Studies of lactate dehydrogenase in the purified state and in intact erythrocytes

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Lactate dehydrogenase in intact erythrocytes was studied by observing isotope exchange between lactate and pyruvate by p.m.r. The inhibition of the enzyme in intact cells by both oxalate and pyruvate was found to be similar to that of the purified enzyme. The activity of the enzyme in intact cells indicates that the free solution $\text{NAD}^+ + \text{NADH}$ concentration in erythrocytes is about $10\text{ }\mu\text{M}$ whereas the total extractable $\text{NAD}^+ + \text{NADH}$ is about 80 nmol/ml of cell water.

In the present paper we describe studies of lactate dehydrogenase in intact erythrocytes and in the purified state. The principle of the method used is similar to that of Silverstein & Boyer (1964), who carried out isotope-equilibration studies on purified lactate dehydrogenase. The method has been described in detail in the preceding paper (Simpson *et al.*, 1982), and involves measuring the rate of equilibration of the methyl groups of pyruvate and lactate.

A simple way to understand the activity of a specific enzyme in intact cells, as measured in a given experiment, is to perform the identical experiment with the purified enzyme and measure the effect of various factors on the enzyme activity. The rate obtained in intact cells may then be correlated with these experiments.

Known effectors of lactate dehydrogenase are lactate, pyruvate, NAD^+ , NADH , pH, buffer composition, ionic strength and inhibitors of the enzyme. Lactate dehydrogenase is not thought to be a membrane-bound enzyme in erythrocytes (Ottaway & Mowbray, 1977; Duchan & Collier, 1971), and thus this possible modulation of enzyme activity in intact cells is not considered. In the present paper the absolute rate of methyl-group equilibration across lactate dehydrogenase in intact human erythrocytes is correlated with the values obtained with the isolated enzyme. The inhibition of the enzyme by oxalate and pyruvate is studied, and the observed effects in the intact cell are compared with those found with the purified enzyme. These effects are discussed in terms of the extrapolation of enzyme kinetic data to the intact cell, particularly with reference to the inhibition of the enzyme by pyruvate.

Experimental

Assays of $\text{NAD}^+ + \text{NADH}$

$\text{NAD}^+ + \text{NADH}$ concentrations were determined by using the cycling method of Nisselbaum & Green (1969) on an extract prepared by heating packed erythrocytes for 5 min in a boiling-water bath and then centrifuging for 5 min in an Eppendorf centrifuge (type 5142).

Alteration of erythrocyte $\text{NAD}^+ + \text{NADH}$ concentrations

Erythrocyte $\text{NAD}^+ + \text{NADH}$ concentrations were elevated by using the method of Tsuboi *et al.* (1966) by incubation at 37°C for 6 h with 4 mM-nicotinic acid, 16 mM-glutamine and 10 mM-glucose in Krebs–Ringer buffer (Krebs & Henseleit, 1932) at 40% haematocrit. The cells were then washed twice in 10 vol. of Krebs–Ringer buffer and depleted of glucose as described in the preceding paper (Simpson *et al.*, 1982). Control erythrocytes were prepared exactly as above except that no glutamine was present in the incubation mixture. Swollen erythrocytes were prepared by washing cells five times with 1 vol. of Krebs–Ringer buffer prepared with 59 mM- NaCl (half the usual concentration of NaCl) in $^2\text{H}_2\text{O}$ and suspending them in this buffer. The resulting erythrocytes were found, by measuring the haemoglobin content versus haematocrit, to be swollen by a factor of 1.4. The methyl-group equilibration rate constants for these and untreated erythrocytes were determined at low haematocrit (12%) in the appropriate buffer made with $^2\text{H}_2\text{O}$.

