Biochem. J. (1973) **133**, 515–520
Printed in Great Britain

Affinity Chromatography of Lactate Dehydrogenase on Immobilized Nucleotides

By C. R. LOWE and P. D. G. DEAN
Department of Biochemistry, University of Liverpool, P.O. Box 147, *Liverpool L*69 3*BX, U.K.*

(*Received* 31 *October* 1972)

The interaction of two isoenzymes of lactate dehydrogenase from pig heart muscle (H_4) and rabbit skeletal muscle (M_4), with immobilized nucleotides was examined: the effects of pH and temperature on the binding of lactate dehydrogenase were studied with immobilized NAD^+ matrices. The influence of substrate, product and sulphite on the binding of heart muscle lactate dehydrogenase to immobilized NAD^+ was investigated. The interaction of both lactate dehydrogenase isoenzymes with immobilized pyridine and adenine nucleotides and their derivatives were measured. The effects of these parameters on the interaction of lactate dehydrogenase with immobilized nucleotides were correlated with the known kinetic and molecular properties of the enzymes in free solution.

In the past affinity chromatography has found considerable application in the purification of enzymes (Cuatrecasas & Anfinsen, 1971*a*,*b*). The wider implications of the technique for the investigation and exploration of complex biological interactions have been largely unrealized until recently (Wilchek, 1972). This appreciation of the potential of the technique has prompted its further application to many novel situations: the study of complex formation between enzymes and other ligands (Akanuma *et al.*, 1971); the separation of regulatory and catalytic subunits (Reimann *et al.*, 1971); the resolution of active and inactive enzyme species (Edmondson *et al.*, 1972) and the investigation of peptide–peptide interactions (Gawronski & Wold, 1972).

In the present study we examine the feasibility of investigating the nature of an enzyme–ligand system in which one of the interacting components is fixed on an insoluble matrix. The use of immobilized coenzymes, besides finding considerable application in the purification of groups of cofactor-dependent enzymes, presents an ideal opportunity to investigate the interaction of a wide range of enzymes with a single immobilized ligand (Arsenis & McCormick, 1966; Collier & Kohlhaw, 1971; Lowe & Dean, 1971; Lowe *et al.*, 1972). In the present paper and elsewhere (Lowe *et al.*, 1973) attempts have been made to correlate the behaviour of the nicotinamide nucleotide-dependent dehydrogenases when interacting with nucleotides covalently attached to an insoluble matrix and in free solution. Lactate dehydrogenase (EC 1.1.1.27) was particularly chosen for study as its behaviour in free solution is well documented (Schwert & Winer, 1963). The interaction of lactate dehydrogenase isoenzymes with immobilized nucleotides and their analogues could thus be compared with an equivalent system in free solution. The binding data could be interpreted tentatively in terms of the nature and mechanism of the interaction with pyridine nucleotides.

Materials and Methods

Materials

Pig heart lactate dehydrogenase (H_4) and rabbit muscle lactate dehydrogenase (M_4) were obtained from the Boehringer Corp. (London) Ltd., London W.5, U.K. CNBr, dicyclohexylcarbodi-imide and ϵ-aminohexanoic acid were obtained from R. N. Emanuel Ltd., Wembley, Middx., U.K. The adenine and pyridine nucleotides were a gift from Whatman Biochemicals, Maidstone, Kent, U.K. and Sepharose 4B was purchased from Pharmacia (G.B.) Ltd., London W.5, U.K. All other chemicals were of the highest purity available from BDH Chemicals Ltd., Poole, Dorset, U.K.

Methods

ϵ-Aminohexanoic acid was coupled to Sepharose 4B by the CNBr activation technique of Axen *et al.* (1967). The immobilized nucleotide derivatives were prepared by coupling the appropriate nucleotide to ϵ-aminohexanoyl-Sepharose by the carbodi-imide reaction exactly as described by Larsson & Mosbach (1971).

