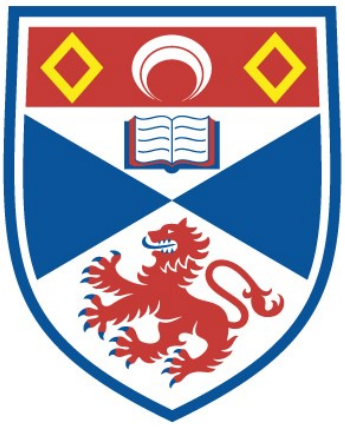
SOME STUDIES ON SOLUBLE AND IMMOBILISED DEHYDROGENASES

Stephen James Bayne

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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Some Studies On

Soluble and Immobilised Dehydrogenases

by

Stephen James Bayne

A thesis

submitted to the University of St. Andrews

in application for the degree of

Doctor of Philosophy

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July, 1974.

CERTIFICATE

I hereby certify that Stephen James Bayne has spent nine terms in research work under my direction, and that he has fulfilled the conditions of Ordinance No. 16 (St. Andrews) and that he is qualified to submit this thesis for the degree of Doctor of Philosophy.

William Ettard

DECLARATION

I hereby declare that the following thesis is based on work carried out by me, that the thesis is my own composition and that no part of it has been presented previously for a higher degree.

The research was carried out in the Department of Biochemistry of the University of St. Andrews, under the direction of Dr. William E. Hornby.

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I should like to thank my supervisor, Dr. W. E. Hornby for his advice and critiscm throughout this work and also Dr. D. L. Morris for helpful discussion. Thanks are also due to Miss Meriel Bayne for typing this thesis.

Academic Record

I matriculated at the University of St. Andrews in October 1967 and graduated with the degree of Bachelor of Science, Second Class Honours (Upper Division) in Biochemistry and Chemistry in June 1971.

In October 1971, I matriculated as a research student at the University of St. Andrews.

Abbreviations & Symbols

YADH	yeast alcohol dehydrogenase
LDH	lactate dehydrogenase (from rabbit muscle unless otherwise stated)
MDH	porcine mitochondrial malate dehydrogenase
CDI	1-cyclohexyl-3-(morpholinoethyl)-carbodimide *p*-toluene sulphonate
DAE	diaminoethane
PEI	polyethyleneimine
OAA	oxalacetic acid
Tris	2-amino-2-hydroxymethylpropane-1,3-diol
BSA	bovine serum albumin
U (unit)	micromoles NADH formed or oxidised per ml per minute under the conditions specified in each case.

Where possible, all other abbreviations and symbols conform with the recommendations in The Biochemical Journal, Instructions to Authors, 1973.

Abstract

1. YADH was immobilised on two aminoethylcelluloses and their properties were compared.
2. Experimental conditions for the coupling of YADH to Cellex-AE were optimised; these conditions were used for immobilising LDH and MDH on Cellex-AE.
3. The effect of sodium borohydride reduction on the stability of YADH, LDH and MDH immobilised on Cellex-AE was studied.
4. The effect of pH on the stability of soluble YADH, LDH and MDH was studied. The results were compared to those for the three enzymes immobilised on Cellex-AE.
5. The effect of temperature on soluble YADH, LDH and MDH was compared to the effect of temperature on several immobilised derivatives of these enzymes.
6. YADH was immobilised on DEAE-cellulose. The pH variation of the kinetic parameters of the immobilised derivatives were compared to those of soluble YADH.
7. LDH was immobilised on both Cellex-AE and PEI. The kinetic parameters and stabilities of these two derivatives were compared.

8. LDH was immobilised on NP/3 Nylon Powder. The pH variation of the kinetic parameters of the immobilised derivatives were compared to those of soluble LDH.
9. The change in equilibrium constant when YADH and LDH were immobilised on macromolecular supports was studied. A theory was developed to explain these changes.

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Introduction

Chapter 1

The study of enzyme immobilisation on macromolecular supports was stimulated by two main considerations. In the technological application of enzymes, the major expense is the cost of the reagents. Any procedure which reduced this cost is advantageous. For instance, the determination of glucose with soluble glucose oxidase involves measurement of hydrogen peroxide. Glucose oxidase, being a catalyst, is not used up during the reaction but time-consuming dialyses are necessary to recover the enzyme. If, however, glucose oxidase is immobilised on a nylon tube (Inman and Hornby, 1974; idem, 1972; J. Campbell, 1974) glucose may be determined by pumping the solution through the tube and analysing the eluant. Because the enzyme is immobilised, it may be washed free of any substrate or product by flushing the tube with buffer before the next glucose sample is measured. Similar procedures have been employed for the production of NADH by immobilised dehydrogenases (Hornby et al., 1972) and for the measurement of glutamate and oxalacetate in blood serum by transaminases (J. Campbell, 1974).

The second consideration was the idea that immobilised enzymes were mimicking the action of bound enzymes in the cell (Blaedel et al., 1972). This hypothesis cannot

easily be tested although the increased stability towards temperature on immobilisation of many enzymes (Sundaram and Hornby, 1970; Barker et al., 1968; Chang, 1972) suggests that enzymes may be attached to the cell wall or to stabilising structures in the cell. Callahan and Kosicki (1967) found that MDH is intimately associated with a mitochondrial lipid.

Enzymes from most of the main groups have been immobilised: oxidoreductases, transferases, hydrolases, lyases and isomerases. Most work has been carried out on hydrolases, particularily trypsin (Habeeb, 1967; Haynes and Walsh, 1969; Gabel, 1973), chymotrypsin (Kay and Lilly, 1970), papain (Goldstein et al., 1970) and urease (Sundaram and Hornby, 1970). Much less work has been carried out on the oxidoreductases and especially the dehydrogenases. YADH has been immobilised by Wieland et al. (1966), by Manecke and Guenzel (1962) and by Hornby et al. (1972). The first two groups reported only its activity when immobilised and its stability; Hornby et al. used YADH immobilised on nylon tube for the continuous production of NADH for autoanalytical circuits. LDH has been immobilised by Wilson et al. (1968); recently Dixon et al. (1973) and Cho and Swaisgood (1974) immobilised LDH on glass to study its catalytic properties and stability. MDH

has been immobilised on Sephadex G-50 (Srere et al., 1973) as one component of an immobilised three-enzyme system. As for YADH, only its activity is reported.

Three main methods have been used for immobilisation of enzymes. The first is simple adsorption of the enzyme on a carrier (Thang et al., 1968); bonding may be ionic, hydrophilic or hydrophobic. The second main method is encapsulation of the enzyme. Chang (1972) immobilised L-asparaginase in semipermeable microcapsules; Chang and Poznansky (1974) compared the enzyme kinetics and immunological properties of free catalase and of catalase immobilised by encapsulation. The substrates and products can diffuse in and out of the microcapsules but larger molecules such as enzymes and antibodies cannot. The third method of immobilising enzymes is by covalent attachment to a macromolecular support, of which there are many examples in the literature (Melrose, 1971). Many support materials such as glass (Cho and Swaisgood, 1974), polystyrene (Hornby et al., 1970) and nylon (Hornby and Filippusson, 1970) to naturally occurring polymers such as cellulose (Wilson et al., 1968) have been used. The chemistry of enzyme attachment is also very varied (Melrose, 1971). An active immobilised enzyme is obtained by selecting the most suitable support material and the best method of

attachment for the enzyme in question.

Many reports on immobilised enzymes quote only the activities of the enzyme-supports and their stability towards temperature. Several reports discuss the changes of Michaelis parameters on immobilisation of enzymes and attempt to account for these changes. Gabel (1973) has discussed how different amino groups stabilise the product towards denaturation by urea. Much emphasis has been placed on immobilising enzymes; there has been little investigation of the mode of attachment. For most coupling reagents, the types of amino acid residue involved in covalent binding can be predicted; what cannot be predicted so easily is at what point on the enzyme surface coupling is occurring. Much work is necessary to establish the locations on the enzyme subunit of the amino acid residues involved in coupling; also the variation of these residues with coupling time and pH.

One of the reasons for immobilising enzymes is to increase their stability at temperatures at which the soluble enzyme is quickly inactivated. In the present work, the relatively unstable enzyme, YADH, was immobilised on Cellex-AE. The effect of immobilisation on its catalytic parameters and stability was investigated. These results were compared with those obtained when LDH, a more stable enzyme, was examined

in the same way. The results for these two enzymes, which are tetramers, were compared with those obtained for MDH, a dimer.

YADH catalyses the following reaction:

$$RCH_2OH + NAD^+ \rightleftharpoons RCHO + NADH + H^+$$

The equilibrium is shifted to the right at pH9.0 and to the left at pH6.0. The reduction of NAD^+ is known as the forward reaction; the oxidation of NADH is known as the reverse reaction. YADH oxidises many primary straight-chain alcohols, but reactivity with branched-chain alcohols is very low. Reactivity with the straight-chain homologous series decreases as the chain length increases (van Eys and Kaplan, 1957). Very little work has been reported on different aldehydes as substrates for YADH.

It is a thiol enzyme with a molecular weight of about 150,000 (Kagi and Vallee, 1960; Hayes and Velick, 1954) and has four very similar, if not identical subunits, each of molecular weight 35,000, (Kagi and Vallee, 1960; Harris, 1964). Since the original work of Hayes and Velick (1954), it has been widely accepted that a molecule of YADH contains four apparently identical active sites between which there is no interaction. Harris (1964) later supported these observations. Recently, Dickinson (1970) has cast some

doubt on these conclusions and has suggested that there is a maximum of three binding sites per molecule. This is explained by negative cooperativity - the affinity of the coenzyme for unfilled sites is lessened by increased saturation of the enzyme. More recently Yamada and Yamato (1973) have also concluded that the coenzyme binding sites interact negatively with each other; they postulate that the maximum number of substrate molecules bound on ternary complexes might be two instead of four. Dickinson (1974) reported that reaction with two equivalents of iodoacetate gave a 90 - 95% loss of activity, these observations suggesting two essential thiol groups per molecule. Measurements of NADH binding indicate a similar number of coenzyme binding sites.

In solutions with pH values below 6.0 and above 8.5, YADH is unstable (van Eys et al., 1957). It is very sensitive to heavy metal ions which cause loss of activity. Because of this, YADH was kept as a lyophilised preparation until required for coupling experiments. When required for soluble enzyme studies, it was stored as a 1mg/ml solution containing 0.1% (w/v) BSA at $4^{o}C$ in phosphate buffer (pH7.4,I0.1).

When YADH is coupled to a macromolecular support using glutaraldehyde as the coupling reagent, amino, sulphydryl, phenolic and imidazole groups are involved (Habeeb and Hiramoto, 1968). The amino groups involved

are on lysine residues. The N-terminal amino acid residue does not react with glutaraldehyde as it is acylated (Jörnvall, 1973).

Sund and Theorell (1963) reported that the rate-limiting step in the YADH reaction was intramolecular hydrogen transfer. Using modern spectrophotometric and fluorimetric techniques, Dickinson and Monger (1973) reported that results for the NADH-acetaldehyde reactions are consistent with a compulsory ordered mechanism. In contrast, the results for the NAD^+-ethanol reactions indicate that some dissociation of the coenzyme from the active enzyme- NAD^+ - ethanol ternary complex must occur, and that the mechanism is not strictly a compulsory order one. The rate-limiting step for both reactions is the dissociation of the of the enzyme-coenzyme complexes.

LDH catalyses the following reaction:

$$\text{Lactate} + NAD^+ \rightleftharpoons \text{Pyruvate} + NADH + H^+$$

As for YADH, NAD^+ reduction is the forward reaction; NADH oxidation is the reverse reaction. The equilibrium lies to the right at pH10.0 and well to the left at pH6.0. Activity rapidly diminishes upon increasing of the chain length of the 2-keto acids (Meister, 1950).

The molecular weight of LDH has been reported as 142,000 ± 3,600 by Jaenicke and Knof (1968) and as 155,000 by Cho (1973). LDH is also a tetrameric thiol protein and dissociation to the dimer is pronounced at concentrations less than 1mg/ml in buffer, pH7.0, (Cho and Swaisgood, 1973). These authors also reported that NADH prevents this dissociation and that strenuous treatment is necessary to dissociate the LDH tetramer to the monomer.

Kinetic studies indicate that both NAD^+ and NADH bind at the same site on the enzyme surface (Alberty, 1953). Anderson and Weber (1966) found that four coenzyme molecules were bound on the tetramer. The reaction mechanism was investigated by Zewe and Fromm (1962, 1965) by using product inhibition studies. These authors have proposed a compulsory addition sequence for LDH. The coenzyme must bind before the substrate; after interconversion of the ternary complexes, the product dissociates first from the enzyme-coenzyme complex.

Compared to YADH, LDH is a stable enzyme with respect to pH. For coupling procedures, however, LDH was kept as a lyophilised preparation until required. When used for soluble enzyme studies, it was dissolved in phosphate buffer (pH7.0,I0.1) containing 0.1% bovine serum albumin at a concentration of 1mg/ml and stored at $4^{o}C$.

MDH catalyses the following reaction:

$$\text{Malate} + NAD^+ \rightleftharpoons \text{Oxalacetate} + NADH + H^+$$

As for YADH and LDH, NAD^+ reduction is the forward reaction. At pH10.0, the equilibrium lies to the right; at pH6.0 it lies well to the left.

MDH is a dimeric thiol enzyme with a molecular weight of 70,000 (Thorne and Kaplan, 1963). It consists of two similar or identical subunits, each of molecular weight 35,000 (Devenyi et al., 1966). MDH binds one molecule of NADH per subunit (Holbrook and Wolfe, 1972). Early work by Raval and Wolfe (1962) suggested a compulsory-ordered reaction mechanism for MDH with the coenzyme binding first. Later work (Harada and Wolfe, 1968) reported a more complex compulsory ordered mechanism. Many properties of porcine heart mitochondrial MDH have been studied by Raval and Wolfe (1962).

A common feature of these three dehydrogenases is the presence of a cysteine residue at the active centre. For MDH, Sequin and Kosicki (1967) indicated that two sulphydryl groups per molecule are involved in substrate binding. Fondy et al. (1965), Dube et al. (1963), Sabato and Kaplan (1963) and Holbrook and Stinson (1970) have all shown the presence of cysteine residues in LDH. Harris (1964) showed there were four essential thiols per mole of YADH; Jörnvall (1973) has shown

that the reactive cysteine residue in YADH is the forty third from the N-terminal.

In the present work, an attempt was made to characterise immobilised derivatives of YADH, LDH and MDH by studying their pH stability, temperature stability and kinetic parameters; comparisons were made with the soluble enzymes.

Materials and Methods

Chapter 2

2.1. Water

Glass distilled water was used for the preparations of all solutions. This water had a pH of approximately 6.0. Double-distilled water was used for all reactions involving soluble YADH, NADH and acetaldehyde.

2.2. Buffers

Whenever buffers of specified ionic strength were used, these were made up according to the data of Datta and Grzybowski (1961). Where data were not available for specific buffers, these were calculated from the equation

$$I = 1/2 \sum i z_i^2$$

The pH of all buffers was checked using a Pye 'Dynacap' pH meter (W. G. Pye & Co. Ltd., Cambridge, UK). For each buffer, the instrument was calibrated using a standard reference buffer of pH6.86 at 25°C (Beckman Instruments Inc., Fullerton, California, USA).