General methods

The sources of materials and most of the methods were as described in the preceding paper (Simpson *et al.*, 1982). The lactate dehydrogenase, as supplied, yielded zero equilibrium velocities in the absence of added NAD^+ . When high concentrations of the enzyme were required, the suspension in $(\text{NH}_4)_2\text{SO}_4$ solution was dialysed twice against 20 vol. of 0.1 M-imidazole buffer, pH*7.4, made with $^2\text{H}_2\text{O}$, before use. Data processing was as described previously, except that, in the experiment to study the effect of varying the concentration of lactate and pyruvate, the initial velocity of exchange of lactate protons was determined from the lactate-peak decay time course. This was done by fitting a quadratic equation to the points (by using routines available on the Nicolet 1180 computer NTCFT software) and taking the first derivative at $t = 0$ of this equation. Concentration refers to free solution concentration. This was calculated by assuming a solution volume of 0.72 ml/ml of cells (Eilam & Stein, 1974). All experiments were performed at 37°C .

Results and discussion

In these experiments lactate, pyruvate, NAD^+ and NADH are at equilibrium and this situation is maintained during the time course by the methods described in the preceding paper (Simpson *et al.*, 1982). Lactate and pyruvate are added in large excess over NAD^+ , NADH and endogenous lactate and pyruvate. The only unknown substrate variable is the sum of the NAD^+ and NADH concentrations. The dependence of the methyl-group equilibration rate constant of human erythrocyte lactate dehydrogenase on NAD^+ + NADH concentration in 0.1 M-imidazole buffer, pH*7.45, is shown in Fig. 1. The experimental points lie approximately on a hyperbola characterized by a ' K_m ' of about $4\ \mu\text{M}$. It is possible to derive an equation describing the methyl-group equilibration rate across lactate dehydrogenase (Silverstein & Boyer, 1964; Yagil & Hoberman, 1969). We may write the mechanism thus (Borgmann *et al.*, 1974):

The corresponding expression for V_e , the equilibrium velocity from lactate to pyruvate, is:

$$V_e = \frac{[\text{E}]_t}{k_{+3} + k_{-2} \cdot \left[\frac{k_{+4}}{k_{-4} \cdot [\text{R}] \cdot [\text{P}]} + \frac{1}{[\text{P}]} + \frac{k_{-3}}{k_{+3}} + \frac{k_{-3} \cdot k_{-2}}{k_{+3} \cdot k_{+2} \cdot [\text{L}]} \right]} \quad (1)$$

Fig. 1. Dependence of the specific methyl-group equilibration rate constant (k_{12}/v) of human erythrocyte lactate dehydrogenase on NAD^+ + NADH concentration at 10 mM-lactate and -pyruvate

Human erythrocyte lactate dehydrogenase (0.8 unit) in 0.5 ml of 0.1 M-imidazole buffer, made with $^2\text{H}_2\text{O}$, pH*7.39 $\pm$ 0.02, the appropriate concentration of NAD^+ and 10 mM-[U- ^3H]lactate were preincubated at 37°C in the p.m.r. spectrometer, and at $t = 0$ 10 mM-[^3H]pyruvate was added. The value of k_{12} and the lactate dehydrogenase activity (v) were determined as described in the preceding paper (Simpson *et al.*, 1982).

where the k_i terms are defined above, R and O are the reduced and oxidized forms of NAD^+ respectively, L and P are lactate and pyruvate respectively and $[\text{E}]_t$ is the total enzyme concentration.

Human erythrocyte lactate dehydrogenase is similar to the bovine heart enzyme in that it has the same mechanism and its affinities for substrates are of the same order of magnitude [Wang (1977) and Nisselbaum & Bodansky (1963) compared with Borgmann *et al.* (1974)]. The individual rate constants for the bovine heart enzyme may be used in eqn. (1) to model the NAD^+ + NADH -dependence of the enzyme. If this is done, a hyperbola, characterized by a ' K_m ' of about $190\ \mu\text{M}$ for NAD^+ + NADH , is obtained. The observed experi-

mental curve might result from a combination of this model curve with an NAD^+ -dependent inhibition