Chromatographic analyses to determine binding (β) were performed as described by Lowe & Dean (1971) with modifications as follows. Except where stated, columns were 5mm × 20mm of the appropriate immobilized nucleotide derivative. The columns were equilibrated by washing with at least 10 volumes of 10mM-KH_2PO_4–KOH buffer, pH7.5. Enzyme samples (50μl) were applied to the top of the moist column and allowed to run into the bed. The enzyme was washed into the column with a small volume of the equilibration buffer. The column was then washed with the same buffer (at least 6ml) to remove non-adsorbed protein before application of a linear gradient of KCl (0–1M) in the same buffer. Fractions (1.6ml) were collected and assayed for protein, KCl (by conductivity) and enzyme activity. Flow rates were maintained between 8–10ml/h.

Lactate dehydrogenase was assayed essentially by the method of Fritz *et al.* (1970) but using 0.1M-potassium phosphate buffer, pH7.0 instead of Tris.

For the effect of pH on the binding of lactate dehydrogenase to immobilized NAD the following buffers were used: 10mM-KH_2PO_4 adjusted to the desired pH with 0.1M-KOH, 10mM-Tris adjusted to the desired pH at 4°C with 0.1M-HCl and 10mM-glycine adjusted to the desired pH with 0.1M-KOH. Enzyme samples were dialysed against the equilibration buffer before application to the columns.

For the effect of temperature, a micro-column (5mm × 15mm) of ϵ-aminohexanoyl-NAD^+–Sepharose was established in a Quickfit Cl/00 microcondenser, the bottom end of which was plugged with glass-wool. The linear-gradient-making apparatus was immersed in a water-bath whose water was circulated by a Churchill (CH/LTC/3) circulating and heating pump through the condenser to maintain the gradient apparatus and affinity adsorbent at the same temperature. The apparatus was assembled in a cold-room at 4°C and ice added as necessary to maintain the lower temperatures. The temperature range used was 7–32°C.

Results and Discussion

Effect of pH

The effect of pH on the binding of rabbit muscle (M_4) and pig heart (H_4) lactate dehydrogenases to ϵ-aminohexanoyl-NAD^+–Sepharose is shown in Fig. 1. The binding of both enzymes changed little when the $[H^+]$ was varied over the pH range 6–8. The strength of binding of pig heart lactate dehydrogenase decreased when the pH was increased until the enzyme was eluted in the void volume of columns equilibrated with buffers of pH9 or above; this behaviour was associated with an apparent pK of about 8.6. The binding of the rabbit muscle enzyme decreased sharply above pH values of 9 and was characterized by an apparent pK of about 9.4.

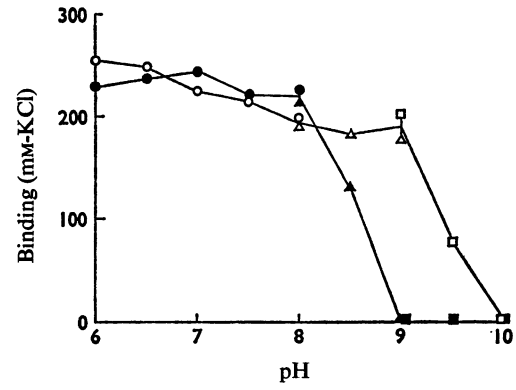

Fig. 1. *Effect of pH on the binding of pig heart muscle and rabbit skeletal muscle lactate dehydrogenases to ϵ-aminohexanoyl-NAD^+–Sepharose*

Full details of the method are given in the appropriate sections. The buffers used were: (○, ●) 10mM-potassium phosphate; (△, ▲) 10mM-Tris–HCl; (□, ■) 10mM-glycine–KOH. Open symbols refer to rabbit muscle lactate dehydrogenase and closed symbols to the pig heart enzyme.

