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Malate Dehydrogenase of the Cytosol

PREPARATION AND REDUCED NICOTINAMIDE-ADENINE DINUCLEOTIDE-BINDING STUDIES

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1. Two methods of preparing pig heart soluble malate dehydrogenase are described. A slow method yields an enzyme composed of three electrophoretically separable subforms. The more rapid method reproducibly gives a high yield of an enzyme that consists predominantly of the least acid subform. 2. The $A^{1\%}_{1cm}$ of the protein was redetermined as 15 at 280nm. By using this value the enzyme molecule was found to contain two independent and indistinguishable NADH-binding sites in titrations with NADH. 3. No evidence was found for the dissociation of the enzyme in the concentration range 0.02–7.2μM. 4. L-Malate (0.1M) tightened the binding of NADH to both pig and ox heart enzyme (2-fold), but, in contrast with the report by Mueggler, Dahlquist & Wolfe [(1975) *Biochemistry* **14**, 3490–3497], did not cause co-operative interactions between the binding sites. 5. Fructose 1,6-bisphosphate had no effect on the binding of NADH to the pig heart enzyme, but with the ox heart enzyme the NADH is slowly oxidized. This slow oxidation explains the 'sigmoidal' binding curves obtained when NADH was added to ox heart soluble malate dehydrogenase in the presence of fructose 1,6-bisphosphate [Cassman (1973) *Biochem. Biophys. Res. Commun.* **53**, 666–672] without the postulate of site–site interactions. 6. It is concluded that neither L-malate nor fructose 1,6-bisphosphate could *in vivo* modulate the activity of soluble malate dehydrogenase and alter the rates of transport of NADH between the cytosol and the mitochondrion. 7. Details of the preparation of soluble malate dehydrogenase have been deposited as Supplementary Publication SUP 50080 (8 pages) at the British Library Lending Division, Boston Spa, Wetherby, West Yorkshire LS23 7BQ, U.K., from whom copies may be obtained under the terms given in *Biochem. J.* (1978) **169**, 5.

The malate dehydrogenase present in the soluble fraction of pig heart extracts has been shown by an X-ray-diffraction study to be a dimer of two identical subunits (Hill *et al.*, 1972). It is thus surprising that there is conflict in the literature as to the number and the natures of the NADH-binding sites in the dimeric mole. Two sites per molecule have been reported by Thorne & Kaplan (1963), Pfleiderer & Auricchio (1964) and Holbrook & Wolfe (1972). Cassman & King (1972) have shown that for ox heart soluble malate dehydrogenase the nature of NADH binding varies with the protein concentration and that at high protein concentrations there is slight positive co-operativity in NADH binding. This was ascribed to a monomer–dimer equilibrium in which only the dimer binds NADH. Koren & Hammes (1975) have also proposed that the enzyme exists in a monomer–dimer system in which NADH binds to both the monomer and dimer with equal affinity, but with differing NADH fluorescence enhancements. This is in contrast with Holbrook & Wolfe (1972), who found no evidence for distinguishable NADH-binding sites.

It has been suggested that soluble malate dehydrogenase is a regulatory enzyme in the transport of reducing equivalents between the cytosol and the mitochondrion. Cassman (1973) reported that L-malate alters the binding constant of NADH to the ox heart soluble enzyme, but has no effect on the reported co-operativity, and fructose 1,6-bisphosphate was found to inhibit NADH binding at low enzyme concentrations. Cassman & Vetterlein (1974) report the isolation of two forms of the ox heart enzyme (s-MDH_a and s-MDH_b); the forms of the enzyme have identical amino acid composition and molecular weights. Mueggler *et al.* (1975) report that negative co-operative interactions between the NADH-binding sites of the pig heart soluble enzyme

Abbreviation used: SDS, sodium dodecyl sulphate.

are induced by L-malate, and that at saturating concentrations of NADH L-malate binds with negative co-operativity.

Both the soluble and mitochondrial malate dehydrogenase are considered to be involved in the transport of reducing equivalents across the mitochondrial membrane (see, e.g., Williamson *et al.*, 1973). If the properties of the soluble enzyme were altered by L-malate and/or fructose 1,6-bisphosphate this would have important regulatory consequences and provide a means of linking cellular energy states to the transport of NADH across the mitochondrial membrane.

To resolve these uncertainties the binding of NADH to the enzyme was re-examined. As a preliminary, the reproducibility of the enzyme preparation was investigated and the $A^{1\%}_{1cm}$ for the enzyme was redetermined.

Methods and Materials

NADH (grade II, disodium salt) was purchased from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany, and was purified by chromatography (Holbrook & Wolfe, 1972). Microgranular ion-exchange celluloses were obtained from H. Reeve Angel and Co., London E.C.4, U.K. Sephadex G-100 was purchased from Pharmacia, Uppsala, Sweden. All other chemicals were purchased from either Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K., or British Drug Houses, Poole, Dorset, U.K.

Enzyme assay

The assay medium contained 0.12M-L-malate, 2mM-NAD^+ and 0.1M-glycine adjusted to pH10.0 with 5M-NaOH. Assays were at 25°C and the reaction was monitored by the change in A_{340}. One unit of enzyme activity caused the production of 1μmol of NADH/min.

Protein assay

Protein concentration of the pure enzyme was calculated by using $A^{1\%}_{1cm} = 15$ at 280nm (this value was determined as described below). Molar enzyme concentrations are calculated from w/v protein concentrations assuming a molecular weight of 70000 for the dimer.

Enzyme preparation

Two methods (1 and 2) were used to prepare the soluble enzyme from pig hearts. Both are modifications of the procedure of Gerding & Wolfe (1969) and are shown in Scheme 1. Method 2 was used to prepare the enzyme from ox heart. Details of the procedure have been deposited as Supplementary Publication SUP 50080 at the British Library Lending Division, Boston Spa, Wetherby, West Yorkshire LS23 7BQ, U.K.

Polyacrylamide-gel electrophoresis

Tubular gels containing 8% (w/v) polyacrylamide were used, either with or without SDS (Tanner & Boxer, 1972). When no SDS was used the samples were loaded in 50% (w/v) sucrose and run at 3mA per gel for 12–24h at room temperature. Protein was detected as described by Berg (1969) by using Coomassie Brilliant Blue. Enzyme activity was revealed by using 1.1mM-NAD^+, 0.5mM-Nitro Blue Tetrazolium and 0.07mM-phenazine methosulphate in 20mM-L-malate, adjusted to pH8.0 with 0.5M-Tris base (this is a modification of the stain used by Thorne *et al.*, 1963).

