<i>Mycobacterium phlei</i>	Aerobic	6	Nil	†	†
<i>Corynebacterium hofmannii</i>	Aerobic	4	Nil	†	†
<i>Neisseria catarrhalis</i>	Aerobic	4	0.025	0.037	Nil

* Specific activity = rate of reduction of 2,6-dichlorophenol indophenol at 600 nm (in o.d. units) by 1 mg protein in 1 min.

† Not tested.

Table 2. *Constituents of media used to grow the bacteria studied*

The bacteria, and the identity of the medium used to grow each one are listed in Table 1.

All constituents are quoted as percentage (w/v) concentration except where indicated.

Constituents	Medium 1	Medium 2	Medium 3	Medium 4	Medium 5	Medium 6
Casein hydrolysate (enzyme digested)	2	2	2	2	2	2
Sodium citrate	1.5	1.5	0.5	—	0.5	1.5
Sodium acetate	0.5	0.5	0.5	—	0.5	0.5
KH ₂ PO ₄	0.1	0.1	0.1	0.1	0.1	0.1
Mg SO ₄ .H ₂ O	0.1	0.1	0.1	0.1	0.1	0.1
Yeast extract* (% by vol.)	8	8	8	8	8	8
MnCl ₂ .4H ₂ O	0.0005	0.0005	0.0005	—	0.0005	0.0005
Glucose	2	2	1	2	1	2
NaCl	—	—	0.5	1	0.5	—
Sodium glycerophosphate	—	—	—	2	—	—
Thioglycolic acid (by vol.)	—	0.1	—	—	—	—
Tween 80 (by vol.)	—	—	—	—	—	0.05
KNO ₃	—	—	—	—	0.05	—
Agar-agar	—	0.05	2	—	—	—
pH	6.8	7.4	6.8	7.0	6.8	6.8

* Yeast extract was prepared by boiling fresh compressed yeast in an equal volume of water for 3 to 5 min and collecting the clear extract by filtration.

Mycobacterium phlei which were grown for 48 h, the latter at 25 °C, and *Streptomyces* species which was grown for 3 weeks at room temperature (about 22 °C). Medium 4 (Table 2) in which maltose replaced glucose was used to grow *Corynebacterium hoffmannii*. The bacteria were removed from liquid medium by centrifuging at 12 000 g (after filtering through gauze to remove agar from cultures grown in medium 2), or were washed off the agar slopes: in both cases the bacteria were washed twice with distilled water.

Preparation of extracts of the organisms. Four g wet wt of bacteria were suspended in 10 ml of 0.01 M-Na⁺K⁺ phosphate buffer, pH 7.0, and transferred to a prechilled capsule containing 7 g of Ballotini beads. The suspension was shaken for 3 × 20 s bursts on an Ultra rapid shaker (Nossal, 1953), allowing 5 min cooling between bursts. The glass beads and whole bacteria were removed by centrifuging at 18 000 g. Protein concentrations of the extracts were measured by the biuret reaction using bovine serum albumin as standard.

Reagents. NAD, NADH₂, sodium pyruvate, alcohol dehydrogenase (alcohol: NAD oxido-reductase (EC. 1.1.1.1)) were obtained from Sigma Chemical Company, St Louis, Missouri, U.S.A., and 2,6-dichlorophenol-indophenol, acetaldehyde and DL-lithium lactate (laboratory and chemical grade) were the products of British Drug Houses Ltd, Poole, Dorset. D(−)- and L(+)-lactates were purchased as the Ca²⁺ salts from Calbiochem, Los Angeles, California, U.S.A., and were converted to lactic acid immediately before use by passing through the ion exchange resin, Dowex 50; the acid was neutralized with NaOH. NADase (NAD glycohydrolase, EC.3.2.2.5) was prepared from *Neurospora crassa* as described by Kaplan (1955).

Enzyme assays

NAD-independent lactic dehydrogenases were assayed as described by Snoswell (1959) using a tris-maleate buffer, pH 6.0 when either DL-lithium lactate or L(+)-sodium lactate was substrate, and pH 6.6 when the enzyme specific for D(−)-lactate was measured.