The similar response to pH of the two isoenzymes may reflect the ionization of a group of the enzyme for the following reasons. First, the interaction of lactate dehydrogenase with immobilized NAD^+ is NAD^+-specific, because lactate dehydrogenase was bound to immobilized NAD^+ and could be eluted with an NAD^+ gradient (Fig. 2). Secondly, bovine serum albumin was not retained by the same adsorbent over the pH range investigated, despite the fact that the overall charge on both proteins was similar (pI = 4.7). This indicates an NAD^+-specific interaction of the dehydrogenase with the immobilized nucleotide. Thirdly, no distinct species that ionized over the pH range 7–10 could be detected, either on the matrix or the nucleotide itself, by direct titration of the adsorbent with 0.01M-KOH. Fourthly, the overall charges on the two isoenzymes are different (Jaenicke, 1970). The pig heart enzyme retained its overall negative charge (pI = 4.7) whereas the overall charge on the rabbit muscle enzyme (pI = 8.0) changed from positive to negative. This would imply that the ionizing species was an integral part of the active site rather than one that contributed directly to the overall surface charge of the enzyme.

The presence of residual negatively charged groups on the matrix arising from incomplete reaction of

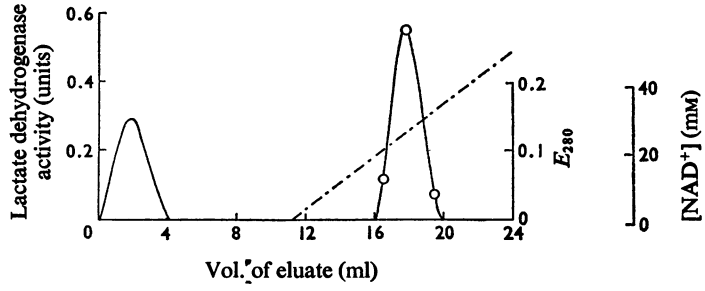

Fig. 2. *Elution of heart muscle lactate dehydrogenase from ε-aminohexanoyl-NAD⁺–Sepharose with a linear gradient of NAD⁺*

A sample (50μl) containing 1.85 units of lactate dehydrogenase and 0.8mg of bovine serum albumin was applied to a column (5mm×20mm) of the immobilized NAD^+ equilibrated with 10mM-potassium phosphate buffer, pH7.5. Non-absorbed protein was washed off with 10ml of the same buffer and the column was eluted with an NAD^+ gradient (0–50mM; 20ml total volume) in 10mM-potassium phosphate buffer, pH7.5. Lactate dehydrogenase (○) and bovine serum albumin (——) were assayed in the effluent. The NAD^+ concentration was measured by its E_{260} (– · – ·).

NAD^+ with ε-aminohexanoyl-Sepharose (Lowe & Dean, 1971; Dean & Lowe, 1972) could influence the $[H^+]$ within the domain of the gel. From polyelectrolyte theory, the response to pH of an enzyme binding to an immobilized ligand embedded in a negatively charged gel should be displaced towards more alkaline pH values. The magnitude of this response will depend largely on the overall charge of the enzyme protein. The apparent p*K* values observed with the interaction of lactate dehydrogenase with immobilized NAD^+ may be shifted to the alkaline side by 2–3 units (Goldstein *et al.*, 1964) particularly at the low ionic-strengths employed in the present study. It is thus difficult to make quantitative comparisons with results obtained when both enzyme and nucleotide are in free solution (Winer & Schwert, 1958).

Effect of temperature

The effect of temperature on the binding of lactate dehydrogenase to ε-aminohexanoyl-NAD^+–Sepharose is shown in Fig. 3. The binding decreased with increasing temperature. The decreased binding in the temperature range 5–10°C is of considerable practical significance since this range of temperature is generally experienced in a typical laboratory cold-room.

The dependence of binding on temperature is also illustrated in the corresponding Arrhenius plot (Fig. 3*b*) for which a heat of adsorption of −28.6kJ/mol (−6.8kcal/mol) can be calculated. This compares well with the energy of binding of nicotinamide nucleotides to dogfish muscle lactate dehydrogenase (McPherson, 1970) and to the bovine heart enzyme (Hakala *et al.*, 1956) when all components are in free solution.