2.3. Inorganic and Organic Reagents

Wherever possible, analytical grade reagents were used without further purification. Ethanolamine and DAE (Fisons Scientific Apparatus Ltd., Loughborough, Leicestershire), and N,N-dimethylaminopropylamine (Koch-Light Laboratories

Ltd., Colnbrook, Bucks.) were distilled before use; fractions distilling within the correct boiling range were collected.

2.4. Enzymes

2.4.1. Yeast Alcohol Dehydrogenase (EC.1.1.1.1)

Two lyophilised YADH preparations (Sigma London Chemical Co. Ltd., Norbiton Station Yard, Kingston-upon-Thames, Surrey) were used. One preparation contained 36%(w/w) of phosphate and sucrose and had an activity of 430 U/mg protein. The other preparation contained 2%(w/w) citrate and had an activity of 450 U/mg protein. Both preparations were dissolved in the required buffer and the protein concentration measured spectrophotometrically (Hayes & Velick, 1954) using the formula

$$E^{1\%}_{280nm} = 12.6$$

When required for coupling, the enzyme was freshly dissolved in the appropriate buffer. Otherwise it was stored for five days at $4^{o}C$ in the phosphate buffer (pH7.4,I0.1) containing BSA (0.1% w/v).

2.4.2. Liver Alcohol Dehydrogenase (EC 1.1.1.1)

Liver alcohol dehydrogenase (The Boehringer Corporation (London) Ltd., Bilton House, Uxbridge Road, Ealing, London) was obtained as an

ammonium sulphate suspension containing 10mg liver ADH/ml (2.7U/mg protein).

2.4.3. Rabbit Muscle Lactate Dehydrogenase (EC 1.1.1.27)

Two LDH preparations (Sigma Chemical Co. Ltd.) were used. One of these was an ammonium sulphate suspension containing 10mg LDH/ml (900U/mg protein), which was purified by gel filtration, on a G-25 Sephadex column as described in Section 3.1.1.1 before use in coupling experiments. The second preparation was a salt-free lyophilised powder which had an activity of 700U/mg protein. The protein concentration of the latter preparation was measured spectrophotometrically (Zewe and Fromm, 1962) using the formula

$$E^{1\%}_{280nm} = 12.9$$

Preparation (of the enzyme) for the coupling and storage were as described for YADH in Section 2.4.1.

2.4.4. Pig Heart Lactate Dehydrogenase (EC 1.1.1.27)

Pig heart LDH (Boehringer) was obtained as an ammonium sulphate suspension containing 10mg LDH per ml (400U/mg protein).

2.4.5. Malate Dehydrogenase (EC 1.1.1.37)

Porcine heart (mitochondrial) MDH

(Boehringer Corporation Ltd.) was obtained as an ammonium sulphate suspension containing 5mg MDH/ml (about 1000 U/mg protein).

2.5. General

When substrates and coenzymes were required for kinetic studies, they were freshly prepared on the day of use.

2.6. Substrates

2.6.1. Ethanol

Absolute ethanol was used in all studies. Dilutions of this reagent for kinetic experiments were made with the required buffer on the day of use.

2.6.2. Acetaldehyde

Acetaldehyde was redistilled on the day required, using a silica-glass distillation apparatus.

2.6.3. Sodium Pyruvate

Sodium pyruvate (Sigma Chem. Co. Ltd.) stock solution (8mg/ml) was made up every three days by dissolving the salt in water and storing at $4^{o}C$. Dilutions, when required for kinetic studies, were made with the appropriate buffer.

2.6.4. Sodium Lactate

Sodium lactate, a 70% (w/w) solution in water (BDH Chemicals Ltd., Poole, Dorset) was used to prepare a stock solution of approximately 1M every three days by diluting with water. Dilutions, when required for kinetic studies, were made using the appropriate buffer.

2.6.5. Oxalacetic Acid

Grade 1 OAA (Sigma Chemical Co. Ltd.) was used to prepare a stock solution of 1mg/ml in distilled water daily. For kinetic studies, the stock solution was diluted with the appropriate buffer.

2.6.6. Malic Acid

Malic acid (Sigma Chemical Co. Ltd.) was used to prepare a stock solution of 15mg/ml in distilled water daily and diluted where required with the appropriate buffer for kinetic studies.

2.7. Coenzymes

2.7.1. β-nicotinamide adenine dinucleotide

β-NAD^+ (Grade III, Sigma Chemical Co. Ltd.) was dissolved in water and used within three days (see Section 3.1.3). The final pH of these solutions was about 3.5. Where NAD^+ was required for soluble YADH kinetic studies, it was purified on a DE50 cellulose

column according to the method of Dalziel and Dickinson (1966).

2.7.2. β-Dihydronicotinamide adenine dinucleotide

β-NADH (Grade III, Sigma Chemical Co. Ltd.) was dissolved in water and used within three days. A few crystals of Tris were added to bring the pH to about 8.5 and the NADH solutions were stored at 4°C.

2.8.1. Measurement of Enzyme Activity

The activity of the various dehydrogenases was measured as the rate of change in absorbance at 340nm at 25°C using a Beckman DBGT spectrophotometer with a water-jacketted cell carriage (Beckman Instruments, Glenrothes, Scotland). Soluble or free enzymes are described as those which have not been chemically modified or attached to any macromolecular supports and their activities are measured in U/mg of free enzyme. An immobilised enzyme is one which has been chemically or physically attached to a macromolecular support and its activity is measured in U/g support enzyme. The immobilised derivatives were assayed in a "stirring cuvette". This is explained in the Experimental chapter (see Section 3.4).

2.8.2. YADH

The activity of both free and immobilised YADH was measured by following the increase at 340nm owing to NADH formation. Unless otherwise stated, activity was determined at 25°C and in pyrophosphate buffer (pH9.0,I0.1). Assays were started by the addition of either 10µl of soluble enzyme or 100µl of a suspension of the immobilised enzyme.

2.8.2.1. Assay Procedure

2.8.2.1.1. Soluble YADH

Into a 1cm-lightpath cuvette was pipetted 2.7ml pyrophosphate buffer (pH9.0,I0.1), 200µl NAD^+ solution (15mg/ml in water - this will be referred to as the NAD^+ stock solution in later sections), and 100µl ethanol, all of which had been previously incubated to 25°C. The contents of the cuvette were thoroughly mixed and the reaction started by the addition of 10µl of the enzyme solution. Results are expressed as U/mg enzyme.

2.8.2.1.2. Immobilised YADH

Into a 1cm-lightpath cuvette was pipetted 3.1ml pyrophosphate buffer (pH9.0,I0.1), 200µl stock NAD^+ solution and 100µl ethanol, all of which had been previously incubated to 25°C.

Stirring was started using the overhead stirrer described in Section 3.4 and in Plate II, and 100µl of the immobilised enzyme suspension added to start the reaction. Results were expressed as U/g enzyme-support.

2.8.3. LDH

The activity of both free and immobilised LDH was measured spectrophotometrically by following the oxidation of NADH at 340nm. Assays were started as described in Section 2.8.2.

2.8.3.1. Assay Procedure

2.8.3.1.1. Soluble LDH

Into a 1cm-lightpath cuvette was pipetted 2.8ml phosphate buffer (pH7.4, I0.1), 100µl NADH solution (4mg/ml dissolved in water with a few crystals of Tris added - this is the stock solution referred to in the later sections), and 100µl pyruvate stock solution, all previously incubated to 25°C. The contents were thoroughly mixed and the reaction started by the addition of 10µl of the soluble enzyme. Results were expressed as U/mg enzyme.

2.8.3.1.2. Immobilised LDH

Into a 1cm-lightpath cuvette was pipetted 3.2ml phosphate buffer (pH7.4, I0.1), 100µl stock

NADH solution, and 100µl stock pyruvate solution, all previously incubated to 25°C. Stirring was started using the overhead stirrer as described in Section 3.4 and 100µl enzyme suspension added to start the reaction. Results were expressed as U/g enzyme-support.

2.8.4. MDH

The activity of both free and immobilised MDH was measured essentially as described in Sections 2.8.3.1.1 and 2.8.3.1.2 for free and immobilised LDH. The only difference was that 100µl of the stock solution was used in place of the 100µl stock pyruvate solution. Results were expressed as described in Sections 2.8.3.1.1 and 2.8.3.1.2.

2.9. Determination of Substrates and Coenzymes

The concentration of substrates and coenzymes, for all three enzymes, were measured enzymatically.

2.9.1 Ethanol

Into a 1cm-lightpath cuvette was pipetted 100µl NAD^+ stock solution, a volume of ethanol, generally 100µl or 200µl, which, when oxidised to acetaldehyde by NAD^+, would give an optical density change of 0.4 - 0.6 units owing to the production of NADH. Glycine

buffer (pH9.8,I0.1) was added to give a final volume of 3.0ml. The contents of the cuvette were thoroughly mixed, a base-line obtained, 10µl of the YADH solution (5mg/ml) added and the optical density increase at 340nm measured. The concentration of ethanol was calculated using a molar absorptivity of 6.22×10^3 for NADH.

2.9.2. NAD^+

The method was as described in Section 2.9.1 but that 100µl ethanol and enough NAD^+ was used, which, when completely reduced, would give an optical density change of 0.4 - 0.6 units. Calculation of the NAD^+ concentration was as in Section 2.9.1.

2.9.3. Acetaldehyde

Into a 1cm-lightpath cuvette was pipetted 100µl stock NADH solution, enough acetaldehyde to give an optical density decrease of 0.4 - 0.6 units, when reduced to ethanol, and acetate buffer (pH5.4,I0.1) to give a final volume of 3.0ml. The procedure was then as described in Section 2.9.1 and the optical density change measured, thus allowing calculation of the acetaldehyde concentration using a molar absorptivity of 6.22×10^3 for NADH.

2.9.4. Pyruvate

The method was as for acetaldehyde in Section 2.9.3 with pyruvate replacing acetaldehyde and phosphate buffer (pH6.0,I0.1) replacing acetate buffer. The reaction was started by addition of 10µl of LDH solution (5mg/ml).

2.9.5. Oxalacetate

The method was as for acetaldehyde in Section 2.9.3 with OAA replacing acetaldehyde. The reaction was started by the addition of 10µl MDH solution (5mg/ml).

2.9.6. NADH

Into a cuvette was pipetted 100µl stock solution, sufficient NADH when completely oxidised to give an optical density change of 0.4 - 0.6 units and phosphate buffer (pH6.0,I0.1) to give a final volume of 3.0ml. The reaction was started by the addition of 10µl LDH solution (5mg/ml) and the NADH concentration calculated as previously described in Section 2.9.).

2.9.7. Lactate

For the determination of lactate, the method was essentially that described by Hohorst (1963). Into a 1cm-lightpath cuvette was pipetted 2.5ml hydrazine-glycine buffer (pH9.5), 0.3ml stock

NAD^+ solution and 100µl sodium lactate solution (~1.5mM). A zero reading was obtained, the reaction started by the addition of 20µl of LDH solution (5mg/ml) and the increase in absorbancy at 340nm measured. The end-point was reached after about 20min and the concentration of the lactate then calculated.

2.9.8. Malate

The method was essentially as described in Section 2.9.7 but malic acid solution (~1.5mM) replaced the sodium lactate, and the reaction was started by the addition of 10µl of MDH (5mg/ml).

2.10. Kinetic Assays

2.10.1. Soluble Enzymes

All soluble enzyme assays were carried out in a cuvette containing a total volume of 3.0ml. Reactions were started by the addition of 10µl enzyme solution, which had been previously diluted to give a rate, under saturating substrate conditions, with a slope of about 45° on the 20mV range of the recorder. All assays were carried out at 25°C and rates were obtained by measuring the rate of increase or decrease of NADH formation at 340nm on a Beckman DRGT spectrophotometer. Rates were linear for at least

2 minutes.

2.10.2. Immobilised Enzymes

All immobilised enzyme assays were carried out in a cuvette containing a total volume of 3.5ml. Reactions were started by the addition of 100µl of enzyme suspension, diluted if necessary with the storage buffer. The substrates and buffers were mixed, equilibrated at 25°C and zero time readings obtained on the Beckman spectrophotometer before addition of the immobilised enzyme.

2.11. Equilibrium Studies

All studies were carried out at 25°C. For both YADH and LDH studies, the amount of NADH formed at equilibrium was measured, and as the initial concentrations of both reactants were known, the equilibrium concentrations of all four products were calculated. The total reaction volume was 6.1ml for the soluble enzyme, and 5.2ml for the immobilised enzyme studies.

2.11.1. YADH Studies

Into a 10ml conical flask was pipetted 200µl aqueous ethanol (~M), varying volumes of NAD^+ (~600µM-fresh stock solution diluted with phosphate

buffer (pH7.0,I0.1)), and the volume made up to 6.0ml with phosphate buffer (pH7.0,I0.1). The solutions were mixed, equilibrated at 25°C, and zero time readings obtained on the Beckman spectrophotometer.

2.11.1.1. Soluble YADH

100µl YADH solution (1mg/ml) was added to start the reaction and the conical flasks were incubated at 25°C for 3h when equilibrium was attained. The final optical density was then read and the pH of the solution measured.

2.11.1.2. Immobilised YADH

5.0ml of the reactant volume was transferred to a thermostatted stirring chamber, 200ul of enzyme suspension was added, and the suspension stirred for 3h at 25°C. The suspension was then filtered through a Sartorius filter (pore size = 1.2µ), the equilibrium optical density read, and the pH of the solution measured.

2.11.2. LDH Studies

The methods involved for the determination of the equilibrium constants were essentially the same as described in Sections 2.11.1.1 and 2.11.1.2 with the following differences.

The reactant solution contained 0.2ml sodium lactate (~1M), varying amounts of NAD^+ (~300μM - fresh stock solution diluted with pyrophosphate buffer (pH9.0,I0.1)) and the volume made up to 6.0ml with pyrophosphate buffer (pH9.0,I0.1).

2.12. Heat Inactivation Studies

A solution of the soluble enzyme or a suspension of the immobilised enzyme in phosphate buffer (pH7.4,I0.1), for YADH and LDH and in pyrophosphate buffer (pH8.4,I0.1) for MDH, was stirred by a submersible stirrer in a waterbath, preheated to the required temperature. 10μl samples of the soluble enzyme or 100μl samples of the immobilised enzyme were removed at various time intervals, pipetted into a cuvette, and assayed for residual enzymic activity.

2.12.1. YADH Assays

These assays were carried out in pyrophosphate buffer (pH9.0,I0.1). For the soluble enzyme, a 3.0ml volume contained 100μl absolute ethanol and 100μl NAD^+ solution (10mg/ml dissolved in water). The immobilised enzyme assay cuvette contained the same quantities of the substrate and coenzyme but the total volume was increased to 3.4ml.

2.12.2. LDH Assays

For the soluble enzyme, the cuvette contained 100µl stock sodium pyruvate solution, 100µl stock NADH solution, and phosphate buffer (pH7.4,I0.1) in a total volume of 3.0ml. The immobilised enzyme assay contained the same volumes of substrate and coenzyme but the final volume was increased to 3.4ml.

2.12.3. MDH Assays

The assays for both the soluble and the immobilised enzymes were identical to those described in Section 2.12.2 for LDH but 100µl stock pyruvate solution was replaced by 100µl stock OAA solution.

2.13. Effect of pH

2.13.1. Short Term Stability

The effect of pH on the stability of soluble YADH, LDH and MDH was studied. Buffers were selected to cover the pH range from 5 to 10 and were made up according to the data of Datta and Grzybowski (1961). 100µl of each enzyme solution (1mg/ml in phosphate buffer (pH7.4,I0.1)) was added to 3.0ml of each of the buffers and the solution kept at 4°C for 2h. The initial activity of each enzyme stock solution was measured and also

the activity remaining after 2h. Two assays were carried out on each solution and the average result used. Standard assays were used as described in Section 2.8.2.1.1 for soluble YADH, Section 2.8.1.1 for soluble LDH and Section 2.8.4 for soluble MDH.