Lactate: NAD oxido-reductases (EC. 1.1.1.27 and EC. 1.1.1.28) were assayed by the method of Kornberg (1955). They were also assayed under the conditions of the NAD-independent lactic dehydrogenase: a 3 ml incubation mixture contained 60 μmoles tris-maleate buffer, pH 6.0, 10 μmoles NAD, 20 μmoles DL-lithium lactate and an appropriate amount of enzyme. The rate of the reaction was measured spectrophotometrically at 340 nm.

Diaphorase. Reduced NAD: lipoamide oxido-reductase (EC. 1.6.43) was measured under the conditions of the NAD-independent lactic dehydrogenase assay. The 3 ml reaction mixture consisted of 60 μmoles tris-maleate, pH 6.0, 0.4 μmole NADH₂, 0.12 μmole 2.6-dichlorophenol indophenol, and crude bacterial extract. The reaction was followed spectrophotometrically at 600 nm.

Pyridine nucleotides were estimated fluorometrically by the method of Ciotti & Kaplan (1957).

NAD was removed from bacterial extracts with NADase as follows: 0.2 μmole acetaldehyde and 10 i.u. alcohol dehydrogenase (dissolved in buffer containing 0.1 % bovine serum albumin) was added to 3 ml of bacterial extract in 10 mM-K⁺ phosphate buffer, pH 7.2. The mixture was incubated at 30 °C for 5 min to oxidize endogenous NADH₂, and then 200 u of NADase was added and the extract reincubated for a further 10 min at 37 °C, when the NAD was hydrolysed.

RESULTS AND DISCUSSION

The NAD-independent lactic dehydrogenases which have been examined catalyse the following reaction (where dye = 2,6-dichlorophenol indophenol)

Also present in many bacterial extracts are the two enzymes NAD: lactate oxidoreductase and diaphorase which catalyse

respectively. The net result of reactions (2) and (3) is indistinguishable from reaction (1) in the assay method employed. The initial work was concerned, therefore, with showing that the reaction being measured in all 20 organisms was the NAD-independent lactic dehydrogenase and not a combination of reactions (2) and (3).

Diaphorase was a widespread and very active enzyme which catalysed the oxidation of NADH_2 with concomitant reduction of 2,6-dichlorophenol-indophenol under the conditions of the NAD-independent lactic dehydrogenase assays. However, without exception the lactate:NAD oxido-reductases of bacteria grown in glucose medium had optimal pH values above 7.6 when catalysing the oxidation of lactate. They were inactive at pH 6.6 and pH 6.0 and therefore it is unlikely that reactions (2) and (3) above contribute to reduction of the dye during the assay of NAD independent lactic dehydrogenases.

Further proof that the measured lactic dehydrogenase activity was NAD-independent was gained after treating extracts of the organisms with NADase before the enzymes were assayed: e.g. after treatment of the crude extract of *Proteus vulgaris* with NADase, the pyridine nucleotide concentration was reduced from more than 50 to 2.4 ng/mg protein (which could be NADP^+), but the specific activity of the NAD-independent lactic dehydrogenase remained at 0.20. The *Shigella* species was the only organism of the twelve in which NAD-independent lactic dehydrogenase was detected (Table 1) where the activity disappeared after treatment with NADase.

Table 1 shows that NAD-independent lactic dehydrogenase activity was found in a number of organisms, some of which were capable of oxidizing both L(+)- and D(-)-lactate. It is clear that this enzyme is not necessarily associated with anaerobiosis: of the two *Clostridium* species tested *Clostridium welchii* could oxidize both D(-)- and L(+)-lactate, but *C. sporogenes* could oxidize neither. *Bacteroides* species could not oxidize lactate via the NAD-independent enzyme. However, it does seem to be present in all the facultative anaerobic bacteria tested after growth in deep cultures, and was only lacking in *Leuconostoc mesenteroides* when it was grown aerobically.