Isoelectric focusing

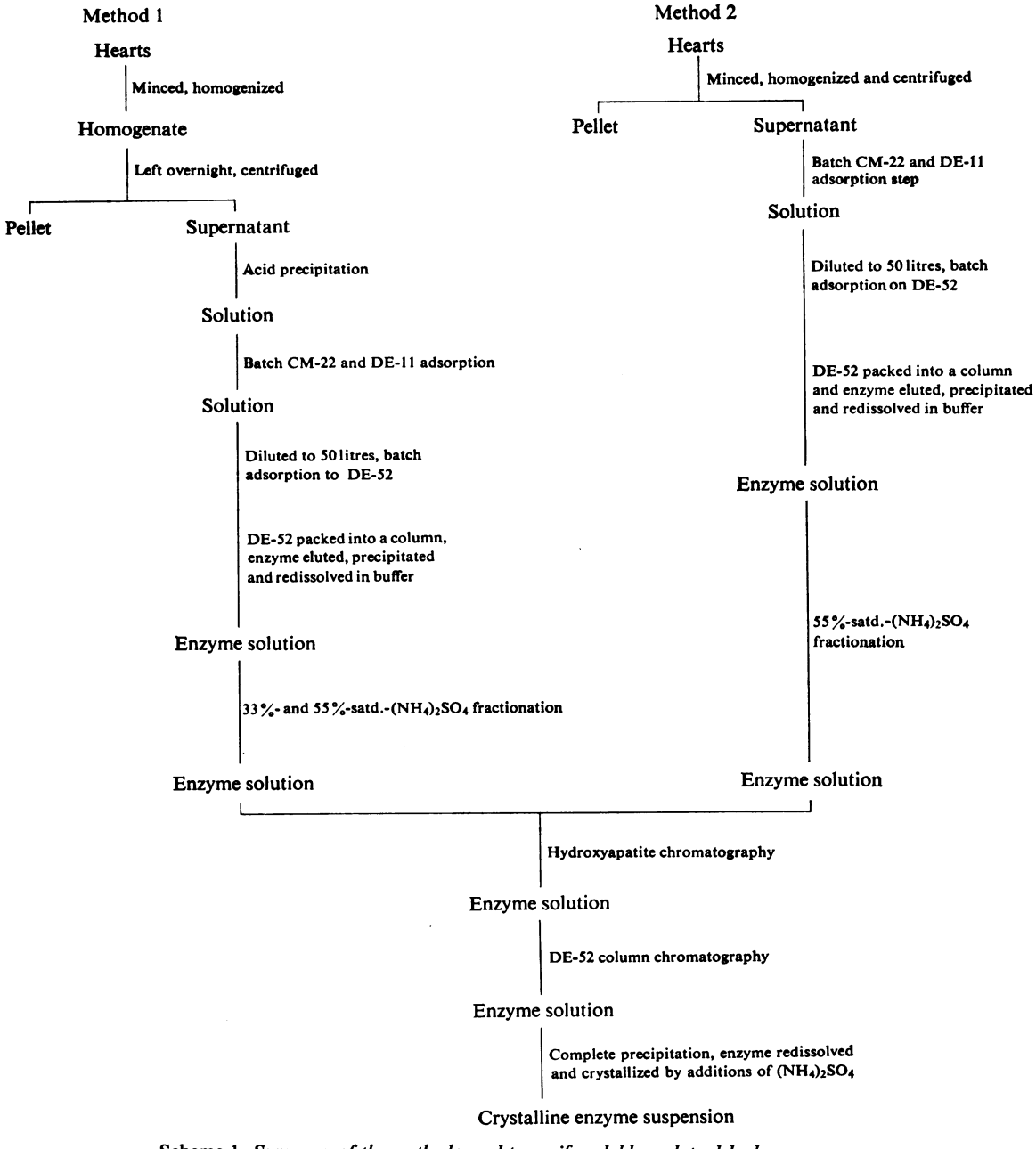
This was done in an LKB (Croydon, Surrey, U.K.) Ampholine PAG plate gel, pH range 3.5–9.5, run according to the manufacturer's instructions. Samples were applied in the centre of the gel. Gels were stained for protein as described by the manufacturer or for enzyme activity as described above.

Gel filtration

A water-jacketed column (2cm×100cm), kept at 13°C and packed with Sephadex G-100, was used to measure the elution volume and to estimate the molecular weight of the enzymes. The column buffer was 50mM-Tris/acetate, pH8.0, or 10mM-sodium phosphate, pH6.0, and the flow rate was maintained at 10ml/h. Samples were applied in 2ml of column buffer. The elution volume was the volume of eluate collected from the point when the sample had half-penetrated the gel. Column fractions were assayed for malate dehydrogenase activity and their A_{280} was measured. The elution volume of soluble enzyme activity was compared with the elution volumes of bovine serum albumin (mol.wt. 67000), myoglobin (mol.wt. 16000) and pig heart lactate dehydrogenase (mol.wt. 144000).

Synthetic-boundary centrifugation

This was used for determining the $A^{1\%}_{1cm}$ for the enzyme. At low speed a synthetic-boundary cell can be combined with the schlieren optical system of the ultracentrifuge to provide a sensitive differential refractometer for evaluating the w/v concentration of protein solutions. The A_{280} of an enzyme solution (previously dialysed against 10mM-sodium phosphate buffer, pH7.0) was measured. Several photographs were taken within 30min after reaching a speed of 3000rev./min. The area (A) under the peak was measured by first enlarging the photograph and drawing around the profile. The photographic paper was then cut out and weighed. This area was propor-



Scheme 1. *Summary of the methods used to purify soluble malate dehydrogenase*

tional to the protein concentration (C_p, g/l), the refractive-index increment and the optical magnification factor. Similar measurements were made for a sucrose solution of known concentration (C_s) when the concentration of protein can be calculated by comparing the area under the protein peak (A_p) with the area under the sucrose peak (A_s) by using:

$$C_p = \frac{A_p \cdot 143 \cdot C_s}{A_s \cdot 185}$$

where 143 g/dl = refractive-index increment for sucrose (Gosting & Harris, 1949) and 185 g/dl = refractive-index increment for proteins (Huglin, 1972).

NADH binding to the enzyme

The enhanced NADH fluorescence (excitation at 297 nm and emission through a Kodak Wratten 98 filter; maximum transmission at 440 nm) and/or the decreased protein fluorescence (excitation at 297 nm and emission through a Kodak Wratten 18A filter; maximum transmission 360 nm) of an enzyme solution (3 ml) was monitored as NADH was continuously added to a stirred protein solution. All measurements were made by using a dual-wavelength-filter fluorimeter/spectrometer, which has been described by Shore *et al.* (1975). When the effect of added compounds was to be studied, the compound, neutralized with 5 M-NaOH where necessary, was added directly to the enzyme solution before the titration began.