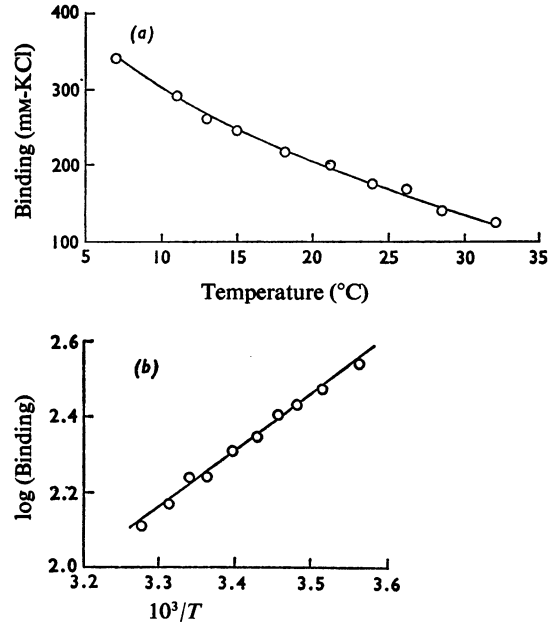

Fig. 3. *Effect of temperature on the binding of pig heart muscle lactate dehydrogenase to ε-aminohexanoyl-NAD⁺–Sepharose*

Full details of the method are given in the appropriate sections. The temperature range used was 7–32°C.

Interaction with cofactor analogues

Table 1 summarizes the results of binding of lactate dehydrogenase to some immobilized coenzymes and coenzyme analogues. Several important points emerge from the essentially similar behaviour of the two isoenzymes. First, the enzymes were specific for their preferred cofactors in that they interacted minimally or were unretarded by ε-aminohexanoyl-$NADP^+$–Sepharose, but were strongly retained by immobilized NAD^+. A similar response to the reduced forms of the cofactors was also shown. Further, the binding of both isoenzymes to adsorbents containing covalently attached 5′-AMP was as strong (H_4) or weaker than (M_4) the equivalent NAD^+ polymer, whereas retention on the immobilized $NADP^+$ analogues, 2′-AMP and 3′-AMP was weak. Secondly, the binding to immobilized AMP analogues demonstrated the effect of the mode of binding of the cofactor to the matrix on the strength of the interaction; neither isoenzyme was eluted from N^6-(6-aminohexyl)-AMP–Sepharose (Lowe *et al.*, 1972; Mosbach *et al.*, 1972) up to salt concentrations of 1M-KCl, but could readily be eluted by a 200μl 'pulse' of 5mM-NADH. In contrast, binding was relatively weak to 5′-AMP immobilized by the method of Larsson & Mosbach (1971), where most of the cofactor was bound in a form unacceptable to the active site of the enzyme (Lowe *et al.*, 1973). Thirdly, both enzymes were virtually unretarded on ε-aminohexanoyl-IMP–Sepharose. These results parallel the observations of McPherson (1970) on an analogous system in free solution.

The interaction of lactate dehydrogenase with immobilized ATP and insolubilized fragments of NAD is shown in Table 2. There was little change in the strength of binding to immobilized fragments of the coenzyme containing adenine until the 5′-phosphate was removed. Binding of the enzymes to immobilized adenosine and adenine was significantly less than that to immobilized 5′-AMP or more complete fragments of the coenzyme molecule. The binding to ADP-ribose was stronger than that to NAD^+.