2.13.2. Long Term Stability

The effect of pH on the long term stability at 4°C of both soluble and immobilised YADH, LDH and MDH was studied. Buffers in the pH range 5.4 to 9.0 were used. For the soluble enzymes, 100µl of stock enzyme solution (1mg/ml) was added to 3.0ml of various buffers. For the immobilised enzymes, 1.0ml of the enzymes suspension was added to 5.0ml of the various buffers. Assays were carried out as described in Sections 2.8.2.1.1, 2.8.2.1.2, 2.8.3.1.1, 2.8.3.1.2 and 2.8.4 for the various enzymes over a period of two months.

2.14. Supports

2.14.1. Carboxymethylcellulose (CM-cellulose)

CM-cellulose (Whatman Chemicals) was modified by the method described in Section 2.14.1.1 as a preliminary to attachment of YADH. The chemical modification is presented in Fig. 1.

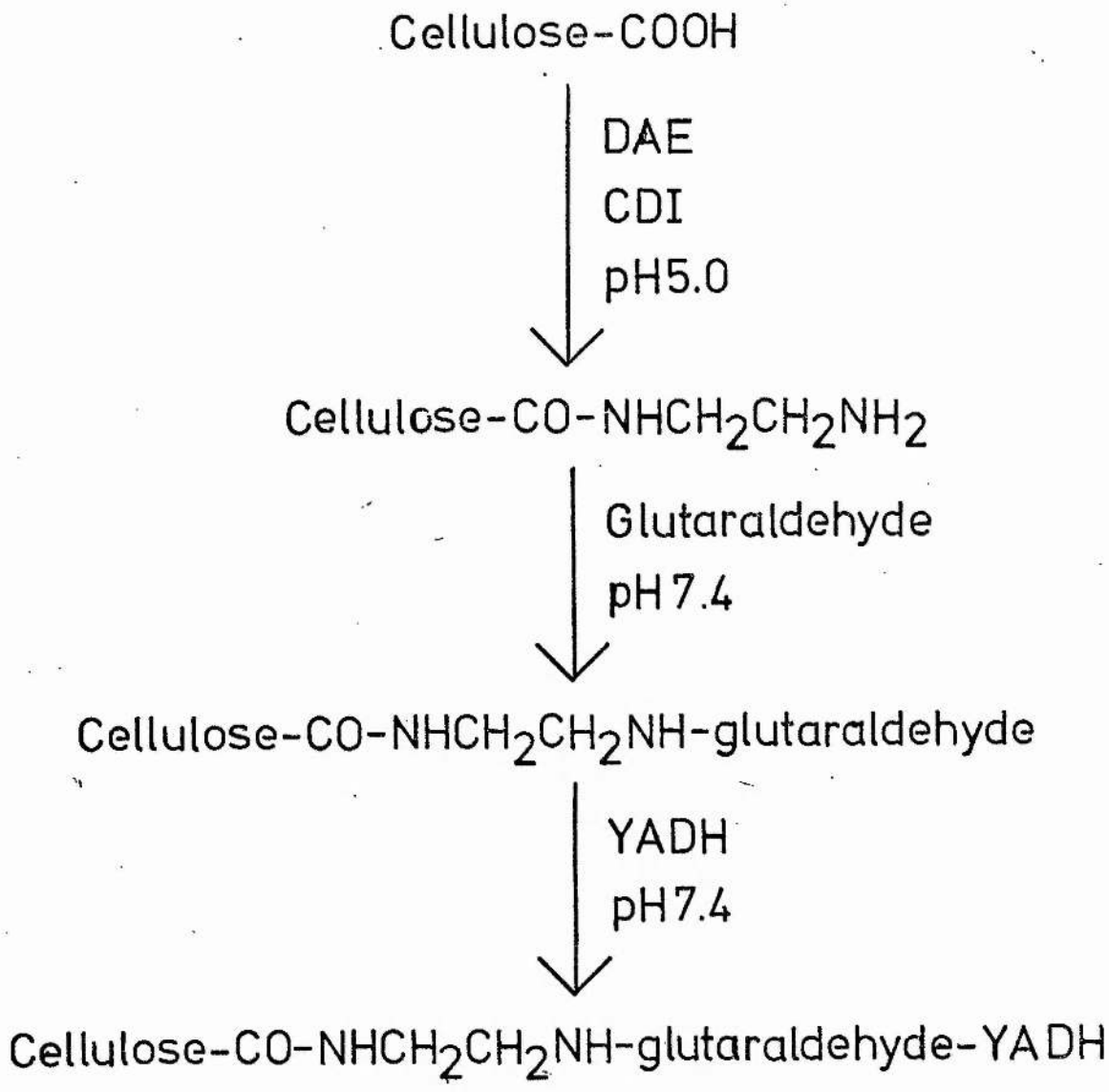


Fig. 1.

Modification of CM-Cellulose, followed by activation with glutaraldehyde and subsequent coupling of YADH.

2.14.1.1. Preparation of "AE-cellulose"

"AE-cellulose" is defined as a microgranular carboxymethylcellulose which has been treated with diaminoethane in the presence of CDI (Ralph Emanuel Ltd., Alperton, Middlesex). In a typical experiment, 0.5g CM-cellulose (containing 0.5mequiv-COOH groups) was suspended in 60ml distilled water at room temperature and stirred continuously. 3.3ml DAE (containing 25mequiv-NH_2 groups) was added and the suspension titrated to pH5.0 with conc. HCl on a pH-stat (Radiometer, Copenhagen). While the pH was maintained at 5.0 by the addition of 0.5M-HCl, 2.5g CDI dissolved in 15ml distilled water was added to the stirred slurry and the reaction allowed to proceed for 16h. The slurry was then centrifuged and washed with water (5 x 100ml). The moist modified Cellulose was finally suspended in 20ml phosphate buffer (pH7.4,I0.1).

2.14.2. Cellex-AE

Cellex-AE (Bio-Rad) was stirred with 0.5M-HCl for 15min then washed on a sintered glass filter with distilled water until the effluent was neutral. Stirring with 0.5M-NaOH for 15min was followed by washing as above with distilled water until the effluent was again neutral. It was

finally suspended in phosphate buffer (pH7.4,I0.1) at a final concentration of about 50mg/ml.

2.14.2.1. Preparation of Aminoethylcellulose-glutaraldehyde derivatives

Glutaraldehyde was coupled to both "AE-cellulose" and Cellex-AE by the following method. 200-250mg modified cellulose was stirred in 5.0ml of phosphate buffer (pH7.4,I0.1) at room temperature. 0.5ml of 25% (w/w) glutaraldehyde (Koch-Light) was added and the suspension stirred for 3min at room temperature. It was then thoroughly washed with phosphate buffer (pH7.4,I0.1) on a sintered glass filter and used immediately for coupling to protein.

2.15. Modification of DEAE-cellulose

The attachment of modified cyanuric chloride, prepared as described in Section 3.3, to microgranular DEAE-cellulose (Whatman) was carried out using a modification of the method of Kay and Lilly (1970). In a typical experiment, 2.5ml distilled water was added to 1g DEAE-cellulose and the suspension stirred at 50°C on a waterbath. 200mg of the cyanuric chloride derivative was dissolved in 5.0ml acetone and 5.0ml distilled water added. This solution was heated to 50°C and

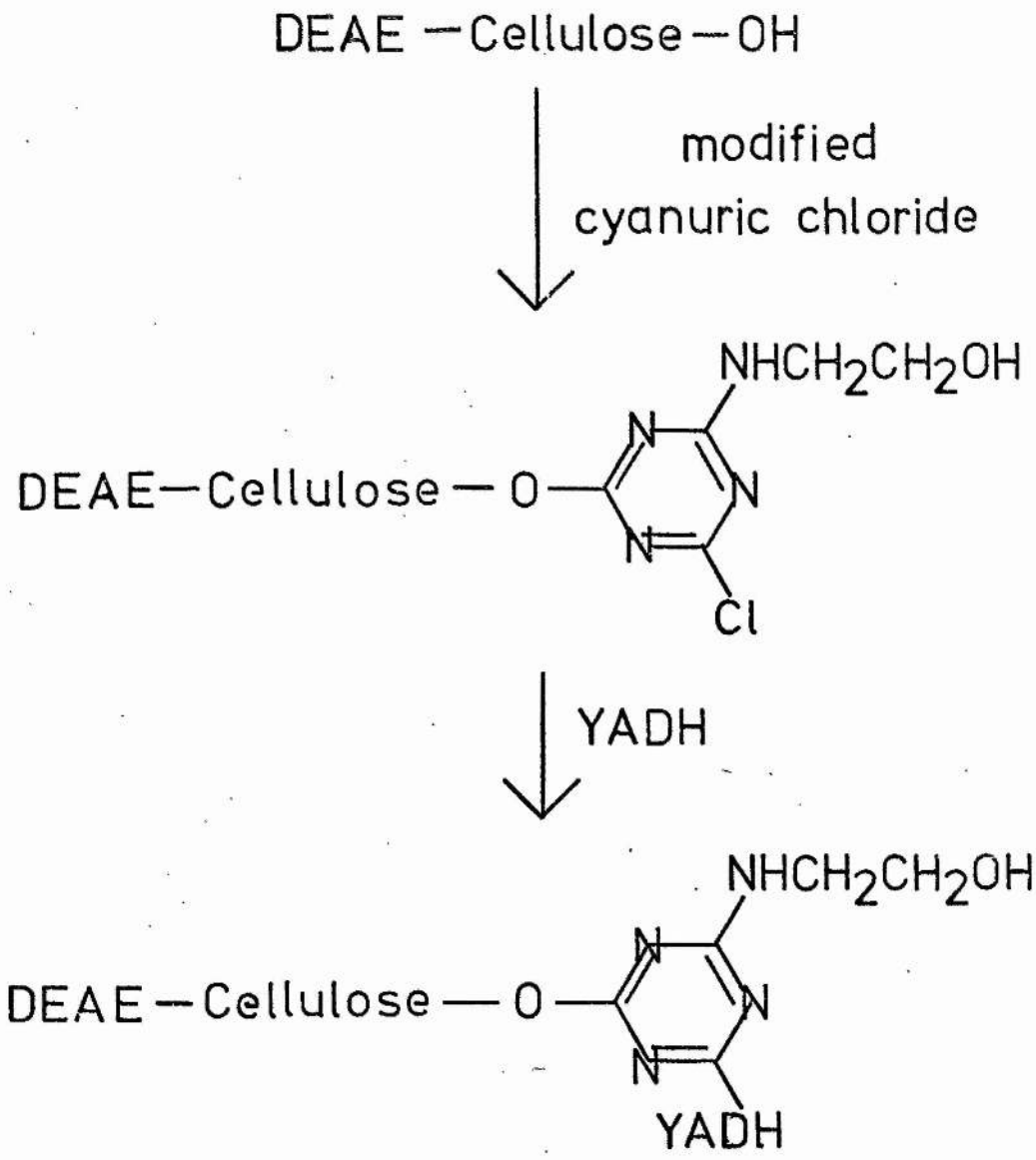
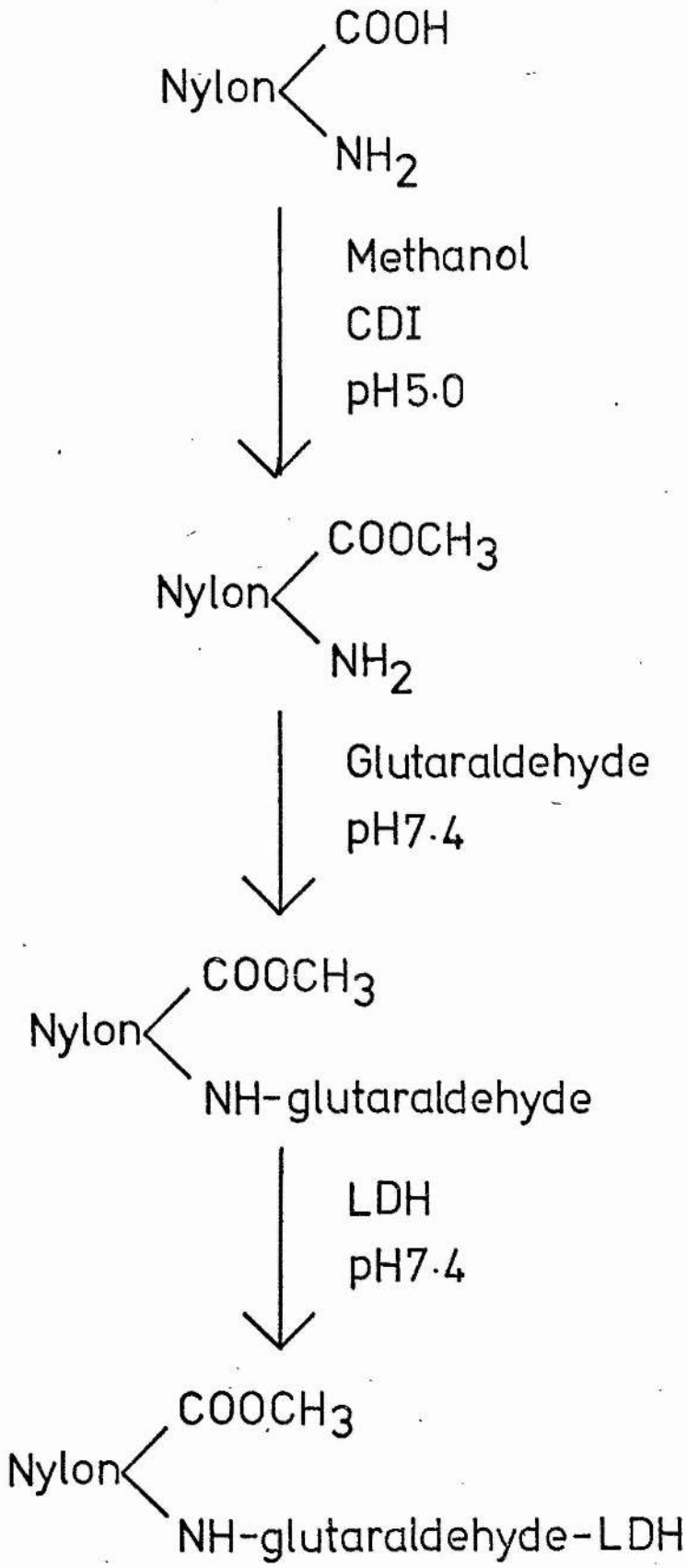


Fig. 1(b).

Activation of DEAE-Cellulose by modified cyanuric chloride and subsequent coupling of YADH.

Fig. 2.

Modification of NP/3 Nylon Powder, followed by activation with glutaraldehyde and subsequent coupling of LDH.


COOH
Nylon
NH2
Methanol
CDI
pH5.0
COOCH3
Nylon
NH2
Glutaraldehyde
pH7.4
COOCH3
Nylon
NH-glutaraldehyde
LDH
pH7.4
COOCH3
Nylon
NH-glutaraldehyde-LDH

a 5.0ml aliquot was transferred to the DEAE-cellulose suspension. The suspension was stirred at 50°C for a further 15min, then 2.0ml of a 15% (w/w) aqueous solution of sodium carbonate, containing 0.6 volumes 1M-HCl, were added and stirring continued for a further 5min at 50°C. The suspension was removed from the waterbath, cooled, and 6M-HCl was added dropwise to bring the pH of the suspension to neutrality. The DEAE-cellulose derivative was quickly filtered on a sintered glass filter and washed successively with 100ml acetone, 100ml 50% (v/v) acetone-water, and finally 200ml water. It was suspended in phosphate buffer (pH7.4,I0.1) and used immediately for coupling to protein. The modification is presented in Fig. 1(b).