Phosphate content of soluble malate dehydrogenase

Forms a and b of the ox heart enzyme are distinguished by their phosphate content and, since they are reported to have different responses to fructose 1,6-bisphosphate (Cassman & Vetterlein, 1974), the phosphate content of the enzyme used in the present study was determined to allow a comparison with this work to be made. The enzyme to be tested was dialysed against two 5-litre changes of 10 mM-NaCl. Trichloroacetic acid (3 M; 9 ml/ml of enzyme solution) was added to the enzyme, and the precipitate sedimented (2000*g*, 2 min) in a bench centrifuge and washed four times by alternate suspension in water (10 ml) and sedimentation. The precipitate was finally resuspended in water (2 ml). Phosphate associated with the protein was converted into P_i by the method of Jones *et al.* (1951) and measured by the colorimetric assay of Allen (1940) (except that one-fifth the volume of all solutions was used).

Results

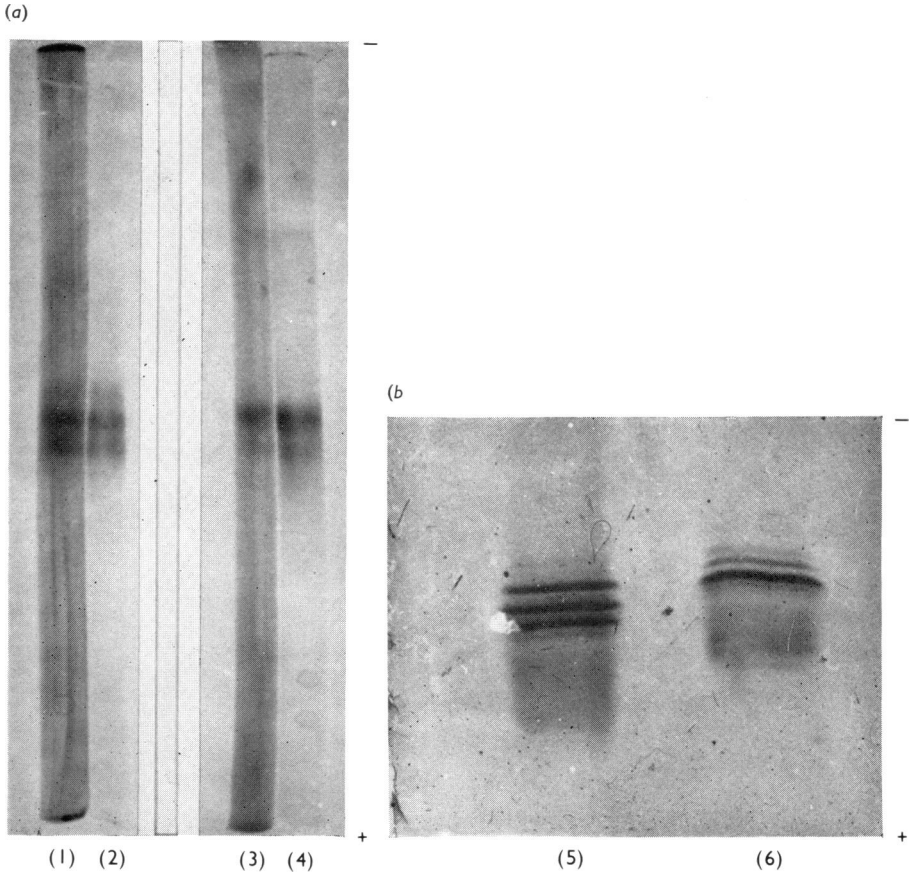
Homogeneity

Typical purification results for soluble malate dehydrogenase by methods 1 and 2 are shown in Table 1. The major differences are that in method 2 the homogenate incubation and acid-precipitation steps are omitted and the procedure is shortened so that within 6 h there is a 27-fold purification of the enzyme. The large loss of activity after DEAE-cellulose chromatography is due to removal of the mitochondrial enzyme. The product of both methods 1 and 2 gave a single protein-staining band on

Table 1. *Purification data for malate dehydrogenase prepared by methods* 1 *and* 2

An outline of the method is given in Scheme 1. Details have been deposited at the British Library (Lending Division), as Supplementary Publication SUP 50080.

Stage of procedure	Volume (ml)	Activity (units)	Total protein (mg)	Specific activity (units/mg)
Method 1				
Extract	14000	990000	195000	5.1
After batch DE-11 adsorption step	14000	900000	190000	4.7
Eluate from batch DE-11 column step	500	70000	2700	25
Hydroxyapatite chromatography	300	50000	700	70
DE-52 column chromatography eluate	250	35000	350	100
Method 2				
Extract	13000	650000	—	—
After batch DE-11 adsorption step	13000	600000	—	—
Eluate from batch DE-52 column step	400	80000	2900	27
Hydroxyapatite chromatography	250	50000	900	55
DE-52 column chromatography eluate	300	30000	400	75



EXPLANATION OF PLATE 1

Comparison of the subforms of pig heart soluble malate dehydrogenase

The subforms were resolved by polyacrylamide-gel electrophoresis (*a*) and isoelectric focusing (*b*). The polyacrylamide gels were run in the absence of SDS; samples of enzyme (about 50 μg) were loaded in sucrose (50%, w/v) and the gels run at 3 mA per gel for 12 h. Before staining the gels were sliced horizontally; one half of the gel was stained for protein (*a*, 1 and 3) by the method of Berg (1969), and the other for enzyme activity (*a*, 2 and 4) by a modification of the procedure of Thorne *et al.* (1963). Gels used in isoelectric focusing, pH range 3.5–9.5, were run and stained for protein according to the manufacturers' instructions (LKB, Croydon, Surrey, U.K.) (*b*, 1 and 2). Pig heart enzyme prepared by method 1 (*a*, 1 and 2, *b*, 5) was resolved into three major subforms, and enzymes prepared by method 2 (*a*, 3 and 4; *b*, 6) into one major subform, which corresponded to the least acidic subform of the enzyme prepared by method 1.

SDS/polyacrylamide-gel electrophoresis. This band was not resolved from pig heart lactate dehydrogenase. In the absence of SDS, the product of method 1 was resolved into three subforms and that of method 2 into two (Plate 1). By slicing the gel longitudinally and staining half for protein and the other for enzyme, it was found that all protein-staining bands were also catalytically active. The major component from method 1 was the most acidic subform, and that from method 2 was the least acidic subform. By using isoelectric focusing it was possible to improve the resolution of the subforms and to identify the major component from method 2 as the minor component from method 1.

Absorbance factor ($A^{1\%}_{1cm}$)

The average value of $A^{1\%}_{1cm}$ at 280nm for the pig heart enzyme determined by differential refractometry was 15 (Table 2). For the ox heart enzyme the value was 10.5. The absorbance factor was also determined by using the biuret method (Beizenherz *et al.*, 1953) and from the absolute amino acid analysis of a solution of known absorbance for pig heart soluble enzyme. A value of $A^{1\%} = 15$, the average of our three determinations, is used below. It compares reasonably well with the values from biuret and amino acid analysis and the values of Kitto & Kaplan (1966) and Glathaar *et al.* (1972). The value of 15 is considerably higher than the value of 9.3 obtained by Gerding & Wolfe (1969). The value for the ox enzyme, determined by the same methods as that for the pig enzyme, is much lower. We have no explanation for this, although we have only limited experience with the ox enzyme.