McPherson (1970) and Adams *et al.* (1970) have examined the kinetics of adenosine, AMP, ADP, ADP-ribose and NAD^+ for dogfish muscle lactate dehydrogenase. The K_I values for dogfish muscle lactate dehydrogenase are in the order: adenosine $\gg$ ADP $\simeq$ AMP > ADP-ribose, suggesting that the 5′-phosphate produced a significant increase in binding affinity. All three phosphate-containing fragments produced the conformation change in crystalline lactate dehydrogenase that was characteristic of NAD^+ and NADH. It was suggested that the 5′-phosphate group anchored by the adenosine moiety interacted with charged groups on the protein to induce the conformation change (Adams *et al.*, 1970). The addition of a third phosphate to ADP to form ATP significantly lessened the ability of that compound to bind to dogfish muscle lactate dehydrogenase.

The results presented in Table 2 lead one to a similar conclusion, although uncertainties as to how the coenzyme analogues were covalently attached to the matrix (Dean & Lowe, 1972; Lowe *et al.*, 1972; Lowe *et al.*, 1973) limited the usefulness of this approach. It may be possible to resolve these problems by studies with chemically defined polymers.

Interaction with substrates and substrate analogues

Three methods were used to test the effect of the second substrate, lactate, and the product, pyruvate,

Table 1. *Binding of lactate dehydrogenase to immobilized coenzyme and coenzyme analogues*

Abbreviations: H_4, pig heart muscle lactate dehydrogenase; M_4, rabbit skeletal muscle lactate dehydrogenase. For details see the text.

	Binding (mM-KCl)	
Compound immobilized	H_4	M_4
NAD^+	295	410
NADH	220	220
N^6-(6-Aminohexyl)-AMP	>1M*	>1M*
ε-Aminohexanoyl-AMP	290	270
$NADP^+$	0	110
NADPH	45	60
2′-AMP	60	0
3′-AMP	60	0
IMP	10	0

* Elution could be effected by a 200μl 'pulse' of 5mM-NADH.

Table 2. *Binding of lactate dehydrogenase to immobilized fragments of the coenzyme molecule*

Columns (5mm×20mm) were equilibrated with 10mM-KH_2PO_4–KOH buffer, pH7.5 and developed according to the procedure outlined in the Materials and Methods section. N.D., not determined.

	Binding (mM-KCl)	
Fragment	H_4	M_4
NAD^+	295	410
ADP-ribose	310	540
ADP	290	290
5′-AMP	290	270
Adenosine	160	0
Adenine	120	0
ATP	225	N.D.

Table 3. *Effect of lactate and pyruvate on the binding of lactate dehydrogenase to immobilized NAD$^+$*

To determine the finite partition coefficient, a 6mm×200mm column of ε-aminohexanoyl-NAD$^+$–Sepharose was equilibrated with 10mM-KH_2PO_4–KOH buffer, pH7.5 containing 250mM-KCl and a sample of lactate dehydrogenase and bovine serum albumin (50μl containing 1.85 units and 0.8mg of bovine serum albumin) in the same buffer was applied to the top. The mixture was washed through with the phosphate–KCl buffer. Fractions (1.5ml) were collected at a flow rate of 8ml/h and assayed for lactate dehydrogenase and bovine serum albumin. The data are expressed as the ratio of the elution volume of lactate dehydrogenase to the elution volume of bovine serum albumin. Additions to eluting buffers were made both to dialysis buffers and throughout column development.

Additions to eluting buffers ...	Binding (mM)		
	No addition	20mM-Lactate	1.0mM-Pyruvate
KCl gradient	290	240	290
NAD$^+$ gradient	10	*	15
Finite partition coefficient	3.86	3.86	3.58

* All enzyme activity was lost.