2.16. Nylon

Nylon powder (NP/3) was obtained as a gift from ICI (Dr. J. Mears, ICI Agricultural Division, Billingham, County Durham). This material had an exchange capacity of 0.0835mequiv amino groups per g and 0.077mequiv carboxyl groups per g. It was modified as described in Section 2.16.1, and as shown schematically in Fig. 2.

2.16.1. Modification of NP/3 Nylon Powder

1g nylon powder (NP/3) was suspended in

25ml distilled water and was titrated to pH5.0 with conc. HCl on the pH-stat. 10mequiv of methanol was added to the slurry followed by 1.5g CDI dissolved in 5ml distilled water. The pH was kept at 5.0 by addition of 50mM-HCl and the slurry was stirred for 2h at room temperature. The slurry was then washed thoroughly on a sintered glass filter with distilled water and resuspended in 20ml phosphate buffer (pH7.4,I0.1).

2.16.2. Preparation of Nylon-glutaraldehyde

To 5.0ml of phosphate buffer (pH7.4,I0.1) containing 200-250mg NP/3 (modified as in Section 2.16.1), was added 0.5ml 25% (w/w) glutaraldehyde solution and the slurry stirred for 3min at room temperature. The nylon-glutaraldehyde derivative was then thoroughly washed on a sintered glass filter with phosphate buffer (pH7.4,I0.1) and used immediately for coupling to protein.

2.17. Polyethyleneimine

Polyethyleneimine was obtained as a 50% (w/w) solution in water from BDH Chemicals Ltd. Before its use for the immobilisation of LDH, the concentration of amino groups was measured by titration (see Section 3.5).

2.17.1. Standard PEI Solution

A standard PEI solution was used for immobilising LDH. This was prepared by dissolving about 2g (weighed accurately) of the 50% (w/w) PEI solution in phosphate buffer (pH7.4,I0.1) and making up to 50ml. This solution was kept at $4^{o}C$ and used within 2 days.

2.17.2. Preparation of PEI-glutaraldehyde

2.0ml of the standard PEI solution prepared as described in Section 2.17.1 was diluted to 5ml with phosphate buffer (pH7.4,I0.1). 1.0ml of 25% (w/w) glutaraldehyde solution was added and the solution stirred for 30sec at room temperature. The reaction of glutaraldehyde and PEI was stopped by the addition of 150µl of conc. phosphoric acid which lowers the pH to 3.5. 1.0ml of this solution was loaded, in 2 x 0.5ml aliquots, on a Sephadex G-25 column previously equilibrated with phosphate buffer, pH3.5. The PEI-glutaraldehyde preparation was separated from unreacted glutaraldehyde by eluting with phosphate buffer (pH3.5). To the void volume, which contained the PEI-glutaraldehyde, was added 150µl of N-ethylmorpholine which raised the pH to 8.5. This solution was used immediately for coupling to LDH.

2.17.3. Attachment of PEI to Cellex-AE

250mg Cellex-AE was activated with glutaraldehyde as described in Section 2.14.2.1. The freshly prepared Cellex-AE-glutaraldehyde was suspended in 5.0ml of the standard PEI solution and stirred for 10min at room temperature. The Cellex-AE-glutaraldehyde-PEI derivative was washed thoroughly with phosphate buffer (pH7.4,I0.1) on a sintered glass filter.

2.17.4. Activation of Cellex-AE-glutaraldehyde-PEI with glutaraldehyde

The moist derivative prepared in Section 2.17.3.was immediately suspended in 5.0ml 25% (w/w) glutaraldehyde solution for 3min at room temperature. The activated derivative was then washed with phosphate buffer (pH7.4,I0.1), and used immediately for coupling to protein.

2.18. Preparation of Immobilised Enzyme Derivatives

All immobilised enzyme derivatives were stored at 4°C unless otherwise stated.

2.18.1.1. "AE-Cellulose"-YADH (Method I)

YADH was attached to a freshly activated suspension of "AE-cellulose" (see Section 2.14.2.1). In a typical coupling, 250mg freshly activated "AE-cellulose" was suspended in 500uM NAD^+ and stirred for 60min in the dark at 4°C. The resulting "AE-cellulose"-glutaraldehyde-YADH was washed on a sintered glass filter with 1.0M-NaCl to remove non-covalently bound protein, and then with phosphate buffer (pH7.4, I0,1). The derivative was suspended in 20ml of this buffer.

2.18.1.2. "AE-Cellulose"-YADH (Method II)

The procedure was as described in Section 2.18.1.1 but, before the final washing with NaCl, the immobilised enzyme was resuspended in 5.0ml of the coupling buffer containing 50mM-sodium borohydride. The suspension was stirred for 15min at 4°C to reduce any free aldehyde groups and double bonds which may be present (see Discussion chapter). The enzyme derivative was then washed and resuspended in buffer as described in Section 2.18.1.1.

2.18.2.1. Cellex-AE-YADH (Method I)

YADH was coupled to freshly prepared Cellex-AE-glutaraldehyde. The coupling was essentially as described in Section 2.18.1.2 but NAD^+

was omitted from this and all subsequent couplings. Before coupling, 100µl of the enzyme coupling solution was assayed as described in Section 2.8.2.1.1 to determine the initial activity of the coupling protein. Typically, 250mg freshly activated Cellex-AE was suspended in 5.0ml phosphate buffer (pH7.4,I0.1) containing 2mg YADH and stirred for 120min at 4°C in the dark. The suspension was then carefully filtered into a small Buchner flask, the derivative washed with 5.0ml coupling buffer before being resuspended in a sodium borohydride solution as described in Section 2.18.1.2. After stirring for 15min at 4°C, the immobilised YADH was thoroughly washed as described in Section 2.18.1.2. The washings from the coupling were pooled and the enzymic activity of the uncoupled protein measured.

2.18.2.2. Cellex-AE-YADH (Method II)

YADH was also coupled to activated Cellex-AE to form derivatives in which the reduction step with sodium borohydride was omitted.

2.19. DEAE-cellulose-YADH

YADH was coupled to freshly activated DEAE-cellulose prepared as described in Section 2.15. The coupling procedure was as described in Section 2.18.1.1. Before final resuspension in coupling

buffer, the immobilised YADH derivative was stirred in 50mM-NH_4Cl/NaOH buffer (pH8.5) for 2h at room temperature to remove any unreacted chloro groups.

2.20. Immobilisation of LDH

LDH was immobilised on three different activated supports.

2.20.1. Cellex-AE-LDH

LDH was coupled to a freshly prepared glutaraldehyde derivative of Cellex-AE prepared as described in Section 2.19.2.1. 100µl of the enzyme coupling solution was assayed as described in Section 2.8.3.1.1 to determine the initial activity of the coupling protein. Typically 250mg activated Cellex-AE was suspended in 5.0ml phosphate buffer (pH7.4,I0.1) containing 1mg LDH. The suspension was stirred at 4°C for 2h in the dark. Collecting and assaying the supernatant, reduction, washing and resuspending in coupling buffer were as described in Section 2.18.2.1.

Cellex-AE-LDH derivatives were also prepared where the reduction step with sodium borohydride was omitted.

2.20.2. NP/3-LDH and derivatives

LDH was immobilised on a glutaraldehyde derivative of NP/3 nylon powder, prepared as described in Section 2.16.2.

250mg freshly activated NP/3 powder was suspended in 3.0ml phosphate buffer (pH7.4,I0.1) containing 300µg LDH (purified as described in Section 3.1.1.1). A 100µl aliquot was removed before addition of the NP/3 and this was assayed for initial activity of the coupling protein. The suspension was stirred at 4°C for 60min in the dark. The supernatant was filtered off carefully and the NP/3-LDH washed with 3ml coupling buffer before being reduced in a sodium borohydride solution (50mM phosphate buffer (pH7.4,I0.1)) for 15min at 4°C. The reduced NP/3-LDH derivative was then washed and resuspended in phosphate buffer (pH7.4,I0.1), as described in Section 2.18.1.1. The washings collected before the reduction step were pooled and assayed for uncoupled LDH activity.

Three other NP/3-LDH derivatives were prepared. The coupling procedure was as described above until after the initial washing. The NP/3-LDH, instead of being reduced, was resuspended in 10ml of pyrophosphate buffer containing 10mmoles of either

N,N-dimethylaminopropylamine or n-butylamine or γ-aminobutyric acid, each of which had been titrated to pH8.4. The NP/3-LDH derivatives were stirred in these solutions for 15min at room temperature before being reduced, washed, and resuspended as described in Sections 2.18.1.1 and 2.18.1.2.

2.20.3.1. PEI-LDH

LDH was coupled to a glutaraldehyde derivative of PEI, prepared as described in Section 2.17.2.

To the activated PEI solution from the column, was added 100µl of LDH solution (1mg/ml) and the mixture stirred for 120min at 4°C in the dark. Sucrose was then added to increase the density of this solution and 0.8ml was layered on the top of a Sephadex G-200 column equilibrated as described in Section 3.1.2. The column was eluted with phosphate buffer (pH7.4,I0.1), at a flow rate of 0.16m/min, to separate the PEI-LDH from "unbound LDH". "Unbound LDH" is defined as LDH which is unreacted or has at the most, only one or two molecules of PEI bound and has a molecular weight close to 1.5×10^5. PEI-LDH is defined as the immobilised preparation of LDH which is eluted in the void volume. The change in absorbance was followed by pumping the eluant through

Fig. 3.

Gel-filtration of PEI-LDH and "unbound" LDH on a Sephadex G-200 column (see Sections 2.20.3.1 and 3.1.2).

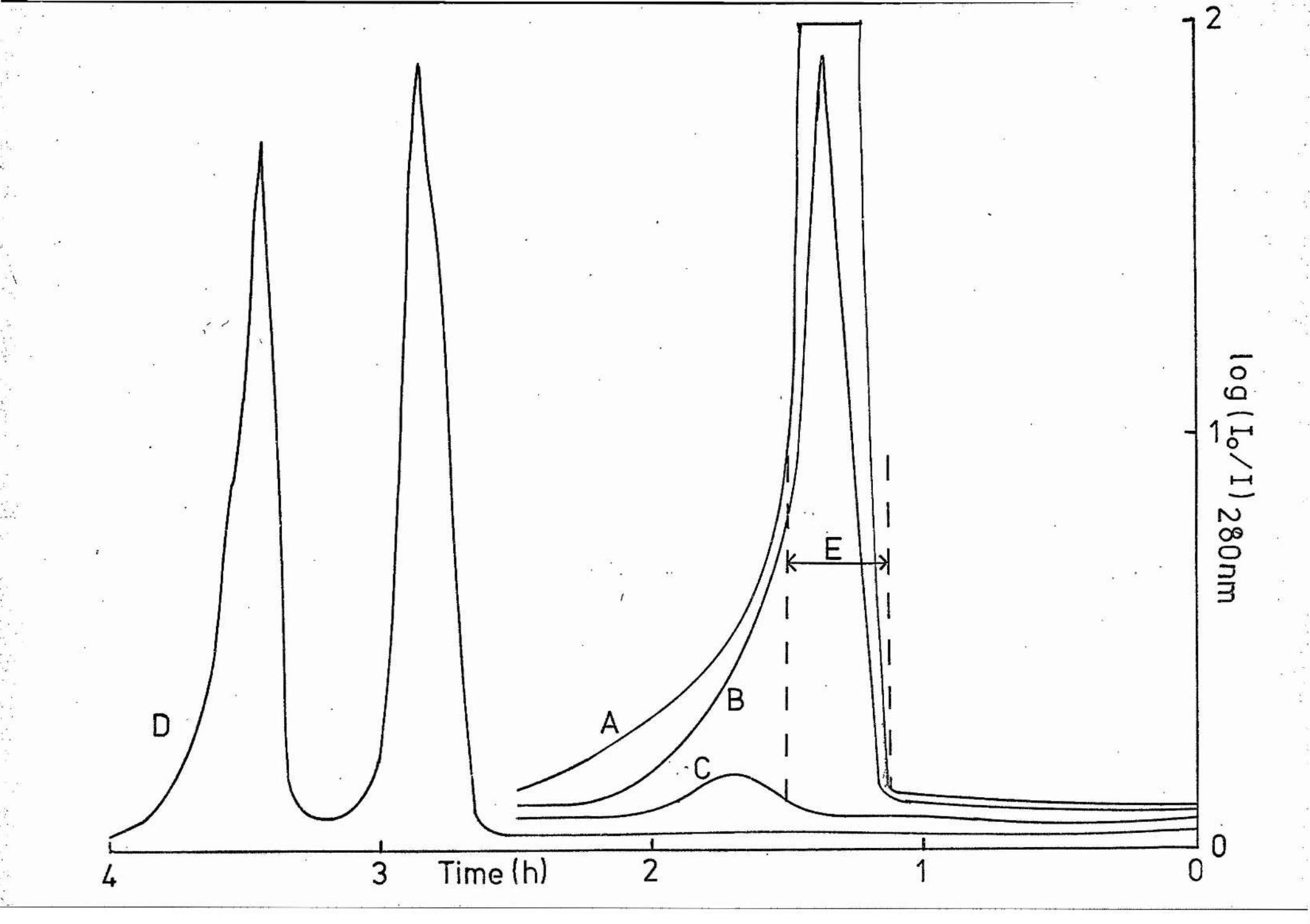
A : Trace obtained for the elution of PEI-LDH and "unbound" LDH.

B : Trace obtained for the high molecular weight marker, Dextran Blue.

C : Trace obtained for free LDH.

D : Trace obtained for unactivated PEI.

E : Volume of PEI-LDH collected.


D
A
B
C
E
4
3
Time (h)
2
1
0
2
1
0
log (I₀/I) 280nm

a Beckman silica flow-cell, subsequently described in Section 3.1.2. Fig. 3 shows the separation of "unbound" LDH from "bound" LDH by gel-filtration on Sephadex G-200.

2.20.3.2. Cellex-AE-(PEI-LDH)

PEI-LDH prepared as described in Section 2.20.3.1 was coupled to freshly activated Cellex-AE which in turn was prepared as described in Section 2.19.2.1.

100mg of freshly activated Cellex-AE was suspended in 2.2ml of PEI-LDH solution. The suspension was stirred for 90min at 4°C in the dark before being washed and resuspended as described in Section 2.18.2.2. The combined supernatant and washings were collected and assayed as before.

2.20.3.3. Cellex-AE-PEI-LDH

LDH was coupled to a freshly activated glutaraldehyde derivative of Cellex-AE-PEI prepared as described in Section 2.17.4.

250mg activated Cellex-AE-PEI was suspended in 5.0ml phosphate buffer (pH7.4,I0.1) containing 1mg LDH. Coupling conditions, collections of the supernatants, washing and final resuspension in buffer were as described in Section 2.18.2.2.

2.21. Cellex-AE-MDH

MDH was coupled to activated Cellex-AE. The coupling conditions for MDH were essentially as for LDH coupling to Cellex-AE as described in Section 2.20.1. Initial and supernatant soluble enzyme activities were measured by the assay procedure described in Section 2.8.4.