Phosphate content of soluble malate dehydrogenase

Values of 0.3 (S.D. $\pm$0.1, $n = 9$) and 0.4 (S.D. $\pm$0.1, $n = 9$) mol of trichloroacetic acid-precipitable phosphate/70000g of pig and ox heart enzyme respectively were obtained. These values are in close agreement with the value for the b form of ox heart soluble malate dehydrogenase reported by Cassman & Vetterlein (1974). We obtained 0.045mol of P_i/mol of trypsin in a control experiment.

Number and nature of the NADH-binding sites on soluble malate dehydrogenase

The equilibrium titration of the binding sites of the enzyme with NADH was followed simultaneously by enhanced nucleotide fluorescence and by the decreased protein fluorescence. If the sites are indistinguishable and equivalent, the fractional saturation of the NADH-binding sites ($\bar{v}$) can be related to the total concentration of NADH added (L_0), the dissociation constant for NADH (K_d) and the total concentration of binding sites (E_0) by:

$$K_d \frac{1}{(1-\bar{v})} = \left(\frac{L_e}{\bar{v}}\right) - E_0$$

A graph of $1/(1-\bar{v})$ against $L_0\,\bar{v}$ will be linear, of slope $1/K_d$, and on extrapolation will give an intercept on the $L_0/\bar{v}$ axis of E_0. At high sites concentration ($> K_d$), accurate information is gained about the concentration of binding sites, whereas at low protein concentrations ($< K_d$) accurate information about K_d is obtained.

The fractional saturation of the ligand-binding sites ($\bar{v}$) was equated to the ratio $\Delta F/\Delta F_{max.}$, where $\Delta F_{max.}$ is the limiting value of the fluorescence difference (ΔF) at high concentrations of added ligands. $\Delta F_{max.}$ was chosen by inspection of the original experimental record (the hyperbolic graph of ΔF against L_0). Errors due to the chosen end point being fractionally too high or low were seen as departures from linearity (Stinson & Holbrook, 1973). The value of $\Delta F_{max.}$ to which ΔF approached asymptotically always compared reasonably with the highest ΔF measured in the titration.

No significant difference in the titration curve was found between enzyme prepared by method 1 or 2. Fig. 1 shows a typical titration curve, with the enzyme prepared by method 2 (1.3 μM), in 50mM-sodium phosphate buffer, pH 6.0. The plot of $1/(1-\bar{v})$ against $L_0/\bar{v}$ is linear (to $\bar{v}$ 0.9); thus the NADH sites occupied are identical and independent. Over a period of 2 years with enzyme prepared by both methods 1 and 2 and

Table 2. *$A^{1\%}_{1cm}$ of malate dehydrogenase*

$A^{1\%}_{1cm}$ at 280nm from the literature is compared with the values determined in the present work. A value of 15 for the pig heart enzyme and 10 for the ox heart enzyme were used in this study.

Species	Method	$A^{1\%}_{1cm}$	Reference
Pig	Differential refractometry	15	The present work
	Amino acid analysis	16$\pm$2	The present work
	Biuret	13.6	The present work
	Dry weight	9.3	Gerding & Wolfe (1969)
	Amino acid analysis	13.1	Glathaar *et al.* (1972)
Ox	Differential refractometry	10.5	The present work
	Differential refractometry	9.0	The present work

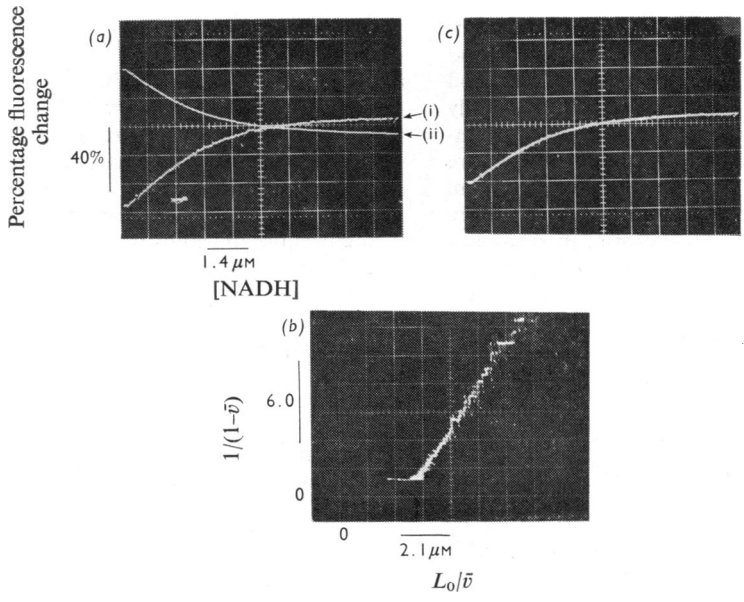


Fig. 1. *Determination of the number of NADH-binding sites on soluble malate dehydrogenase*
(*a*) Binding of NADH was monitored by the decrease in protein fluorescence (ii) and the increase in nucleotide fluorescence (i) on adding NADH to a solution of the enzyme. Determinations were performed in 50mM-sodium phosphate buffer, pH6.0; the binding curve shown was obtained by using pig heart enzyme (1.35μM) prepared by method 1. (*b*) Plots of $1/(1-\bar{v})$ against $L_0/\bar{v}$ were linear to $\bar{v} = 0.9$. (*c*) Superimposition of curves (i) and (ii) from (*a*).

Table 3. *Protein-concentration-independence of NADH-binding parameters*
The K_d and number of NADH-binding sites on pig heart malate dehydrogenase at various enzyme concentrations were determined in 50mM-sodium phosphate buffer, pH6.0. At intermediate protein concentrations (1.55–3.95μM), where binding curves contain information about both K_d and the concentration of NADH-binding sites (see the text), there is no change in K_d or the concentration of bound NADH. Over the whole range K_d changes by less than a factor of 2 compared with a value of $0.6\pm0.2\mu$M determined for the K_d at this pH.

[Enzyme] (μM)	K_d (μM)	NADH bound (μM)	NADH bound (mol/70000g of enzyme)
0.15	0.3	0.15	1.0
0.45	0.3	0.6	1.3
0.8	0.3	1.0	1.3
1.55	0.6	2.8	1.8
3.95	0.6	7.7	1.9
4.7	0.8	9.4	2.0
5.95	0.9	11.9	2.0
7.25	0.8	12.4	1.7

with specific activities varying from 75 to 110 units/mg, it was found that 1.8 ± 0.2 (28) mol of NADH was bound/70000g of enzyme. This result was supported by a determination using gel filtration (Pfleiderer & Auricchio, 1964) (1.6mol of NADH bound/70000g of enzyme of specific activity 70units/mg). Equilibrium titrations at low protein concentrations (about 0.4μM) enabled the K_d to be determined in 50mM-sodium phosphate buffer, pH6.0. A value of $0.4\pm0.1\mu$M ($n = 12$) was determined for the enzyme prepared by method 1 and $0.6\pm0.2\mu$M ($n = 12$) for enzyme prepared by method 2. Where binding of NADH to the enzyme was monitored simultaneously by the change in nucleotide fluorescence and protein fluorescence, the two binding curves were completely superimposable (Fig. 1*c*).