curve, since the experiments are performed at an inhibitory pyruvate concentration. The mechanism of this pyruvate inhibition is twofold (Burgenor *et al.*, 1978). Part of the inhibition is caused by a ternary complex of enzyme, pyruvate and NAD^+ and part by a pyruvate– NAD adduct. The shape of the curve in Fig. 1 (apparent ' K_m ' approx. $4\mu\text{M}$) can be obtained by assuming that there is a competitive inhibition by the pyruvate– NAD adduct that has an NAD^+ -dependence described by a K_i of about $4\mu\text{M}$ (see Trommer *et al.*, 1979). It is noteworthy that Lowry *et al.* (1961) observed an anomalously low ' K_m ' for $\text{NAD}^+ + \text{NADH}$ when studying the $\text{NAD}^+ + \text{NADH}$ -dependence of enzymic cycling with lactate dehydrogenase.

The pH^* -dependence of the methyl-group equilibration rate in 0.1 M-imidazole buffer was studied. It was found to be approximately horizontal over the pH^* range encountered in the experiments with intact erythrocytes [$\text{pH}^* 7.3\text{--}7.5$, monitored by the histidine resonances of haemoglobin (Brown *et al.*, 1977)].

Lactate dehydrogenase activity has been reported to show a dependence on buffer (Winer & Schwert, 1958), halide ion concentration (Anderson, 1981) and ionic strength (Silverstein & Boyer, 1964; Rivedal & Sanner, 1979). The ionic-strength effect seems to be small for the equilibration reaction, however (Silverstein & Boyer, 1964), and the buffer effect also appears to be small for buffers resembling the intracellular milieu. Imidazole buffer (0.1 M) was selected as a suitable buffer for the studies with the purified enzyme because the principal buffer system within erythrocytes is supplied by the histidine residues of haemoglobin. As shown in Table 1, there is no significant difference between the methyl-group equilibration rates in 0.1 M-imidazole, 0.1 M-imidazole/0.12 M-KCl and Krebs–Ringer buffers at $100\mu\text{M-NAD}^+ + \text{NADH}$. Phosphate has been reported to affect the rate constants for lactate dehydrogenase, so the $\text{NAD}^+ + \text{NADH}$ -dependence of the equilibration rate in 0.1 M-imidazole/0.12 M-KCl/1 mM- K_2HPO_4 buffer was determined. There was no significant effect on the curve (see also Fig. 4).

The inhibition, by oxalate, of purified human erythrocyte lactate dehydrogenase in imidazole buffer and in intact erythrocytes was investigated, and the results are shown in Fig. 2. Oxalate, like pyruvate and lactate, permeates erythrocytes very rapidly at 37°C (Halestrap, 1976; Brindle *et al.*, 1979; Hubbard *et al.*, 1980) and competes with lactate for lactate dehydrogenase (Novoa *et al.*, 1959). Lactate is not present at saturating concentrations in these experiments, and hence oxalate shows a fairly potent inhibition in the equilibration experiment. There is a small $\text{NAD}^+ + \text{NADH}$ -dependence of the inhibition, but the spread of inhibition values over the $\text{NAD}^+ + \text{NADH}$ concentration range $5\text{--}60\mu\text{M}$ at 2 mM-oxalate is within the error bar indicated in Fig. 2. It may be concluded that oxalate inhibition of the enzyme is the same in the intact cell and in the purified state, the apparent inhibition constant being about 0.9 mM.