Experimental

Chapter 3

3.1. Column Techniques

3.1.1. Sephadex G-25

Sephadex G-25 (Pharmacia (GB) Ltd., Paramount House, 74 Uxbridge Road, Ealing, London W.5) was allowed to swell for 2 days at room temperature in phosphate buffer (pH7.4,I0.1). It was then packed into a small glass column (10 x 1cm) and equilibrated with 100ml of the same buffer. The column was calibrated using ρ-nitrophenol and Dextran Blue as low and high molecular weight markers respectively. It was used for separating ammonium sulphate from LDH (Section 3.1.1.1), and also for separating glutaraldehyde from PEI-glutaraldehyde prepared as described in Section 2.17.2.

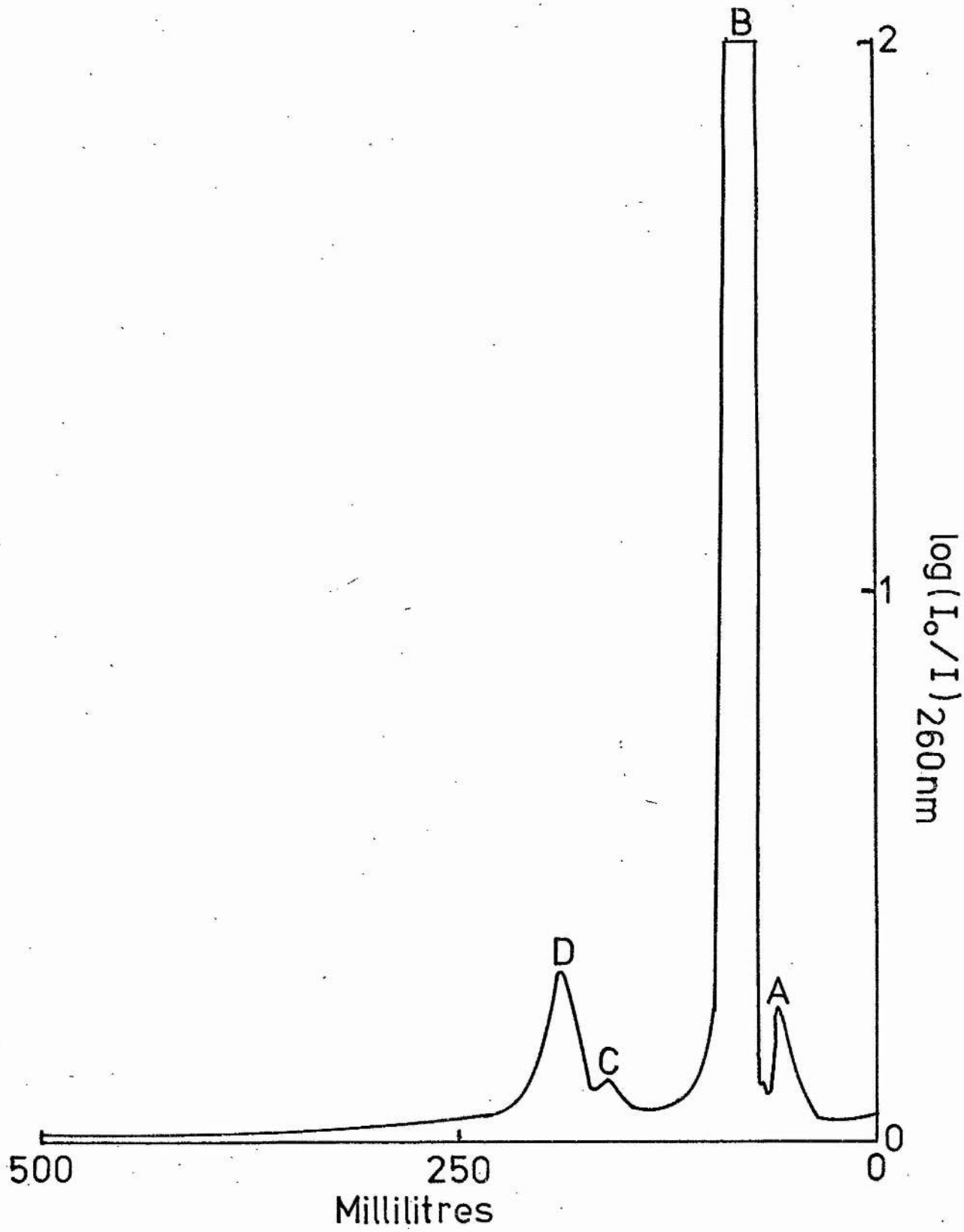
3.1.1.1. Purification of LDH

0.2ml LDH ammonium sulphate suspension was added to 0.8ml phosphate buffer (pH7.4,I0.1) and applied in 2 x 0.5ml aliquots to the equilibrated Sephadex G-25 column. The LDH was then eluted with the same buffer and the void volume collected. The protein concentration was measured spectrophotometrically using the equation (Zewe and Fromm, 1962)

$$E^{1\%}_{280nm} = 12.9.$$

Fig. 4.

Purification of NAD^+ on a DE50 column (see Section 3.1.3). B is the pure NAD^+ peak; A, C and D are the impurities in the commercial sample tested.


B
2
1
0
log(I₀/I)260nm
D
C
A
500
250
0
Millilitres

3.1.2. Sephadex G-200

Sephadex G-200 (Pharmacia) was allowed to swell for seven days at room temperature in phosphate buffer (pH7.8,I0.1). A Pharmacia column (40 x 0.8cm) was packed with the swollen gel and equilibrated for 16h at a flow rate of 0.16ml/min with the same buffer. This flow rate was used in all operations. The column was calibrated with Dextran Blue to give the void volume, and with LDH to locate the free LDH elution volume. The column was then used to separate unreacted LDH from LDH which had been bound to PEI by glutaraldehyde, as described in Section 2.20.3.1. In all column operations, sucrose was dissolved in the solution to be added to the column in order to facilitate sample application. The absorbance of the eluant was followed at 280nm using a Beckman flow-through silica cuvette (1cm lightpath; interval volume = 0.12ml) (Beckman Ltd.).

3.1.3. NAD^+ Purification

NAD^+ was purified by chromatography on cellulose (DE-50) according to the method of Dalziel and Dickinson (1966). Fig. 4 shows a trace of the elution pattern which was followed by measuring the absorbance at 260nm using a Beckman flow-through silica cuvette. To test the purity of the largest

peak, assays were carried out at pH6.0, using a saturating concentration of coenzyme, for both liver and yeast alcohol dehydrogenases. The liver enzyme showed a 25% increase in activity whereas the yeast enzyme showed only a 2.5% increase in activity. Fig. 4 also shows that there are far fewer impurities in comparison with the NAD^+ originally purified by Dalziel and Dickinson (1966). As a rule, the main peak contained 99% of all nucleotides. The impurities are due to the breakdown products of NAD^+ and these inhibit the liver enzyme much more than the yeast enzyme (Dalziel, 1963).

3.2. Determination of Total Nitrogen

Experiments were carried out to determine the amino group of four modified celluloses using the micro-Kjeldahl procedure described by Bruel et al. (1941):

(a) Fibrous AE-cellulose

(b) CM-cellulose (CM 32 μgranular)

(c) "AE-cellulose"

(d) Cellex-AE.

(a) and (b) were obtained from Whatman Chemicals, Maidstone, Kent. (c) was a modified Whatman CM-cellulose (see Section 2.14.1.1). (d) was obtained from Bio-Rad Laboratories, Richmond, California. Each estimation was carried out in duplicate and the

Table 1.

Cellulose	Amino group content (mequiv/g)
CM-cellulose	0
Fibrous AE-cellulose	0.81
"AE-cellulose"	0.81
Cellex-AE	0.35

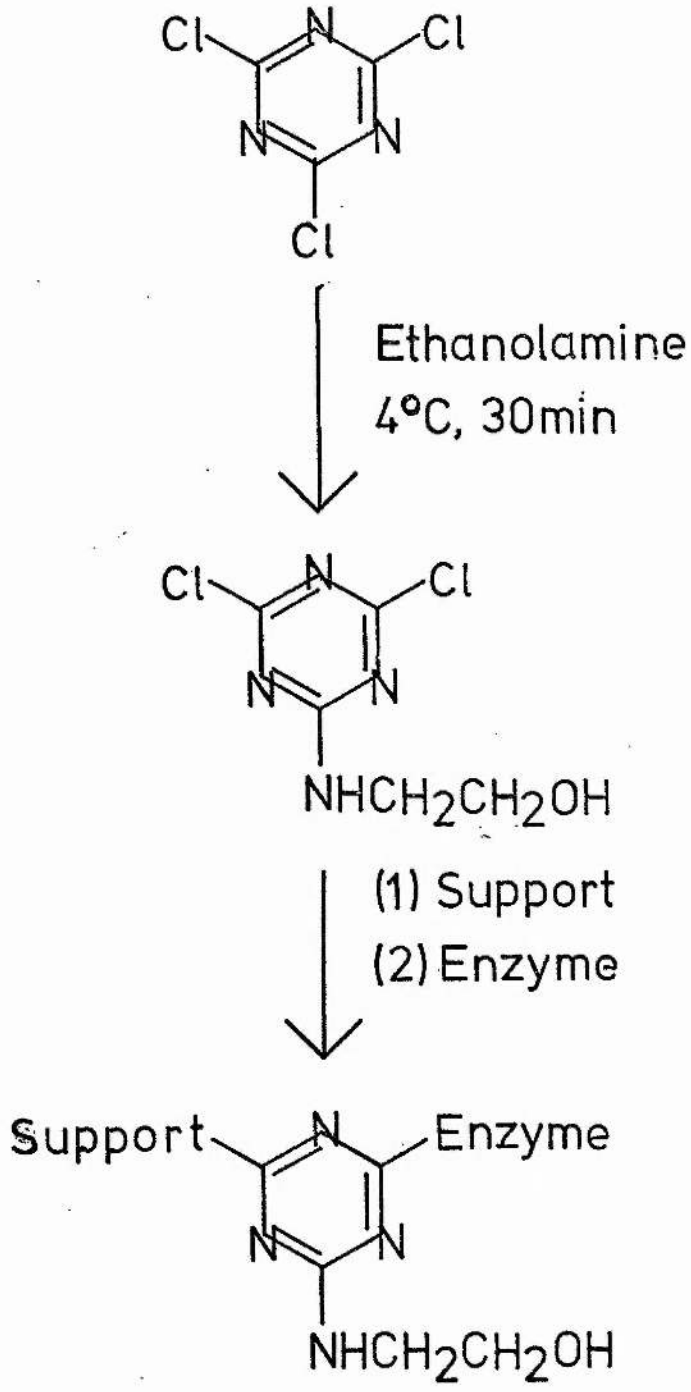


Fig. 5.

Chemical modification of cyanuric chloride with ethanolamine.

Table 2.

Derivative	m.p. Range (°C)	Elemental Analysis
Cyanuric chloride	153 - 154	(a) 19.5%C, 22.8%N
		(b) 20.3%C, 21.9%N
Modified cyanuric chloride	118 - 120	(a) 29%C, 2.9%H, 26.9%N
		(b) 31%C, 3.5%H, 25.9%N

(a) Theoretical Composition

(b) Experimental Composition

Plate Ia

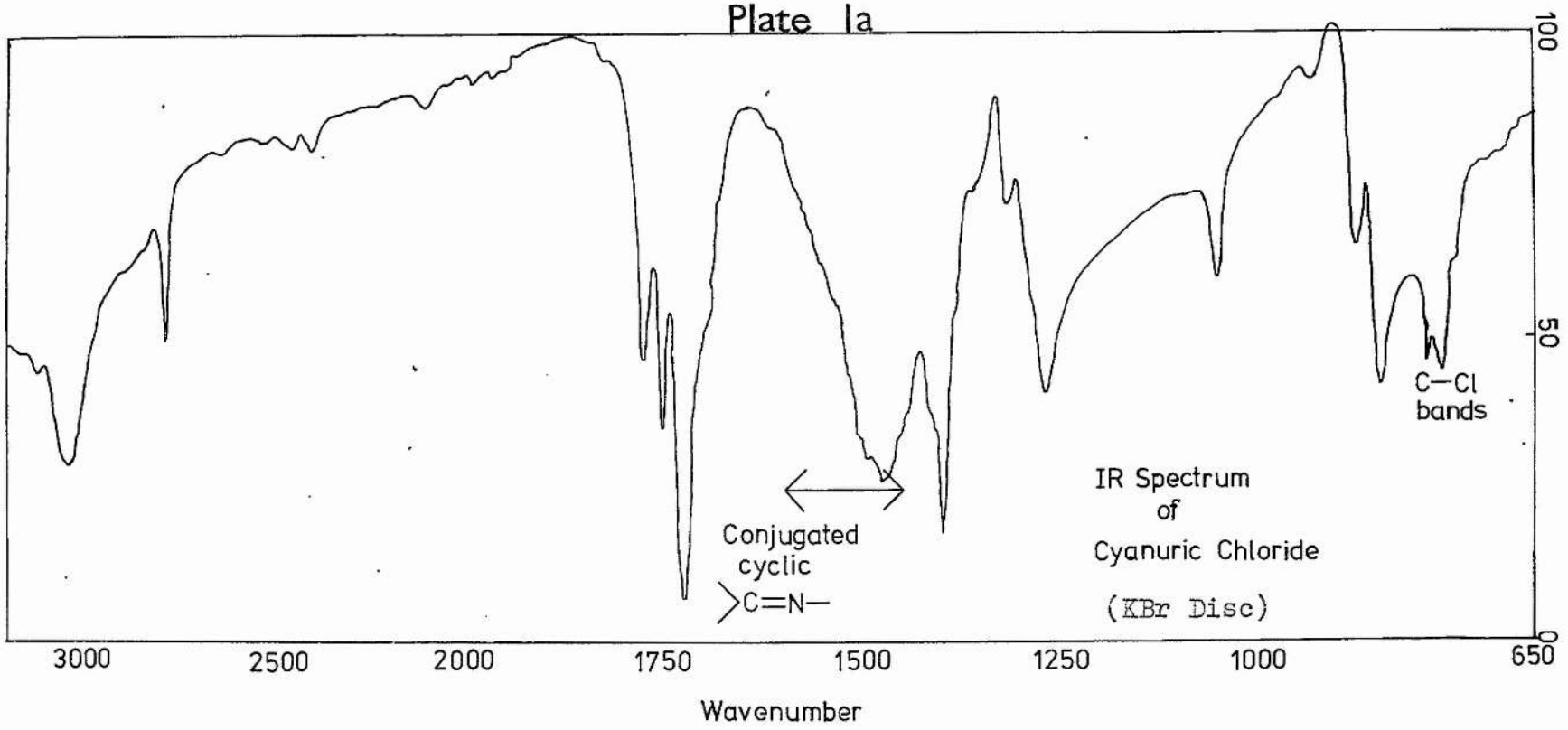
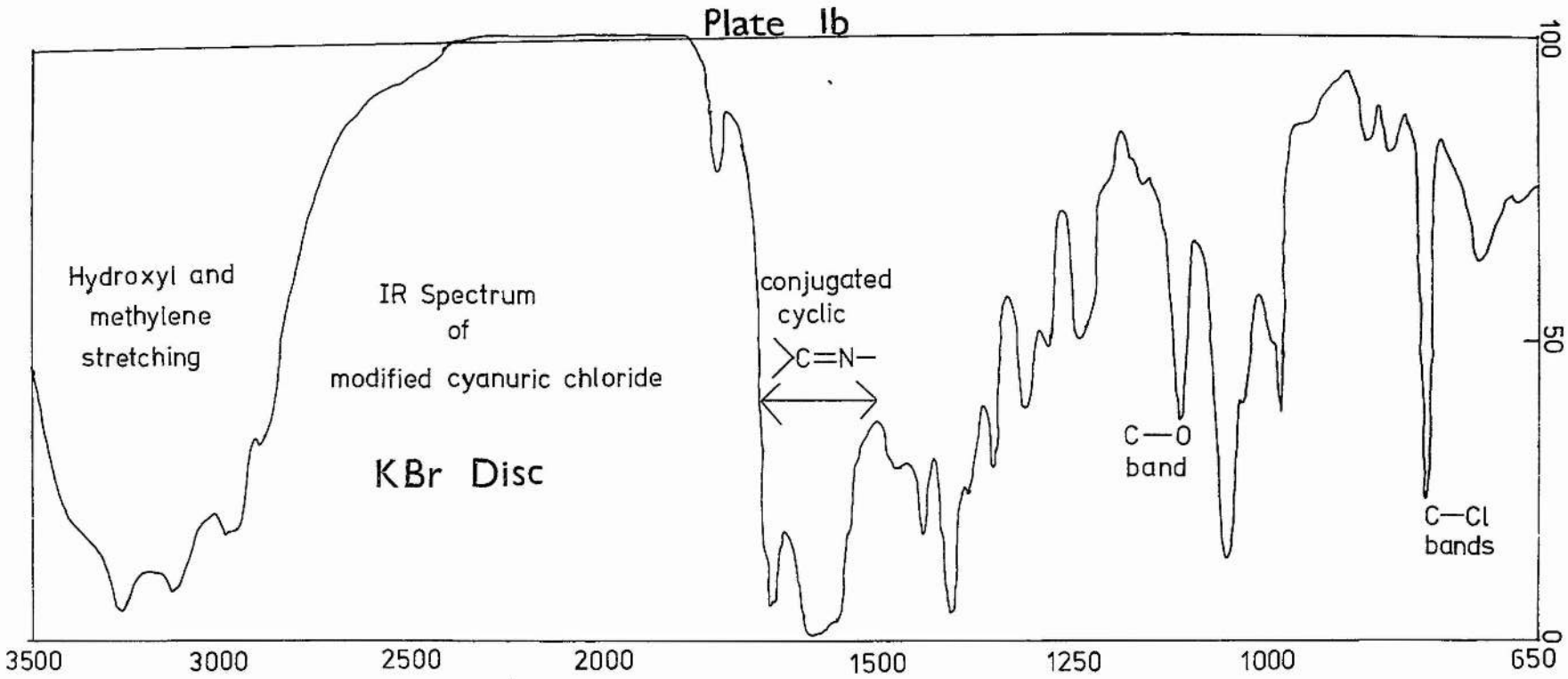