Protein-concentration-independence of NADH-binding parameters

Table 3 reports determinations of K_d and number of sites for NADH at pig heart malate dehydrogenase concentrations from 0.15 to 7.25μM. Over the whole range, K_d changes by less than a factor of 2 compared with the average value of $0.6\pm0.2\mu$M obtained at the more reliable low protein concentrations. This change is not significant. The values at high protein concentrations are less accurate. At the intermediate protein concentrations (1.55 and 3.95μM), where the curves contain information about both K_d and E_0, the results are in excellent agreement.

The slight trend in K_d with protein concentration probably does not reflect a monomer–dimer equilibrium. The enzyme activity was eluted from a calibrated Sephadex G-100 column at an elution volume (just after bovine serum albumin) that was independent of protein concentrations in the range 0.02–4.6 μM-enzyme. This confirms the result of Bleile *et al.* (1977). The elution volume did not change when 1 mM-NADH was included in the buffer used to equilibrate the column. This lack of dissociation was confirmed, since, in contrast with the mitochondrial enzyme (Shore & Chakrabarti, 1976), a sample of soluble malate dehydrogenase covalently labelled with fluorescein isothiocyanate showed unchanged polarization of fluorescein fluorescence (P 0.218) over the concentration range 0.03–3 μM (J. J. Holbrook & J. D. Shore, unpublished work).

Does soluble malate dehydrogenase dissociate in the presence of NADH?

Cassman & King (1972) reported that NADH binds to the ox heart enzyme with slight positive co-operativity with Hill coefficient of 1.33 at 0.56 mg of protein/ml and 1.1 at 0.01 mg of protein/ml. They suggested that the enzyme exists as a monomer–dimer equilibrium and that NADH binds very tightly to the monomer. Koren & Hammes (1975) repeated these experiments. When NADH binding was measured by forced dialysis they found two equivalent NADH-binding sites ($K_d = 14\,\mu$M); when binding was monitored by nucleotide fluorescence enhancement they obtained a non-linear binding plot.

With the pig soluble enzyme in 20 mM-sodium phosphate, pH 6, at enzyme concentrations of 0.15–7.25 μM we measured NADH binding by NADH fluorescence enhancement. The binding curves always gave linear plots of $1/(1-\bar{v})$ against $[\text{NADH}]_{\text{total}}/\bar{v}$ showing that a single dissociation constant ($0.6 \pm 0.2\,\mu$M) could describe both NADH-binding sites over this whole enzyme concentration range. Thus the pig enzyme does not exist as a monomer–dimer equilibrium at pH 6 with greatly different affinities for NADH [Cassman & King (1972) used dissociation constants of NADH from the monomer of 0.25 μM and of 25 μM from the dimer].

Fig. 2 shows results in 20 mM-phosphate buffer/1 mM-EDTA, pH 6.9: exactly the conditions used by Cassman & King (1972) and Koren & Hammes (1975), but for the pig soluble enzyme. The nucleotide-fluorescence enhancement (fluorescence at the end point) increases linearly with enzyme concentration, and the inset shows that both NADH-binding sites may be described by a single dissociation constant. Similarly, with the ox heart enzyme we found under the same conditions linear plots of $1/(1-\bar{v})$ against $L_0/\bar{v}$ at enzyme concentrations of 0.6 μM, 2.4 μM and 4.8 μM. The single dissociation constant of NADH at each concentration was 1.1 μM, 1.0 μM and 1.2 μM respectively. Again there was no indication of

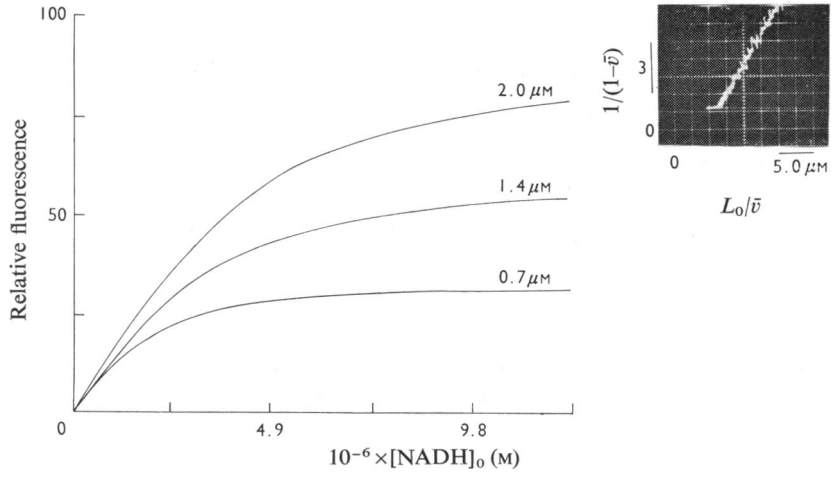


Fig. 2. *Effects of changing malate dehydrogenase concentration on the binding of NADH*
Binding of NADH to the enzyme was monitored by the change in nucleotide fluorescence associated with NADH binding in 20 mM-potassium phosphate buffer/1 mM-EDTA, pH 6.7, while the concentration of pig heart enzyme was varied from 0.7 to 2.0 μM. The inset shows a typical analysis with 2 μM-enzyme. The nucleotide fluorescence enhancement increases linearly with enzyme concentration and both NADH-binding sites are described by a single dissociation constant at all enzyme concentrations.

dissociation of the enzyme or of non-identical environments at each NADH site on the dimeric enzyme.

Changed protein fluorescence of soluble malate dehydrogenase is proportional to the number of NADH-binding sites occupied

Fig. 1 shows that changes in protein fluorescence are proportional to the enhanced fluorescence of NADH. This is but one example of such results obtained with both pig and ox enzyme, at pH 6 and pH 8 and over a wide range of protein concentrations. It confirms the results of Cassman & King (1972) and of Mueggler *et al.* (1975). Linear changes in protein fluorescence when NADH binds to the oligomeric dehydrogenase are unusual (Holbrook, 1972) and require explanation. Non-linear decreases in protein fluorescence arise when the Förster (1959) mechanism is the main cause of the decreased protein fluorescence. Linear protein fluorescence changes occur when either there is only one NADH site per protein molecule or when the change in tryptophan fluorescence results from a protein rearrangement induced by NADH binding. Since with both the pig and the ox enzymes we have shown that two NADH residues are bound to each molecule and that under the measurement conditions the enzyme remains dimeric, we are forced to choose the second alternative, namely that NADH binding induces a local conformation change that results in a changed quantum yield of neighbouring tryptophan residues. This conclusion is not novel. Cassman (1973) noted that below pH 7.5 the quenching of tryptophan fluorescence in ox soluble malate dehydrogenase arose from processes other than Förster (1959) energy transfer.