The inhibition of H-type lactate dehydrogenase by millimolar concentrations of pyruvate has been well studied (Everse & Kaplan, 1973), and from this work one would expect the velocity of the human erythrocyte enzyme (80% H-type; Everse & Kaplan, 1975) to be higher at 2 mM- than at 10 mM-pyruvate (see Latner *et al.*, 1966). It is impossible to vary the concentration of a single substrate in isolation in an equilibrium isotope-exchange experiment. Lactate and pyruvate concentrations, however, may be varied at constant ratio without affecting NAD^+ and NADH concentrations. If the initial velocity of methyl-group equilibration is considered, two competing effects occur as the lactate and pyruvate concentration increases from 2 to 10 mM, namely (1) the velocity of exchange increases because the lactate is not saturating and (2) the velocity decreases as the pyruvate inhibition increases. The ratio of initial rate of loss of protons from lactate at two concentrations of pyruvate and lactate (2 mM and 10 mM) was measured for the purified enzyme at two different $\text{NAD}^+ + \text{NADH}$ concentrations and in whole cells (see Table 2). As can be seen, the initial rate of equilibration increases by about 3-fold both *in vitro* and *in situ*. It is

Table 1. *Specific methyl-group equilibration rates for human erythrocyte lactate dehydrogenase*

These rates were determined in 0.5 ml of the buffer containing (*v*) units of lactate dehydrogenase, 10 mM- $[\text{}^2\text{H}_3]$ -pyruvate, $[\text{U-}^1\text{H}]$ lactate and $100\mu\text{M-NAD}^+$. The pH^* was 7.4 ± 0.1 . Buffers were prepared with $^2\text{H}_2\text{O}$. Values are given as means $\pm$ S.E.M.

Sample	$10^{-3} \times$ Specific methyl-group equilibration rate constant (k_{12}/v) (μmol^{-1})
(a) 0.1 M-Imidazole buffer	12.5 ± 1.0
(b) 0.1 M-Imidazole/0.12 M-KCl buffer	11.4 ± 1.0
(c) Krebs–Ringer buffer	13.4 ± 1.0

possible to calculate [by using eqn. (1) above] that the ratio would be 4.9 in the absence of the pyruvate inhibitory effect (at $5\mu\text{M-NAD}^+ + \text{NADH}$). There thus appears to be significant yet similar inhibition by pyruvate in intact erythrocytes and in the purified enzyme.

This result is in agreement with the findings obtained by Everse & Kaplan (1973) with chicken

Fig. 2. Inhibition of human erythrocyte lactate dehydrogenase by oxalate in 0.1M-imidazole buffer, $\text{pH}^* 7.4 \pm 0.1$, and in intact erythrocytes in Krebs-Ringer buffer

The haematocrit in the experiments with intact cells ($\circ$ and $\bullet$) was 80%, and the oxalate, pyruvate and lactate additions were corrected for the loss of solution volume due to haemoglobin. The experiments with pure enzyme were performed as described in Fig. 1 legend with $40\mu\text{M-}$ (Δ) and $100\mu\text{M-}$ ($\blacktriangle$) $\text{NAD}^+ + \text{NADH}$. The symbol (plus error bars) at 2 mM-oxalate covers the values obtained at $\text{NAD}^+ + \text{NADH}$ concentrations in the range $5\text{--}60\mu\text{M}$. All buffers were made with $^2\text{H}_2\text{O}$. The line is:

$$(\% \text{ inhibition}) = \frac{100 [\text{oxalate}]}{(K_{i(\text{app.})} + [\text{oxalate}]})$$

with the apparent $K_i = 0.9\text{ mM}$.

heart but not with those reported by Burgenor *et al.* (1978) with rat heart. The method used by these groups to observe inhibition of lactate dehydrogenase in intact tissue was to detect the enzyme·pyruvate- NAD^+ complex in tissue extracts. Burgenor *et al.* (1978) could not detect such a complex in heart tissue perfused with 10 mM-pyruvate. As is demonstrated in Fig. 3, however, the complex can be observed in erythrocytes. Fig. 3 shows spectrophotometric lactate dehydrogenase assay time courses for portions of samples treated at 37°C for 45 min with 10 mM-pyruvate and 10 mM-lactate. A lag phase in these assays is noted if pyruvate (to start the assay) and the portion of enzyme (or cells) are added to the NADH simultaneously (this lag can still be discerned when whole cells are added, although it is somewhat obscured by the time taken for the clarification that occurs as the cells lyse in the assay buffer). If the assay mixture plus enzyme are preincubated at 37°C for 4 min before addition of the pyruvate to start the assay, then the reaction proceeds linearly. The lag phase is due to the slow dissociation of the enzyme·pyruvate-NAD complex.