100
50
0
3000
2500
2000
1750
1500
1250
1000
650
Wavenumber
C—Cl
bands
Conjugated
cyclic
>C=N—
IR Spectrum
of
Cyanuric Chloride
(KBr Disc)

Plate Ib


Hydroxyl and
methylene
stretching
IR Spectrum
of
modified cyanuric chloride
KBr Disc
conjugated
cyclic
>C=N-
C—O
band
C—Cl
bands
100
50
0
3500
3000
2500
2000
1500
1250
1000
650

results are presented in Table 1.

3.3. Chemical Modification of Cyanuric chloride

23g cyanuric chloride was stirred in a solution containing 250ml acetone and 50ml toluene for 16h at room temperature. It was then stirred on an icebath for 15min, 15ml of freshly distilled ethanolamine in 25ml acetone added dropwise over 15min and the slurry stirred for a further 15min. The solution was filtered to remove any unreacted cyanuric chloride and then evaporated under pressure on a rotary evaporator to remove acetone and toluene. The crystalline product was dissolved in 50ml acetone, 50ml distilled water added, the acetone removed by rotary evaporation and the resulting white precipitate filtered on a sintered glass filter and dried. The chemical modification of cyanuric chloride is presented in Fig. 5. The cyanuric chloride and its derivative were characterised by melting point, elemental analysis, and IR spectra. Table 2 presents the results of the former two tests, and the IR spectra are presented in Plate I(a) and I(b).

3.4. Assay of Immobilised Enzyme Derivatives

For assay of the immobilised enzyme derivatives, the contents of a cuvette were stirred

Fig. 6.

Relative positions of the overhead stirrer, the light-path and the cuvette for assay of the immobilised enzyme derivatives.(see Section 3.4).

A : Overhead stirrer.

B : Perspex base.

C : Teflon paddle.

D : Lightpath.

E : Cuvette.

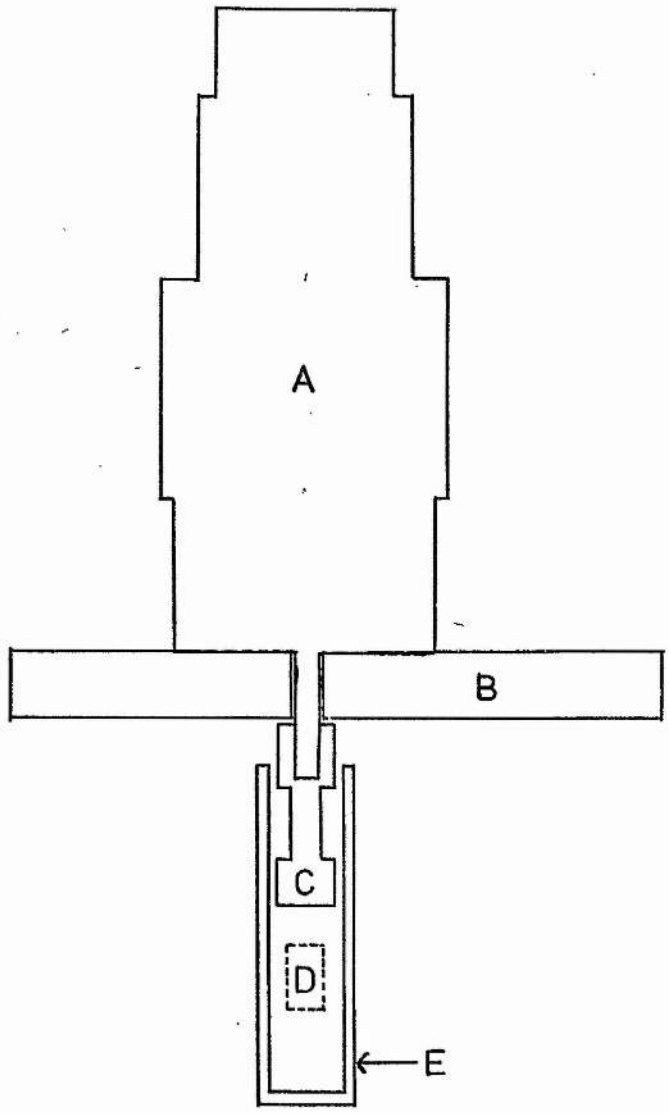

A
B
C
D
E

Plate II

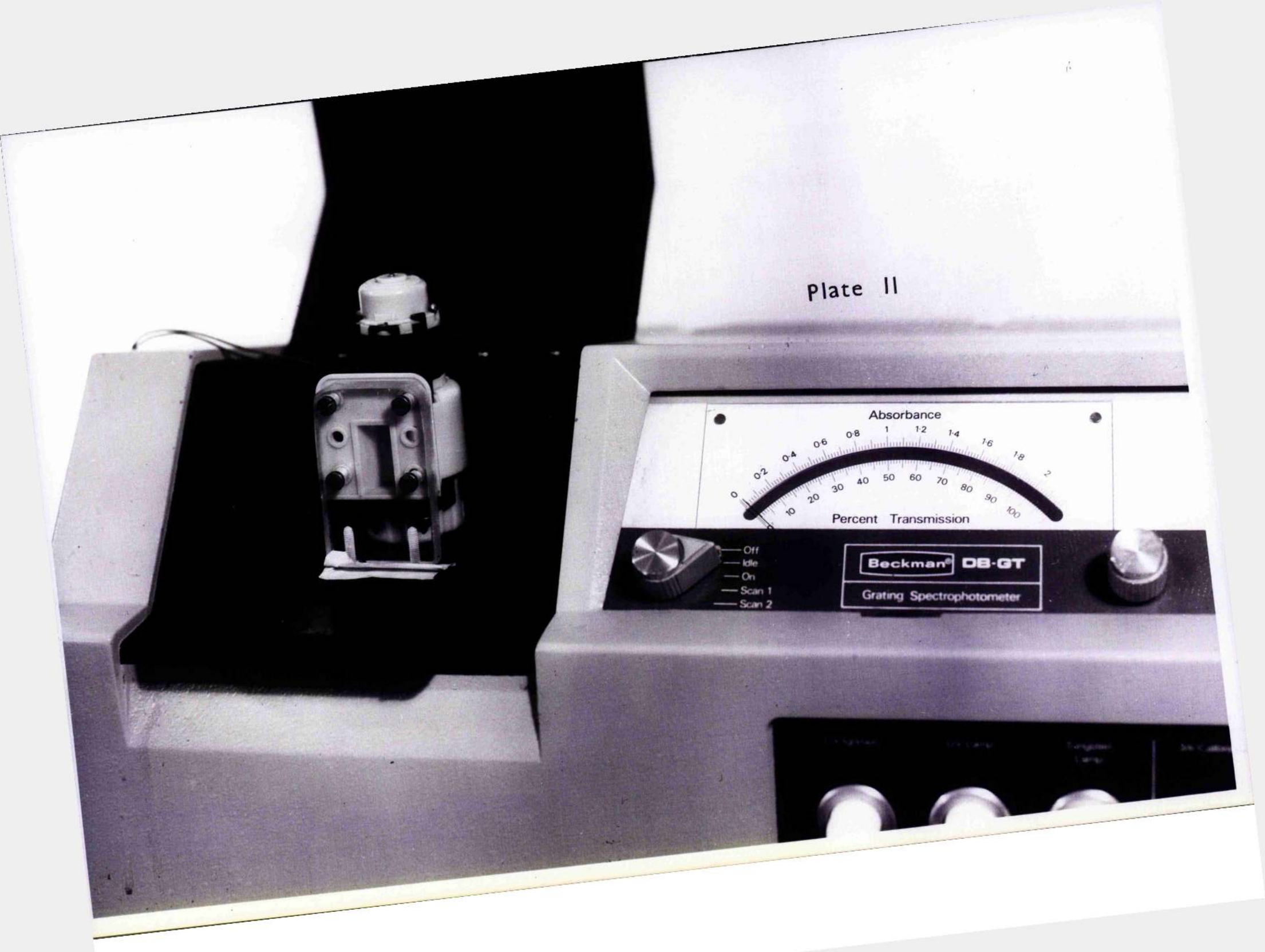
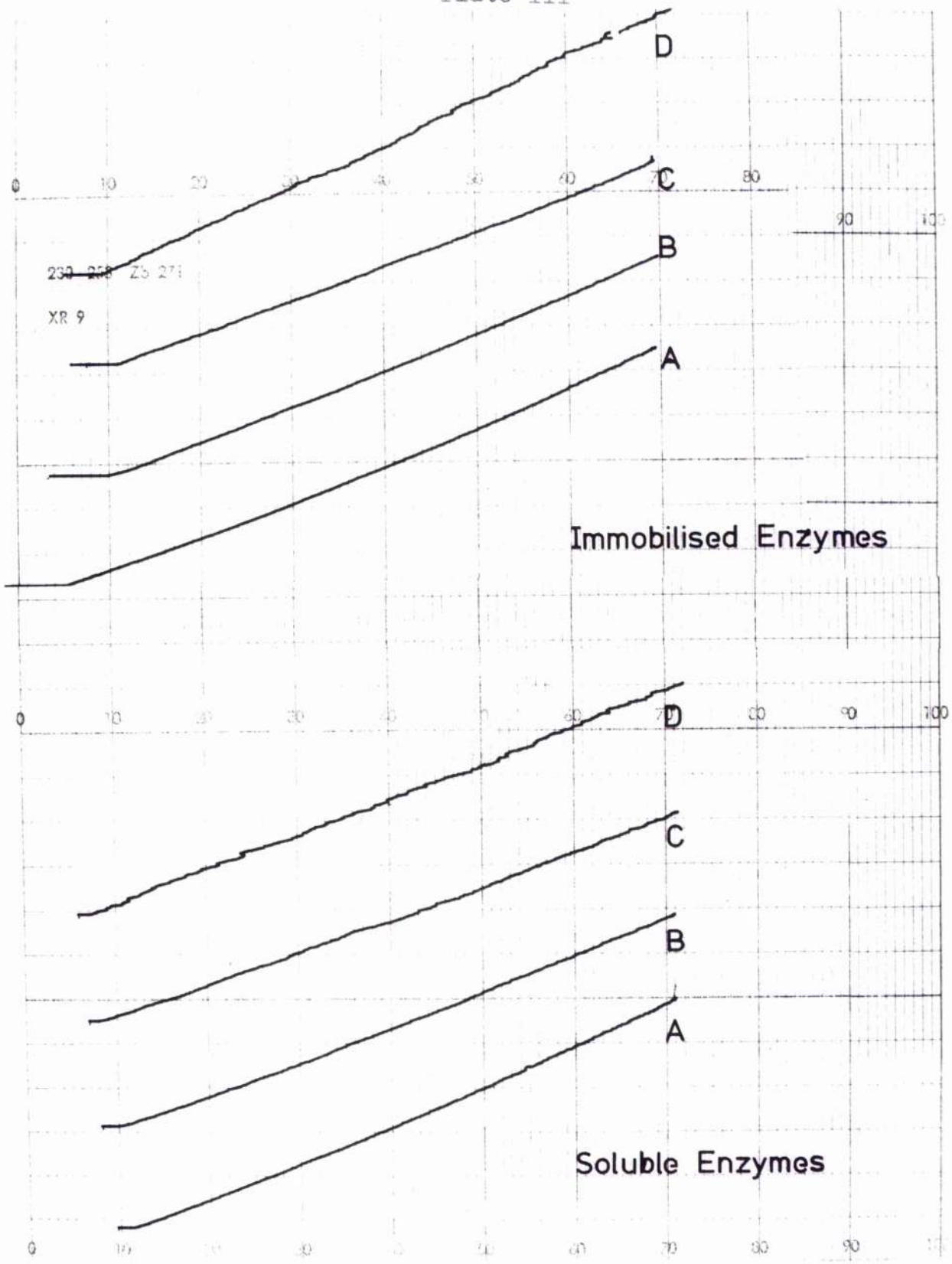

Absorbance
0
0·2
0·4
0·6
0·8
1
1·2
1·4
1·6
1·8
2
10
20
30
40
50
60
70
80
90
100
Percent Transmission
Off
Idle
On
Scan 1
Scan 2
Beckman
DB-GT
Grating Spectrophotometer

Plate III

Traces obtained at various chart ranges for soluble and immobilised enzymes (see Section 3.4). In this instance the traces were obtained for soluble and immobilised YADH. The assay procedure was as described in Sections 2.8.2.1.1 and 2.8.2.1.2. A, B, C and D refer to traces obtained using the 100mV, 50mV, 20mV and 10mV chart expansion ranges respectively. The 0 - 100 scale on Plate III corresponds to one optical density unit on the 100mV range, 0.5 OD units on the 50mV range etc.