Effects of L-*malate on NADH binding*

Mueggler *et al.* (1975) have reported that NADH binding to the pig heart enzyme at pH 8.0 in Tris/acetate buffer (0.5 M in acetate) and in the presence of 0.1 M-L-malate shows negative co-operativity. Preliminary experiments at pH 6.0 showed that, in agreement with Cassman (1973), 5 mM-L-malate lowered the dissociation constant for NADH (K_d) from 0.5 μM (3) to 0.2 μM (3) and that the nucleotide fluorescence was enhanced. The form of the binding curve showed that both NADH-binding sites were indistinguishable and independent.

The binding of NADH to pig and ox heart malate dehydrogenase was monitored by the decreased protein fluorescence at pH 8.0 in 0.1 M-L-malate as described by Mueggler *et al.* (1975). However, NADH binds only weakly at pH 8.0 (Holbrook & Wolfe, 1972), and the decrease in protein fluorescence caused by added NADH must be resolved into two components, (*a*) the 'trivial' decrease caused by absorption of the incident (297 nm) and fluorescent (340 nm) light by the added soluble NADH ('inner filter effect') and (*b*) the decrease caused by NADH bound to the protein.

To determine the extent of the 'trivial' quench of fluorescence, a series of proteins that have not been reported to bind NADH were titrated with NADH. The proteins were bovine serum albumin, trypsin, chymotrypsin, duck ovotransferrin. The initial fluorescence and the measured fluorescence were related to the concentration (c, mM) of NADH by:

$$\text{Initial fluorescence} = \text{measured fluorescence} \times 10^{\varepsilon c 0.5} \quad \text{(i)}$$

where ε is the apparent millimolar absorption coefficient and 0.5 cm is the effective path length for excitation and emission. By using an analogue simulator (Clayton, 1973) values of E were selected such that the 'initial fluorescence' from eqn. (i) remained unchanged as NADH increased to 0.1 mM. As expected, the values for E did not depend on the protein (tryptophan, 10.9; bovine serum albumin, 12.7; trypsin, 11.7; chymotrypsin, 12.6; duck ovotransferrin, 12.9). The average of E is 12.5 ± 1.6 (15) litre·mmol^{-1}·cm^{-1}. This is as expected from the sum of the approximate absorption coefficients of NADH at the exciting (297 nm; 6 litre·mmol^{-1}·cm^{-1}) and fluorescent (340 nm; 6.22 litre·mmol^{-1}·cm^{-1}) wavelengths. The curves in Fig. 3(*a*) are an example of the effectiveness of the correction.

Equilibrium titrations of either the pig or the ox enzyme were performed in the presence or absence of 0.1 M-L-malate, over a concentration range of 1.9–9.5 μM-protein. Typical results are also shown in Fig. 3, where the binding curve has been corrected for 'trivial' quenching due to NADH absorption. Plots of $1/(1-\bar{v})$ against $L_0/\bar{v}$ are linear, showing that the NADH-binding sites are identical and independent. Failure to correct for 'trivial' quenching produces non-linear plots. Uncorrected curves were interpreted by Mueggler *et al.* (1975) to mean that L-malate produces interaction between the NADH-binding sites on the enzyme. This interpretation is not justified. Table 4 summarizes a series of experiments under conditions described by Mueggler *et al.* (1975). No evidence of interaction between the two NADH-binding sites on each enzyme molecule was obtained. Mueggler *et al.* (1975) would have observed apparent site–site interaction because they failed to correct for inner-filter effects. The precise value of E used to correct results must be determined anew for each pair of excitation and emission wavelengths and for different instrument geometry. In fact, the concentration of buffer used in the experiments reported by Mueggler *et al.* (1975) was 0.05 M and not the 0.5 M stated in the paper (R. Wolfe, personal communication). Thus there is no quantitative difference between the results without L-malate from their and our laboratories.

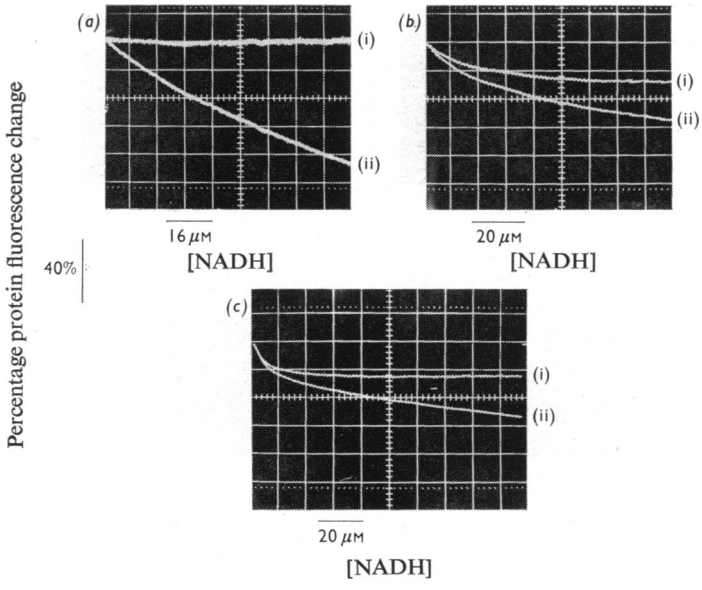


Fig. 3. *Effect of* 0.1 M-L-*malate on the binding of NADH to malate dehydrogenase*
Determinations were performed in 0.5 M-Tris solution adjusted to pH 8.0 with acetic acid. Binding of NADH was followed by the decrease in protein fluorescence. In (*a*) is shown a typical titration (with bovine serum albumin) used to determine the correction factor for 'trivial' quenching by NADH: (i) shows the corrected data and (ii) the uncorrected data. Similarly in (*b*) and (*c*) are the corrected (i) and uncorrected (ii) NADH-binding curves for pig heart enzyme (1.9 μM) in the presence (*c*) and absence (*b*) of 0.1 M-L-malate. When the binding curves are corrected for 'trivial' quenching they are consistent with identical and independent NADH-binding sites.

Table 4. *Effect of* 0.1 M-L-*malate on the binding of NADH to malate dehydrogenase*
Binding was monitored by the decrease in protein fluorescence on adding NADH. All results were corrected for 'inner-filter' effects caused by added NADH by using $E = 12.5$ litre·mmol^{-1}·cm^{-1}. The buffer was prepared by adjusting a solution of 0.5 M-Tris with or without 0.1 M-L-malate to pH 8.0 with acetic acid. Plots of $1/(1-\bar{v})$ against $L_0/\bar{v}$ were linear. n.d., Not determined.