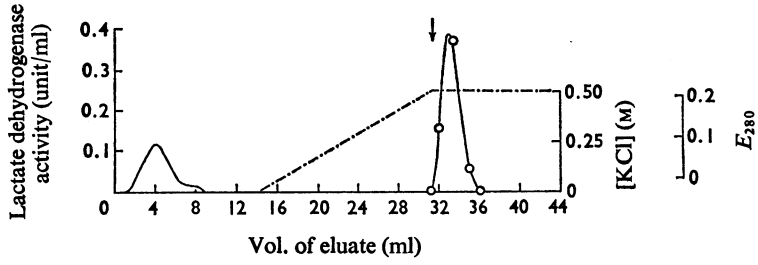

Fig. 4. *Effect of sulphite on the binding of heart muscle lactate dehydrogenase to ε-aminohexanoyl-NAD$^+$–Sepharose*

A sample (50μl) containing 1.85 units of lactate dehydrogenase and 0.8 mg of bovine serum albumin was applied to a 5mm×20mm column of the immobilized NAD$^+$ equilibrated with 10mM-potassium phosphate buffer, pH7.5 containing 1mM-sodium sulphite. Non-absorbed protein was washed off with 10ml of the same buffer and the column eluted with a linear KCl gradient (0–0.5M; 20ml total volume) in the same buffer. Lactate dehydrogenase (○), bovine serum albumin (——) and KCl concentration (– · –) were assayed in the effluent. A 'pulse' (200μl) of 5mM-NADH was added where indicated by the arrow.

on the binding of lactate dehydrogenase to ε-aminohexanoyl-NAD$^+$–Sepharose. Table 3 demonstrates that the inclusion of a constant concentration of the substrate in all buffers and elution of the enzyme with either a salt or cofactor gradient did not affect the binding of lactate dehydrogenase to immobilized NAD. This result was confirmed when lactate dehydrogenase was chromatographed on ε-aminohexanoyl-NAD$^+$–Sepharose under conditions where the concentration of KCl was such that the enzyme was eluted after several void volumes of starting buffer had been passed through the column. It was envisaged that under these conditions substrate or product might promote further retardation of the enzyme. Table 3 also shows that neither pyruvate nor lactate had any effect on this elution volume. The lack of effect of pyruvate and lactate on the binding of lactate dehydrogenase to immobilized NAD$^+$ is, at first sight, difficult to reconcile with the observations of Ohlsson *et al.* (1972), who used combinations of cofactor and substrate or product to effect the elution of biospecifically-adsorbed lactate dehydrogenase from immobilized AMP. The relatively long equilibration time (10–16h) that those authors found necessary for there to be any significant amount of desorption could account for the apparent difference, since our experiments were completed in 2h or less.

The effect of inclusion of 1mM-sodium sulphite in all buffers on the binding of lactate dehydrogenase to ε-aminohexanoyl-NAD$^+$–Sepharose is shown in Fig.

4. It is known that NAD^+ forms an adduct with sulphite and that the binding constant of the NAD^+–sulphite complex is about 3 orders of magnitude lower than that of NAD^+ alone for pig heart muscle lactate dehydrogenase (Holbrook, 1966). This was reflected in the greatly increased binding of lactate dehydrogenase to immobilized NAD^+ in the presence of 1 mM-sodium sulphite. Further, NADH is known to reverse the binding of the NAD^+–sulphite complex to lactate dehydrogenase (Holbrook, 1966); this was confirmed by the fact that although the enzyme could not be eluted by salt gradients of up to 0.5 M-KCl, it was readily and quantitatively eluted by a 200 μl 'pulse' of 5 mM-NADH.

General discussion

The aim of the present study was to correlate the interaction of lactate dehydrogenase with immobilized nucleotides and their analogues with its known behaviour when all components were in free solution. The microenvironment created by the insoluble lattice may superimpose parameters that make the direct interpretation of some of these results difficult. Thus, for example, the results of the pH and temperature studies may reflect the considerable influence of the insoluble support medium on the nature and strength of the interaction. However, where the strength of binding was more dependent on chemical (nucleotide species) rather than physical (pH and temperature) considerations, the behaviour of the soluble macromolecule–insoluble ligand interaction reflected more nearly its counterpart in free solution. Thus the correspondence between the interaction of lactate dehydrogenase with immobilized coenzymes, coenzyme analogues and coenzyme fragments and their equivalents in free solution was almost as predicted from a knowledge of the appropriate kinetic constants. This was perhaps best illustrated with reference to the effect of sulphite on the binding of lactate dehydrogenase to ϵ-aminohexanoyl-NAD^+–Sepharose. Other workers (Ohlsson *et al.*, 1972; Mosbach *et al.*, 1972) have also demonstrated similar correlations.