Plate III



by using an overhead Meccano motor (Meccano Triang Ltd., Binns Road, Liverpool). This was driven by a 6-volt d.c. supply and had an output speed of 300 rev./min. Plate II shows the position of the stirrer on the spectrophotometer and Fig. 6 shows the relative positions of the stirrer, the lightpath and the cuvette. The inset on Plate II shows the teflon paddle used for stirring the slurry in the cuvette.

The stirring speed stated above was used throughout all assays. A lower speed resulted in sedimentation of the support material but this could possibly have been overcome by using a paddle with a better fluting. No vortex or bubbles were caused by this paddle. It was found that either of the substrates or the immobilised enzyme could be added last to the cuvette to start the reaction without changing the initial rate. Having a support material present could result in very uneven traces being obtained when the 10mV range of the recorder was used but as Plate III shows, there is very little "noise" on the 10mV range. A comparison of soluble and immobilised enzyme traces at various chart ranges is also shown in Plate III.

3.5. Titration of PEI

This was in effect, a back titration. The amino groups were neutralised by a known excess of HCl, the excess acid being backtitrated with the standard sodium hydroxide solution using bromothymol blue as the indicator (pK = 7.0). The molarity of the HCl was determined by titration against a standard borax solution using phenolphalein as an indicator.

Amino groups per g PEI = 5.85mMoles.

Yeast Alcohol Dehydrogenase:

Chapter 4

4.1. Attachment of YADH to "AE-Cellulose"

4.1.1. The Effect of Sodium Borohydride Reduction on the Activity of "AE-cellulose"-YADH

YADH was coupled to "AE-cellulose" as described in Section 2.18.1.1. Two identical preparations were made, and one was reduced with sodium borohydride as described in Section 2.18.1.2. Table 3 shows the activity of these preparations.

The lower activity of the "reduced" preparation is probably due to reduction by sodium borohydride. Wallenfels and Sund (unpublished result, The Enzymes (2nd edition), Vol. 7, p. 61) found that the turnover number of a soluble preparation of YADH decreased on reduction with sodium borohydride although the number of -SH groups increased.

4.1.2. The Long Term Stability of "AE-cellulose"-YADH

The stability of the derivatives prepared in Section 4.1.1 was studied at 4°C and 25°C over a period of 13 days. The results are presented in Fig. 7 which shows that there is only a little difference in the stability of both derivatives at both temperatures, the "unreduced" derivative losing activity more quickly at the higher temperature. As expected, activity of both "unreduced" and "reduced" preparations is lost more quickly at the higher temperature.

Table 3.

Derivative	Activity * (U/g)
"Unreduced"	82
"Reduced"	50

* For all immobilised YADH preparations, activity is defined as the number of micromoles NADH per ml per min per g of enzyme-support under the conditions defined in Section 2.8.2.1.2.

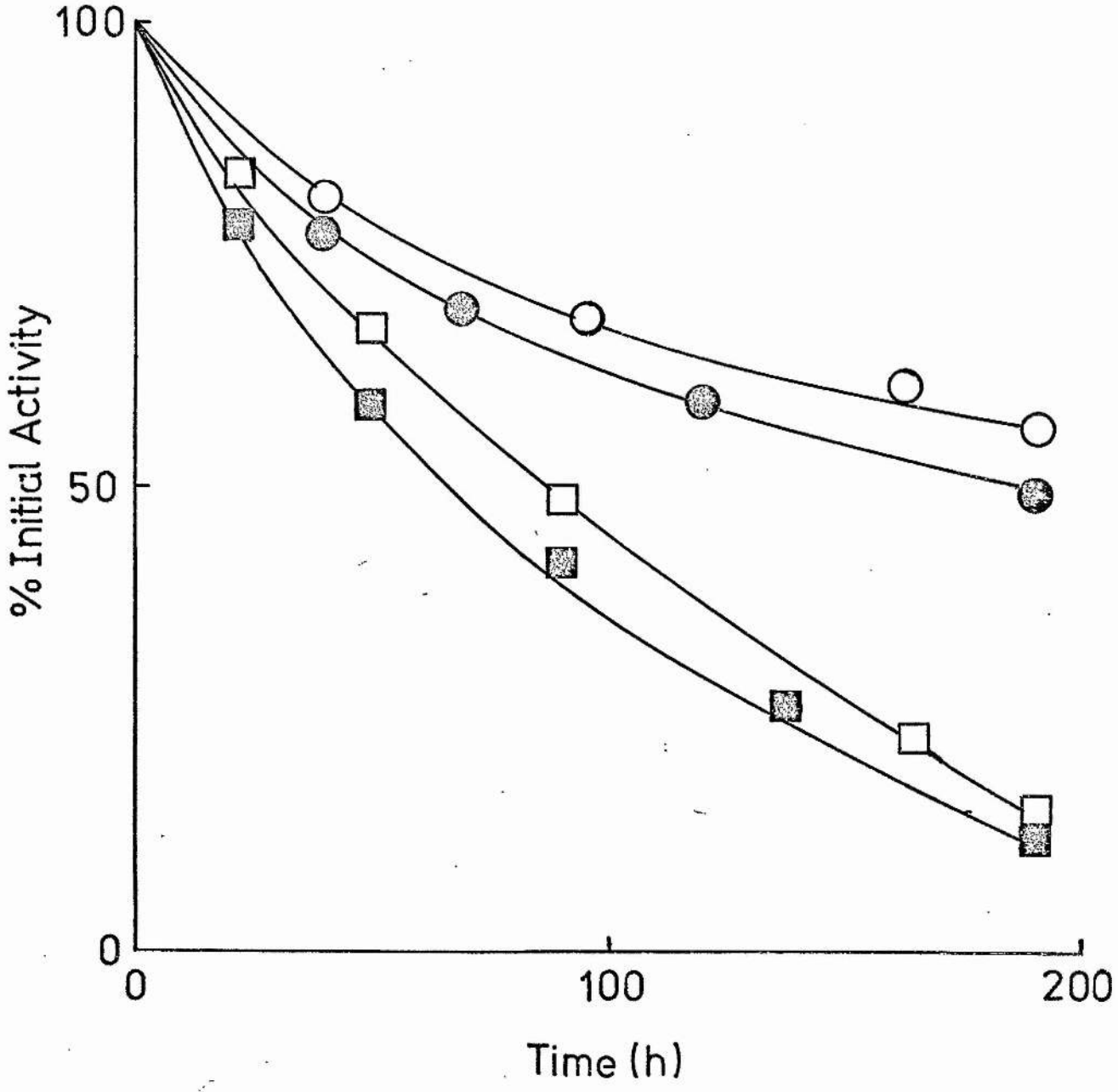


Fig. 7.

Long term stability of YADH attached to "AE-cellulose" at 4°C (○) and at 25°C (□). Unfilled symbols represent the "reduced" derivative; filled symbols represent the "unreduced" derivative.

4.1.3. The Effect of Time on the Coupling of YADH to "AE-cellulose"

The coupling time of YADH to "AE-cellulose" was varied and the results are presented in Fig. 8. This shows that a maximum activity of 68U/g enzyme-support was attained after 75min.

4.1.4. Stability

The stability at 4°C of some of the derivatives prepared in Section 4.1.3 was studied. The results are presented in Fig. 9 and suggest that a shorter coupling time gives a more stable derivative.

4.2. Attachment of YADH to Cellex-AE

YADH was immobilised on Cellex-AE as described in Section 2.18.2.1. In an attempt to optimise conditions required for the coupling of YADH to this support with glutaraldehyde, a number of experiments were performed in which various parameters of the immobilisation procedure were varied.

4.2.1. The Effect of Time on the Coupling of YADH to Cellex-AE

The coupling time of YADH to Cellex-AE was varied and the results are presented in Fig. 10.

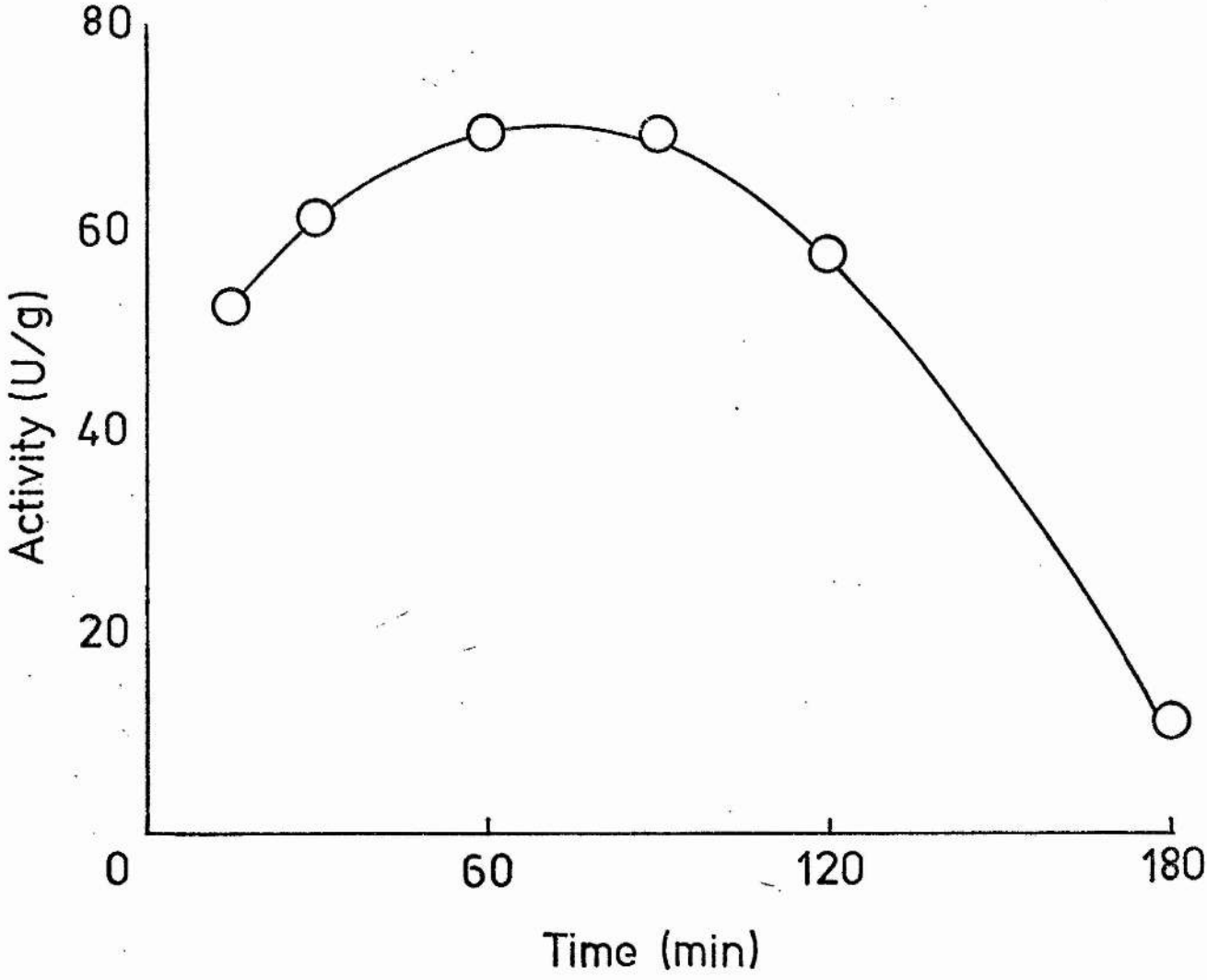


Fig. 8.

Effect of the time of the coupling of YADH to "AE-cellulose".

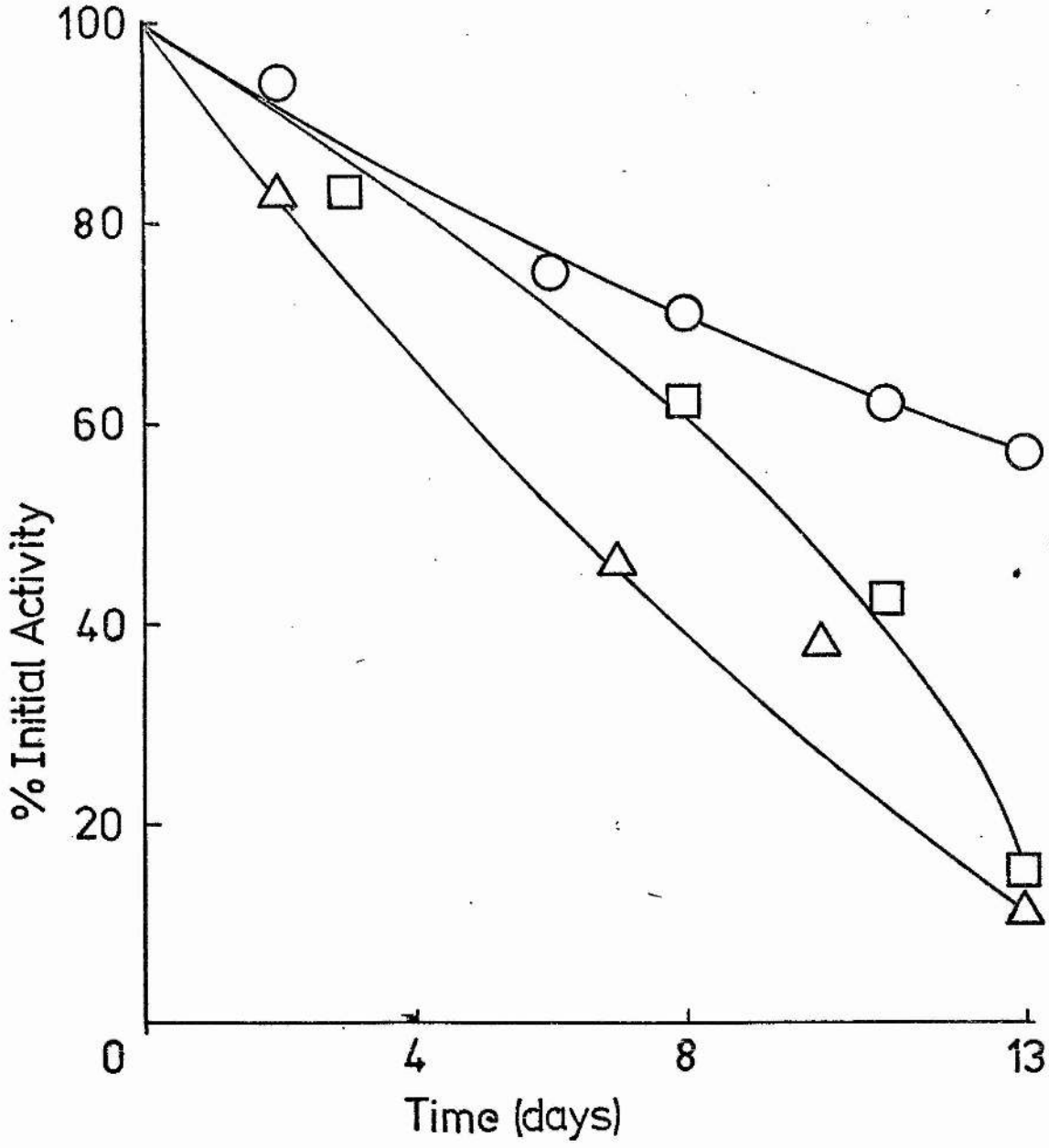


Fig. 9.

Stability at 4°C of some of the derivatives prepared in Section 4.1.4.

○, 30min coupling time.

□, 60min coupling time.

△, 90min coupling time.