The absolute rate constant of methyl-group equilibration across lactate dehydrogenase in intact human erythrocytes was measured from ten experiments, with blood from six individuals, to be $0.0082 \pm 0.0022 (\text{mean} \pm \text{s.d.}) \text{min}^{-1}$ per unit of lactate dehydrogenase per 0.5 ml sample at 10 mM-lactate and 10 mM-pyruvate. This corresponds to the rate obtained with the pure enzyme at roughly $10\mu\text{M-NAD}^+ + \text{NADH}$ (Fig. 1). This value for the free solution $\text{NAD}^+ + \text{NADH}$ concentration in erythrocytes is considerably less than all the reported values, after correction for the excluded haemoglobin volume (Table 3 in Hasart *et al.*, 1972; Steinbrecht & Kunz, 1972; Marshall & Omachi, 1974). Three explanations for this decrease in lactate dehydrogenase activity may be considered.

It is possible that the free solution concentration

Table 2. Ratio of the initial velocity of equilibration from lactate to pyruvate (V_e^0) catalysed by lactate dehydrogenase at 10 mM-lactate and 10 mM-pyruvate to V_e^0 at 2 mM-lactate and 2 mM-pyruvate in intact erythrocytes and with purified lactate dehydrogenase

The measurements with whole cells were made at low haematocrit (less than 10%) in Krebs-Ringer buffer made with $^2\text{H}_2\text{O}$. The measurements with pure enzyme were made in 0.1M-imidazole buffer made with $^2\text{H}_2\text{O}$ ($\text{pH}^* 7.4$), with the specified $\text{NAD}^+ + \text{NADH}$ concentration. Values are given as means $\pm$ S.E.M.

Sample	V_e^0 at 10 mM-lactate and 10 mM-pyruvate
	V_e^0 at 2 mM-lactate and 2 mM-pyruvate
Pure enzyme with $5\mu\text{M-NAD}^+ + \text{NADH}$	2.9 ± 0.2
Pure enzyme with $50\mu\text{M-NAD}^+ + \text{NADH}$	3.0 ± 0.2
Whole cells	3.1 ± 0.2

Fig. 3. Illustration of lactate dehydrogenase-pyruvate-NAD inhibitor formation in intact erythrocytes and with the purified enzyme

Time courses from spectrophotometric lactate dehydrogenase assays of two samples used in methyl-group equilibration experiments are shown. The two 0.5 ml samples were incubated for 45 min with 10 mM-lactate and 10 mM-pyruvate at 37°C for the determination of the methyl-group equilibration rate. The first sample contained 2.4 units of human erythrocyte lactate dehydrogenase and 100 μ M-NAD⁺ + NADH. At $t = 0$ in the enzyme assay, 2 μ l of this sample was added to the NADH/buffer assay mixture (time courses A and B). In A, the enzyme assay was started at $t = 0$ by the simultaneous addition of 1 mM-pyruvate. The broken line is an extrapolation of the linear portion of the curve. The horizontal portion (0–1 min) is a spectrophotometer artifact. In B, the mixture plus sample were incubated for 4 min before the addition of the pyruvate (arrow). The slow loss of absorbance during the 4 min incubation is due to the 20 nmol of pyruvate carried over to the assay in the sample. Time courses C, D and E show analogous assays performed on the second sample. This sample contained intact erythrocytes (haematocrit 9%) in Krebs-Ringer buffer. In C, 2 μ l of this was added directly to the assay mixture along with 1 mM-pyruvate at $t = 0$. For D and E, the erythrocyte sample was lysed by freezing and thawing and used to obtain these time courses in a manner identical with A and B respectively.