Snoswell (1959) suggested that the presence of an NAD-independent lactic dehydrogenase might be advantageous for growth since it would allow lactate, which otherwise accumulates, to be used for synthetic purposes without further utilization of NAD. This hypothesis is supported by the work of Kline & Mahler (1964) and of Kemp (1973) who found that NAD-independent lactic dehydrogenases were induced in *Escherichia coli* and *Pseudomonas aeruginosa* when grown in the presence of DL-, L(+) or D(-)-lactate. Perhaps the enzyme is induced in facultative anaerobes in response to lactate which accumulates during glycolysis. In contradiction to this hypothesis is the fact that NAD-independent lactic dehydrogenase was more active in *Aerobacter aerogenes* grown aerobically than when grown anaerobically (Table 1), a result which agrees with that of Pascal & Pichnoty (1963). This might be

Table 3. *The specific activity of lactic dehydrogenases and NAD concentrations in Staphylococcus albus grown in the presence and absence of lactate*

NAD-independent lactic dehydrogenase was assayed spectrophotometrically in a 3 ml reaction mixture which contained 60 μ moles tris maleate buffer, pH 6.0, 20 μ moles DL-lithium lactate, 0.12 μ mole 2,6-dichlorophenol indophenol and an appropriate amount of enzyme. The reaction was followed at 600 nm. One unit of enzyme activity was defined as that amount causing reduction in optical density of 0.010 in 1 min, and specific activity as units/mg protein. NAD was hydrolysed and measured as described in the text.

	Before treatment with NADase	After treatment with NADase
NAD-independent lactic dehydrogenase in glucose grown bacteria (u)	0.83	0.88
NAD-independent lactic dehydrogenase in lactate grown bacteria (u)	2.73	1.1
NAD concentration in glucose grown bacteria (ng/mg)	112	7.2
NAD concentration in lactate grown bacteria (ng/mg)	200	9.6

explained in our experiments by the fact that the medium supporting aerobic growth had only half the glucose content of that used for anaerobic growth, leading to the rapid exhaustion of this substrate and the consequent necessity to use an alternative carbon source. Lactate may accumulate during aerobic growth either because the organism, which grows very rapidly, uses oxygen more quickly than it can become available, or because the Krebs cycle is not used in some facultative aerobes when glucose is present (Wilson, 1965).

The presence of an NAD-independent lactic dehydrogenase might simply be a protective device for the removal of an acid product of glycolysis.

Not all organisms which can oxidize lactate via an NAD-independent dehydrogenase respond to the presence of lactate in their medium by increasing the specific activity of the enzyme. *Staphylococcus albus*, which was richly endowed with NAD-independent lactic dehydrogenase when grown on a complete glucose medium (Table 1), responded to the presence of lactate (1 % lactate replaced the glucose in medium 4) by an increase in the specific activity of lactate:NAD oxido-reductase, and in NAD concentration (see Table 3). This lactate:NAD oxido-reductase catalysed the oxidation of lactate at pH 6.6. Other strictly aerobic bacteria could not reduce 2,6-dichlorophenol indophenol in the presence of lactate at pH values below 7.

Recently Kaback & Milner (1970) showed that D(–)-lactate dehydrogenase (NAD-independent) was in some way involved with proline uptake into membranes prepared from *Escherichia coli*: the enzyme did not alter the proline *per se*. Many micro-organisms lack NAD-independent lactic dehydrogenase activity (Table 1), so either this is not a universal mechanism for proline uptake, or many organisms synthesize the amino acid even in a complete medium.

We could not demonstrate NAD-independent lactic dehydrogenase activity at pH 6.0 or 6.6 in rat liver, kidney, ovary or red blood cells. Where demonstrated in animal tissues this enzyme has always been measured at pH levels above neutrality (Tubbs & Grenville, 1964): this activity may be associated with non-specific amino acid oxidases.

It has been shown that NAD-independent lactic dehydrogenases are widely distributed amongst bacteria, but are not necessarily associated with either aerobiosis or anaerobiosis. However, they are demonstrable in all the facultative anaerobes tested.

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