Species	[Enzyme] (μM)	L-Malate	K_d (μM)	NADH bound (μM)	NADH bound (mol/70000 g of enzyme)
Pig	1.9	−	11	n.d.	
	1.9	+	5.5	n.d.	
	5.7	−	8.8	11	1.9
	5.7	+	5.5	11	1.9
	9.5	−	11	19.8	2.1
	9.5	+	6.8	19.8	2.1
Ox	1.9	−	10	n.d.	
	1.9	+	4.2	n.d.	
	5.7	−	8.5	10.6	1.9
	5.7	+	4	10.6	1.9
	9.5	−	10	18.5	1.9
	9.5	+	4.2	17	1.8

Effect of fructose 1,6-*bisphosphate*

This metabolite is reported to be an allosteric inhibitor of the binding of NADH to ox heart malate dehydrogenase (Cassman, 1973), and we have confirmed that the NADH titration of ox heart enzyme in the presence of 5 mM-fructose 1,6-bisphosphate (Fig. 4*b*) is highly anomalous. The plot of $1/(1-\bar{v})$ against $L_0/\bar{v}$ was non-linear and at first sight suggests

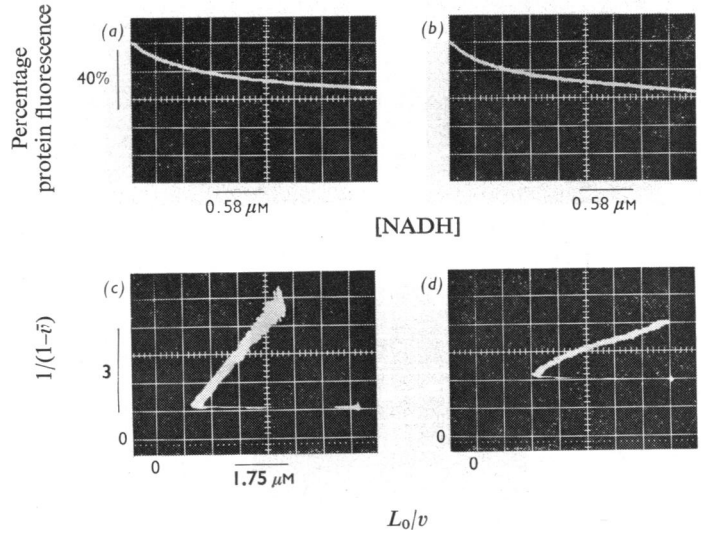


Fig. 4. *Effect of 5 mM-fructose 1,6-bisphosphate on the binding of NADH to malate dehydrogenase*
Binding of NADH to the enzyme was followed by quenching of protein fluorescence. Experiments were performed in 50mM-sodium phosphate buffer, pH6.0. For pig heart enzyme (0.19 μM) the binding curve (*a*) was hyperbolic and the plot of $1/(1-\bar{v})$ against $L_0/\bar{v}$ (*c*) is linear, as expected of independent and identical sites. The binding curve generated by the ox heart enzyme (*b*) appeared to be composed of two phases and the plot of $1/(1-\bar{v})$ against $L_0/\bar{v}$ (*d*) was not linear.

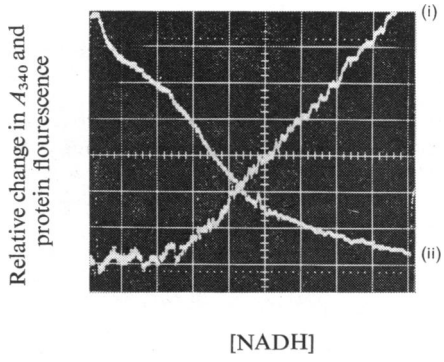


Fig. 5. *Apparent sigmoidal NADH binding to ox heart malate dehydrogenase in the presence of 5 mM-fructose 1,6-bisphosphate*
The quenching of protein fluorescence (ii) and appearance of A_{340} (i) were simultaneously monitored as NADH was slowly added (0.14 μM-NADH·min^{-1}) to a solution of ox heart enzyme (0.85 μM) in 50mM-sodium phosphate buffer, pH6.0. From the quenched fluorescence curve (ii) there is apparent sigmoidal NADH binding. However, no A_{340} (i) is apparent during the first portion of the titration; from the point of appearance of A_{340} (i) the binding curve is hyperbolic, as predicted for identical and independent NADH-binding sites. The apparent interaction observed when NADH is bound to ox heart enzyme in the presence of 5mM-fructose 1,6-bisphosphate is due to a slow oxidation of NADH.

negative site–site interactions. Titrations of pig heart enzyme under exactly the same conditions and with the same solutions as used for the ox enzyme on the same day showed no measurable effect of added fructose 1,6-bisphosphate (Fig. 4*a*).

However, the explanation of the anomalous titration is not NADH site–site interactions as suggested by Cassman (1973). Fig. 5 shows a very slow titration in which A_{340} was monitored at the same time as the protein fluorescence. Under these conditions the decrease in the protein fluorescence is roughly sigmoidal, but the absorbance of the added NADH, which should have increased from the start, showed a lag phase. From the point of appearance of A_{340} (that is NADH), the binding curve for NADH is hyperbolic, as expected of identical and independent NADH-binding sites. As an important control a titration of the pig enzyme recorded on the same day with the same buffer, NADH and fructose 1,6-bisphosphate solutions is also shown in Fig. 4. This does not contain the anomaly.

Titration of an enzyme in which NADH is destroyed in the presence of fructose 1,6-bisphosphate would lead to an incorrect identification of site–site interactions (Cassman, 1973). Nevertheless, the present results do not allow us to put forward an explanation of the oxidation of the initially added NADH. Possible explanations are restricted by the observation that the oxidation is catalysed by the ox enzyme preparation, but not by the pig preparation,

and that the oxidation requires fructose 1,6-bisphosphate.

Discussion

Both methods used to prepare soluble malate dehydrogenase reproducibly give results similar to those reported in the present paper. Method 1 yields an enzyme with three main electrophoretically distinguishable subforms, with the most acidic forms predominant, as reported in earlier work (Thorne *et al.*, 1963; Gerding & Wolfe, 1969). Method 2, which is rapid and avoids prolonged exposure of the extract to low pH, yields in the main the least acidic subform. The subforms are all enzymically active and have the same subunit and molecular weights. The electrophoresis and electrofocusing experiments suggest that each subform differs from the next by a small, but constant, difference in charge. Deamidation of the enzyme in the crude homogenate or in the acid precipitation step would explain our findings, especially the increase in the most acidic subforms. Deamidation of amino acid amides was also suggested by Glathaar *et al.* (1972) to explain subforms of the mitochondrial enzyme prepared from a similar homogenate. Since method 2 is milder and more rapid than method 1, we assume that the least acidic subform most closely reflects the form of the enzyme in the living tissue (although this assumption may change when a molecular explanation of the subforms is available).