Table 3. Effect of changing NAD⁺ + NADH concentrations in erythrocytes on the activity of lactate dehydrogenase
The lactate dehydrogenase activity was determined by preparing glucose-depleted cells from the treated samples and determining the specific methyl-group equilibration rate in duplicate at low haematocrit (approx. 12%) in Krebs-Ringer buffer. The NAD⁺ + NADH assays were performed on the stock, glucose-depleted, packed cells (see the Experimental section). The methyl-group equilibration rates are given as means $\pm$ S.E.M.

Sample treatment	Assayed concn. of NAD ⁺ + NADH expressed as free concentration in cell water (μ M)	Specific methyl-group equilibration rate (% of untreated control)
(a) Untreated cells	92	100 $\pm$ 4
(b) Cells incubated to elevate NAD ⁺ + NADH concentrations (see the Experimental section)	187	117 $\pm$ 4
(c) Cells incubated as (b) but without glutamine	115	103 $\pm$ 4
(d) Swollen cells (see the Experimental section)	83*	86 $\pm$ 6

* Calculated from (a) and the measured degree of swelling (see the Experimental section).

of NAD⁺ + NADH is in the plateau region of Fig. 1 in erythrocytes but that some inhibition lowers the observed rate of the reaction catalysed by lactate dehydrogenase to the value reported above. Such inhibition could result from the formation of compounds such as NADH-X from NADH in the intact cell (Oppenheimer & Kaplan, 1974; Yoshida, 1977) or from some other inhibition (see, for example, Duffy & Sanderson, 1971). Secondly,

some modulator of lactate dehydrogenase may change the affinity of the enzyme for NAD⁺ + NADH. The third possibility is that the free NAD⁺ + NADH concentration is less than the total assayed in normal extracts of erythrocytes. Solti & Friedrich (1979) demonstrated that the NAD⁺ + NADH in concentrated erythrocyte lysates could be divided into 'pools' as judged by accessibility to an NAD⁺ nucleosidase (EC 3.2.2.5) located on the

exterior surface of the cell membrane. Their results imply that less than 50% of the extractable NAD^+ + NADH in erythrocytes is in free solution. These findings make it feasible that the NAD^+ + NADH concentration is in fact the rate-determining factor in the intact cell. In order to test this, cells were prepared with elevated NAD^+ + NADH concentrations by the method of Tsuboi *et al.* (1966) (see the Experimental section). As shown in Table 3, the rate of the reaction catalysed by lactate dehydrogenase can be raised and the increase correlates with an increase in the NAD^+ + NADH concentration in the cells. In another experiment it was observed that swelling the cells, and thus diluting the free NAD^+ + NADH within, produces a decrease in the lactate dehydrogenase equilibration rate (Table 3). These results are difficult to explain if the NAD^+ + NADH concentration is in the plateau region of Fig. 1 and an inhibitor is present.

The NAD^+ + NADH -dependence in concentrated erythrocyte lysates was examined by using packed cells that had been lysed by repeated freezing and thawing (Simpson *et al.*, 1982) and incubated at 37°C for 45 min to destroy the free NAD^+ + NADH by contact with the cell-surface NAD^+ nucleosidase. The NAD^+ nucleosidase was inhibited by the addition of 40 mM-nicotinamide (Alivisatos & Denstedt, 1951) during the methyl-group equilibration experiment. These lysates yield zero methyl-group equilibration rates, indicating that no free NAD^+ + NADH is available to lactate dehydrogenase. It is still possible, however, to measure up to 5 μM NAD^+ + NADH in boiled extracts of these lysates. It is possible to calculate, by using data from Solti & Friedrich (1979), that the binding constant for this tightly bound NAD^+ + NADH must be of the order of 10^{-8}M . This is an extremely tight binding, and it may correspond to a single tightly bound NAD^+ molecule on glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12) (Bell & Dalziel, 1975), since the concentration of glyceraldehyde 3-phosphate dehydrogenase tetramers in erythrocytes is about 5 nmol/ml of cell water (Marshall & Omachi, 1974). The response of the methyl-group equilibration rate to added NAD^+ in these depleted lysates was examined in the presence of oxalate in order to lower the rates to values that could be accurately determined without having to dilute the sample (see Simpson *et al.*, 1982). As shown in Fig. 4, this response is different from that of the pure enzyme in a model buffer. It should be emphasized that the two curves in Fig. 4 are highly reproducible.