We are indebted to the Science Research Council for financial support and to Whatman Biochemicals for the generous donation of nucleotides used in this investigation. The authors wish to thank Mr. D. B. Craven for expert technical assistance and Dr. J. J. Holbrook for stimulating discussions and advice.

References

Adams, M. J., McPherson, A., Rossmann, M. G., Shevitz, R. W., Smiley, I. E. & Wonacott, A. J. (1970) in *Pyridine Nucleotide Dependent Dehydrogenases* (Sund, H., ed.), p. 157, Springer-Verlag, Heidelberg

Akanuma, H., Kasuga, A., Akanuma, T. & Yamasaki, M. (1971) *Biochem. Biophys. Res. Commun.* **45**, 27–33

Arsenis, C. & McCormick, D. B. (1966) *J. Biol. Chem.* **241**, 330–334

Axen, R., Porath, J. & Ernbäck, S. (1967) *Nature (London)* **214**, 1302–1304

Collier, R. & Kohlhaw, G. (1971) *Anal. Biochem.* **42**, 48–53

Cuatrecasas, P. & Anfinsen, C. B. (1971*a*) *Annu. Rev. Biochem.* **40**, 259–278

Cuatrecasas, P. & Anfinsen, C. B. (1971*b*) *Methods Enzymol.* **22**, 345–378

Dean, P. D. G. & Lowe, C. R. (1972) *Biochem. J.* **127**, 11P–12P

Edmondson, D., Massey, V., Palmer, G., Beacham, L. M. & Elion, G. B. (1972) *J. Biol. Chem.* **247**, 1597–1604

Fritz, P. J., Morrison, W. J., White, E. L. & Vesell, E. S. (1970) *Anal. Biochem.* **36**, 443–453

Gawronski, T. H. & Wold, F. (1972) *Biochemistry* **11**, 442–448

Goldstein, L., Levin, Y. & Katchalski, E. (1964) *Biochemistry* **3**, 1913–1919

Hakala, M. T., Glaid, A. J. & Schwert, G. W. (1956) *J. Biol. Chem.* **221**, 191–209

Holbrook, J. J. (1966) *Biochem. Z.* **344**, 141–152

Jaenicke, R. (1970) in *Pyridine Nucleotide Dependent Dehydrogenases* (Sund, H., ed.), p. 71, Springer-Verlag, Heidelberg

Larsson, P.-O. & Mosbach, K. (1971) *Biotechnol. Bioeng.* **13**, 393–398

Lowe, C. R. & Dean, P. D. G. (1971) *FEBS Lett.* **14**, 313–316

Lowe, C. R., Mosbach, K. & Dean, P. D. G. (1972) *Biochem. Biophys. Res. Commun.* **48**, 1004–1010

Lowe, C. R., Harvey, M. J., Craven, D. B. & Dean, P. D. G. (1973) **133**, 499–606

McPherson, A. (1970) *J. Mol. Biol.* **51**, 39–46

Mosbach, K., Guilford, H., Ohlsson, R. & Scott, M. (1972) *Biochem. J.* **127**, 625–631

Ohlsson, R., Brodelius, P. & Mosbach, K. (1972) *FEBS Lett.* **25**, 234–238

Reimann, E. M., Brostrom, C. O., Corbin, J. B., King, C. A. & Krebs, E. G. (1971) *Biochem. Biophys. Res. Commun.* **42**, 187–194

Schwert, G. W. & Winer, A. D. (1963) *Enzymes*, 2nd edn., **7**, 127

Wilcheck, M. (1972) *Biochem. J.* **127**, 7P–8P

Winer, A. D. & Schwert, G. W. (1958) *J. Biol. Chem.* **231**, 1065–1083