In a previous study Holbrook & Wolfe (1972) actually measured only 1.4 NADH-binding sites/enzyme molecule. This value was thought reasonable at that time because the enzyme preparation used had a specific activity of 80 units/mg (compared with the 110 units/mg for the enzyme prepared by Gerding & Wolfe, 1969). However, the purest enzyme samples prepared by methods 1 and 2 contained only active enzyme molecules, and yet were found to bind only 1.1 mol of NADH/70000 g of protein with specific enzyme activities in the range 80–100 units/mg (by using $A^{1\%}_{1cm} = 9.3$ at 280 nm). Because a value of 1.1 NADH-binding sites/dimer might have reflected in solution the differences between the NAD^+-binding sites observed in the crystal (Hill *et al.*, 1972), we critically examined the assumptions involved in this determination. The absorbance factor at 280 nm used by Gerding & Wolfe (1969) was found to be much too low. The new value of $A^{1\%}_{1cm}$ at 280 nm (15 ± 1) yields 1.8 ± 0.2 NADH-binding sites/70000 g of enzyme. We conclude that both subunits in the molecule can bind NADH. This is in agreement with the number of NAD^+-binding sites on the malate dehydrogenase molecule (A. Lodola & J. J. Holbrook, unpublished work).

In our many experiments we have never obtained results from titrations of the enzyme with NADH monitored by either decreased protein fluorescence or enhanced NADH fluorescence that suggest that the two sites in the pig heart enzyme are anything but identical and indistinguishable (as an example, see Fig. 2). This is in agreement with previous titration experiments (Holbrook & Wolfe, 1972) and with a steady-state kinetic study of the pig heart enzyme (Frieden & Souza, 1975). It is noteworthy that, in a symposium paper by Weininger *et al.* (1977), the unequal occupancy of NAD^+-binding sites in pig heart enzyme crystals were found to be due to crystal-lattice forces, which of course would be absent from solution. There is thus no longer a conflict between the hyperbolic binding of NADH observed by Koren & Hammes (1975) using equilibrium dialysis and the results of NADH binding monitored by fluorescence.

We have shown in this paper that (i) the apparent negative co-operativity (Mueggler *et al.*, 1975) in the presence of 0.1 M-L-malate at pH 8.0 was not observed when NADH-binding curves were corrected for inner-filter effects and that (ii) the site–site interactions observed by Cassman (1973) when NADH binds to ox heart enzyme in the presence of fructose 1,6-bisphosphate are due to the oxidation of the added NADH. From all our results it is clear that both NADH-binding sites on the soluble malate dehydrogenase molecule are identical. It was the apparent non-identity of the sites that led Cassman & Vetterlein (1974) to propose a regulatory role for this enzyme. Since in the present paper we have shown that the enzyme is dimeric in solution, and that the two NADH-binding sites are occupied in a random manner with or without added L-malate or fructose 1,6-bisphosphate, we conclude that there is no equilibrium evidence to support the proposed regulatory role of the enzyme.

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References

Allen, R. T. L. (1940) *Biochem. J.* **34**, 858–865
Beizenherz, G., Boltze, M. J., Bucher, T., Czok, R., Garbade, K. H. & Meyer-Arendt, E. (1953) *Z. Naturforsch. Teil B* **8**, 555–562
Berg, H. C. (1969) *Biochim. Biophys. Acta* **183**, 65–78
Bleile, D. H., Schulz, R. A. & Harrison, J. H. (1977) *J. Biol. Chem.* **252**, 755–758
Cassman, M. (1973) *Biochem. Biophys. Res. Commun.* **53**, 666–672
Cassman, M. & King, R. C. (1972) *Biochemistry* **11**, 4937–4944
Cassman, M. & Vetterlein, D. (1974) *Biochemistry* **13**, 684–689
Clayton, G. B. (1973) *Wireless World* **79**, 33–35
Förster, T. H. (1959) *Discuss. Faraday Soc.* **27**, 7–16

Frieden, C. & Souza, J. F. (1975) *J. Biol. Chem.* **250**, 2106–2113
Gerding, R. K. & Wolfe, R. G. (1969) *J. Biol. Chem.* **244**, 1164–1172
Glathaar, B. E., Banaszak, L. J. & Bradshaw, R. A. (1972) *Biochem. Biophys. Res. Commun.* **46**, 757–765
Gosting, L. J. & Harris, M. S. (1949) *J. Am. Chem. Soc.* **71**, 1998–2006
Hill, E., Tsernoglou, D., Webb, L. & Banaszak, L. J. (1972) *J. Mol. Biol.* **72**, 577–589
Holbrook, J. J. (1972) *Biochem. J.* **128**, 921–931
Holbrook, J. J. & Wolfe, R. G. (1972) *Biochemistry* **11**, 2499–2502
Huglin, M. B. (1972) *Light Scattering from Polymer Solutions*, pp. 165–203, Academic Press, New York
Jones, F. J., Lee, W. A. & Peacocke, A. R. (1951) *J. Chem. Soc.* 623–624
Kitto, G. B. & Kaplan, N. O. (1966) *Biochemistry* **5**, 3966–3980
Koren, R. & Hammes, G. G. (1975) *Biochemistry* **14**, 1021–1025
Mueggler, P. A., Dahlquist, F. W. & Wolfe, R. G. (1975) *Biochemistry* **14**, 3490–3497
Pfleiderer, G. & Auricchio, F. (1964) *Biochem. Biophys. Res. Commun.* **16**, 53–59
Shore, J. D. & Chakrabarti, S. K. (1976) *Biochemistry* **15**, 875–879
Shore, J. D., Weidig, C. F., Lodola, A., Parker, D. M. & Holbrook, J. J. (1975) *Proc. FEBS Meet.* **40**, 63–73
Stinson, R. A. & Holbrook, J. J. (1973) *Biochem. J.* **131**, 719–728
Tanner, M. J. A. & Boxer, D. H. (1972) *Biochem. J.* **129**, 333–347
Thorne, C. J. R. & Kaplan, N. O. (1963) *J. Biol. Chem.* **238**, 1866–1872
Thorne, C. J. R., Grossman, L. I. & Kaplan, N. O. (1963) *Biochim. Biophys. Acta* **73**, 193–203
Weininger, M., Birktoft, J. J. & Banaszak, L. J. (1977) *Int. Symp. Pyridine Nucleotide Dependent Dehydrogenases 2nd* (Sund, H., ed.), Verlag Walter de Gruyter, Berlin, in the press
Williamson, J. R., Safer, B., La Noue, N. F. Smith, C. H. & Walattys, E. (1973) *Symp. Soc. Exp. Biol.* **27**, 241–281