In the experiment with purified enzyme the enzyme concentration was approximately one-fifth of that in the lysate experiment, in which there are about 4 μM active sites (Marshall & Omachi, 1974). To test whether these concentration differences were significant, theoretical simulations with a model

system characterized by a ' K_m ' of 190 μM and a competitive substrate inhibition with a K_i of 4 μM (see above) were performed. These calculations indicated that there was no significant effect of enzyme concentration in the relevant range (1–5 μM) on the substrate-versus-rate curve. This was confirmed by experiments that showed that the NAD^+ + NADH -dependence at a lactate dehydrogenase concentration equal to that in intact erythrocytes is indistinguishable from that at a 5-fold lower concentration of enzyme.

The observed difference in NAD^+ + NADH -dependence between lysates and pure enzyme, shown in Fig. 4, would be expected if the added NAD^+ + NADH partitions between free and other bound pools not accessible to lactate dehydrogenase. A crude Scatchard-type analysis can be performed by using the curves shown in Fig. 4. Curve *B* may be used to measure the free NAD^+ + NADH concentration at a given total concentration of added NAD^+ + NADH in the lysate experiment (curve *A*). The result is consistent with there being 10–15 μM of binding sites with a K_d of about 4 μM . A likely candidate for the bound pool is glyceraldehyde 3-phosphate dehydrogenase, for which the concentration of NAD^+ -binding sites of appropriate affinity in erythrocytes may be about 10–15 μM . Other possibilities are various other dehydrogenases, for example malate dehydrogenase (EC 1.1.1.37), and reductases, for example methaemoglobin reductase (EC 1.6.99.3).

The rate of equilibration in intact cells obtained from the same stock sample as in Fig. 4 before lysis

Fig. 4. NAD^+ + NADH -dependence of human erythrocyte lactate dehydrogenase in concentrated erythrocyte lysates and a model buffer

In *A* glucose-depleted erythrocytes (haematocrit 89%) were depleted of NAD^+ + NADH by incubation at 37°C (in the presence of 5 mM-NaF; Simpson *et al.*, 1982) for 45 min before the methyl-group equilibration (V_e) determination. In *B* the buffer was 0.1 M-imidazole, $\text{pH}^* 7.4 \pm 0.02$, containing 0.12 M-KCl and 1 mM- K_2HPO_4 . In both *A* and *B* 3.9 mM-oxalate and 40 mM-nicotinamide were present during the isotope-equilibration experiment.

was determined in the presence of the same concentration of oxalate. This rate was found to be 85% of the plateau rate obtained in the lysate.

The results presented cannot eliminate the possibility that some unknown factor modulates the affinity of lactate dehydrogenase for NAD^+ + NADH . It seems much more likely, however, in the light of the evidence presented above, that the free NAD^+ + NADH concentration is lowered by binding to proteins and thus becomes the rate-determining factor in the intact cell.

Conclusions

(1) The inhibition of lactate dehydrogenase in intact erythrocytes by oxalate and pyruvate is similar to that of the purified enzyme in imidazole buffer, $\text{pH}^* 7.4$.

(2) The activity of lactate dehydrogenase in intact erythrocytes is limited by the free NAD^+ + NADH concentration, which is about 10% of the total extractable NAD^+ + NADH .

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