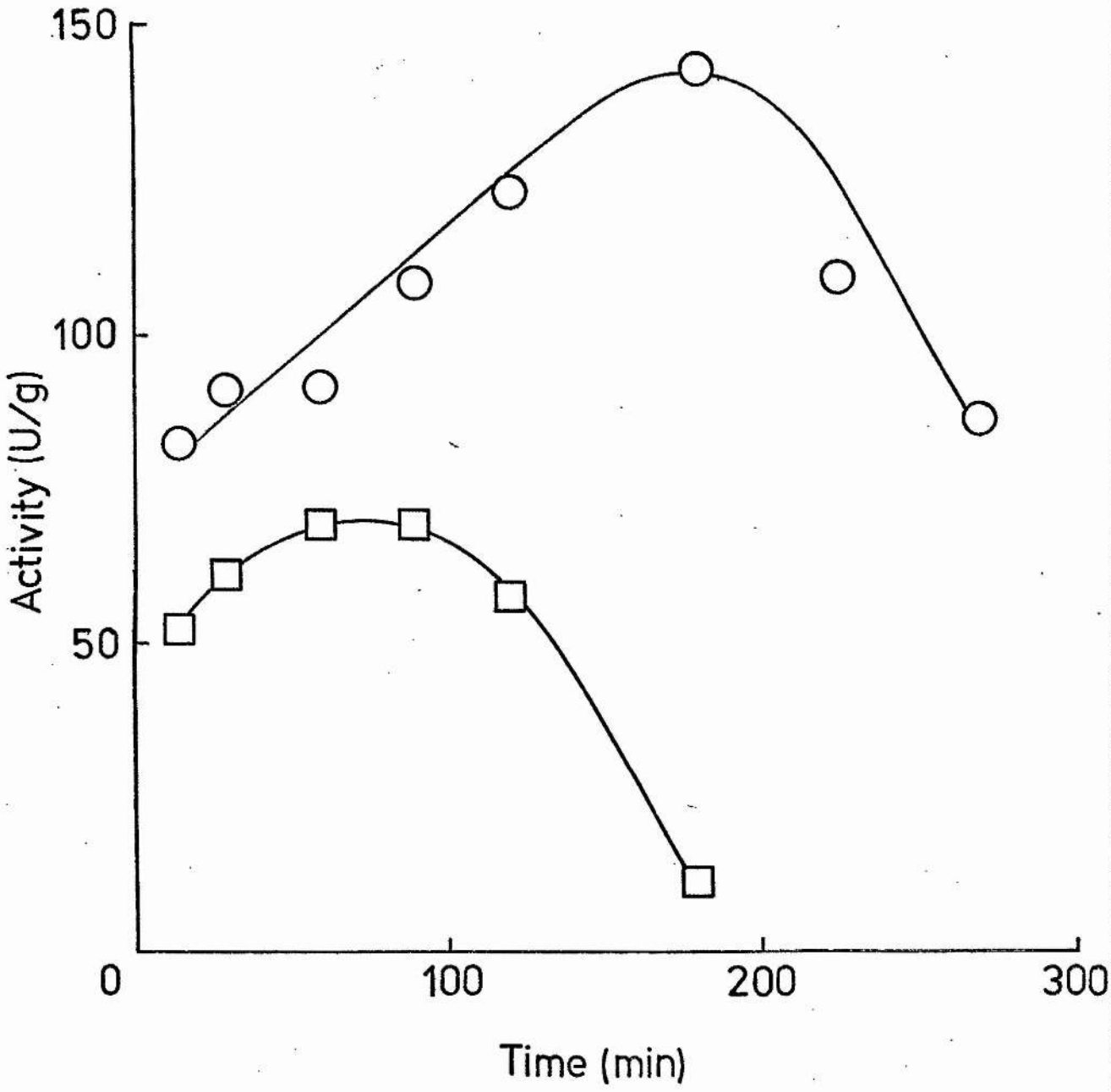


Fig. 10.

Comparison of the effect of time on the coupling of YADH to "AE-cellulose" (□) and to Cellex-AE (○).

The corresponding variation for YADH attachment to "AE-cellulose" is also shown for comparison. The results show that a maximum support specific activity of 142U/g was attained for a coupling time of 180min.

4.2.2. The Effect of pH on the Coupling of Glutaraldehyde to Cellex-AE

Activation of Cellex-AE by glutaraldehyde was carried out over a range of pH; YADH was then coupled to the activated support (Section 2.18.2.1) and the activity of the immobilised YADH measured. 250mg quantities of Cellex-AE were suspended in a range of buffers from pH5 to pH9. Glutaraldehyde activation (Section 2.14.2.1) and YADH attachment (Section 2.18.2.1) was then carried out. The results are presented in Fig. 11, which shows that glutaraldehyde activation of Cellex-AE for the immobilisation of YADH should be carried at about pH7.0, where a maximum support specific activity of 48U/g was attained.

4.2.3. The Effect of pH on the Coupling of YADH to Cellex-AE

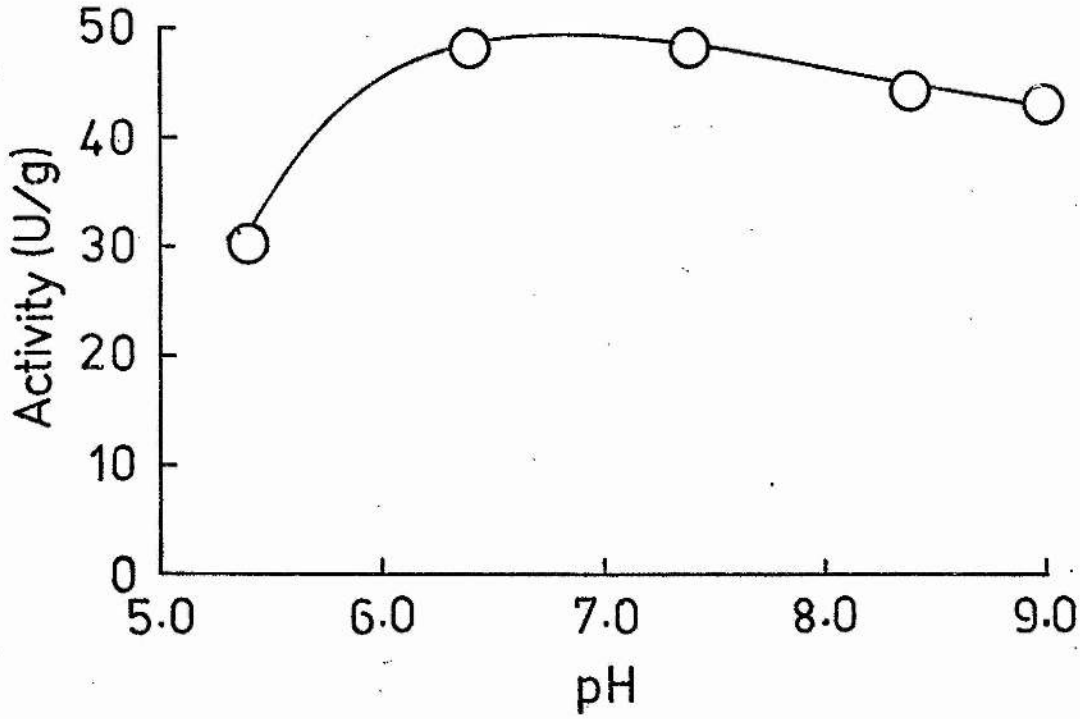
Cellex-AE was activated as described in Section 2.14.2.1. It was then resuspended in a range of buffers from pH5 to pH9. 200µl of YADH solution (10mg/ml) was added and coupling proceeded as described in Section 2.18.2.1. The loss of soluble

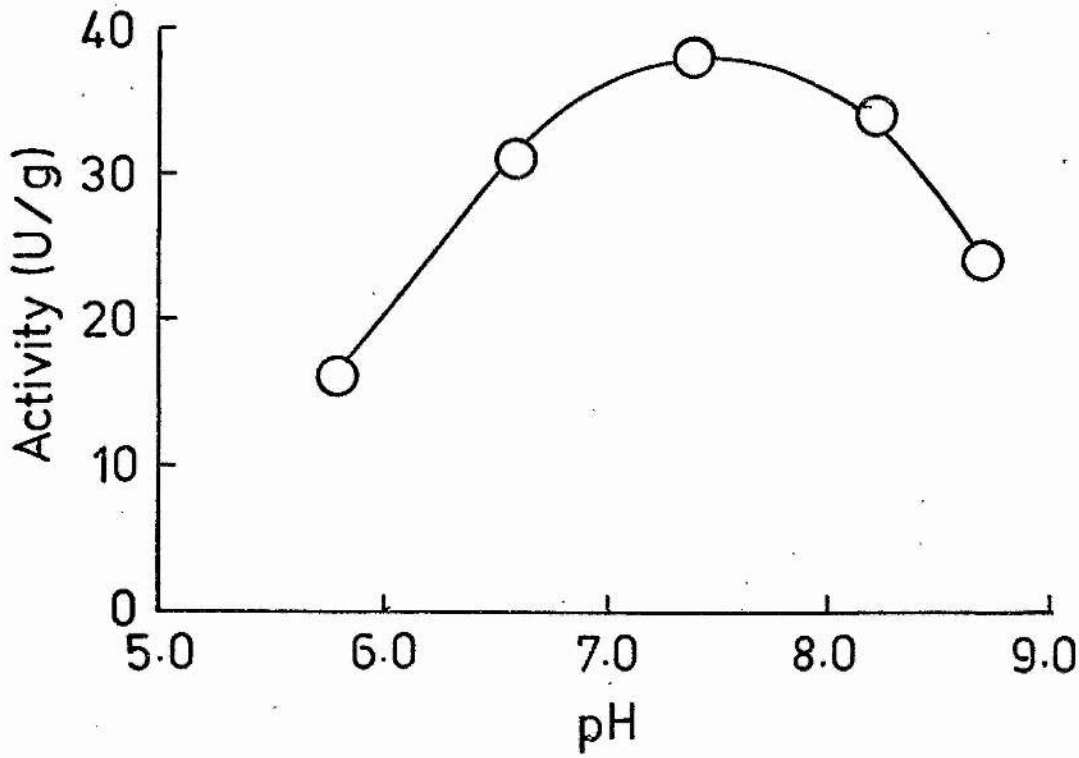
Fig. 11.

Effect of pH on the coupling of glutaraldehyde to Cellex-AE.

Fig. 12.

Effect of pH on the coupling of YADH to Cellex-AE.


Activity (U/g)
50
40
30
20
10
0
5.0
6.0
7.0
8.0
9.0
pH


Activity (U/g)
40
30
20
10
0
5.0
6.0
7.0
8.0
9.0
pH

YADH activity with pH was calculated by the method described in Section 2.13.1. The results are presented in Fig. 12 and show that coupling at pH7.4 gives a maximum support specific activity of 38U/g.

4.2.4. The Effect of Ionic Strength on the Coupling of YADH to Cellex-AE

Cellex-AE was activated as described in Section 2.14.2.1. It was then resuspended in buffers (pH7.4) of different ionic strengths each containing the same amount of protein. The reactions were allowed to proceed for 180min then the support specific activity was measured. The results are presented in Table 4.

These results were expected, since if there is a high salt concentration present, then the support reactive groups and the enzyme reactive groups tend to be masked and so reduce the possibility of chemical bond formation. Another fact which must be considered is the isoelectric point of YADH (5.4, Sund and Theorell, (1963)). At pH7.4, the protein is negatively charged and so will be attracted towards the positively charged support. As the ionic strength increases, there will be less electrostatic attraction between the support and protein. Hence the possibility of coupling and the support specific activity will be lessened.

Table 4.

Ionic Strength of Coupling Buffer	Activity (U/g)	% Highest Activity
0.05	91	100
0.10	63	70
0.20	48	53

4.2.5. Effect of Varying the Glutaraldehyde concentration in the Activation Procedure

In any experiment, the cost of materials is always an important consideration. Glutaraldehyde is no exception and so the concentration of glutaraldehyde in the activation of Cellex-AE procedure was varied to find out what minimum concentration of glutaraldehyde gave a maximum support specific activity under defined conditions. The results are presented in Fig. 13, and show that a 2% (w/w) solution of glutaraldehyde gave a support specific activity of 50U/g enzyme-support. Increasing the glutaraldehyde concentration three-fold gave no increase in activity.

4.2.6. Effect of Varying the Glutaraldehyde Activation Time of Cellex-AE

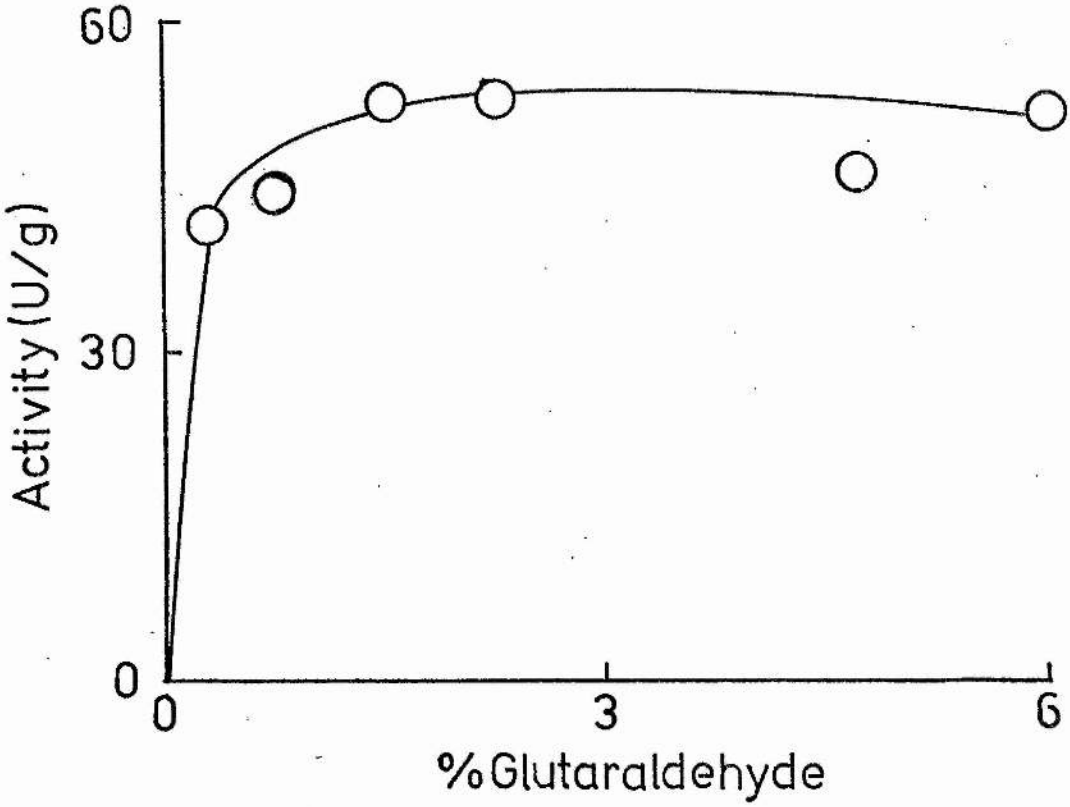
Using the concentration of glutaraldehyde described in Section 4.2.5, the time of the activation of Cellex-AE with glutaraldehyde was varied. The results are presented in Fig. 14 and indicate that 5min is sufficient time for maximum activation of the support. An activation time of 60min gives a derivative with an activity similar to that of a derivative activated for 5min.

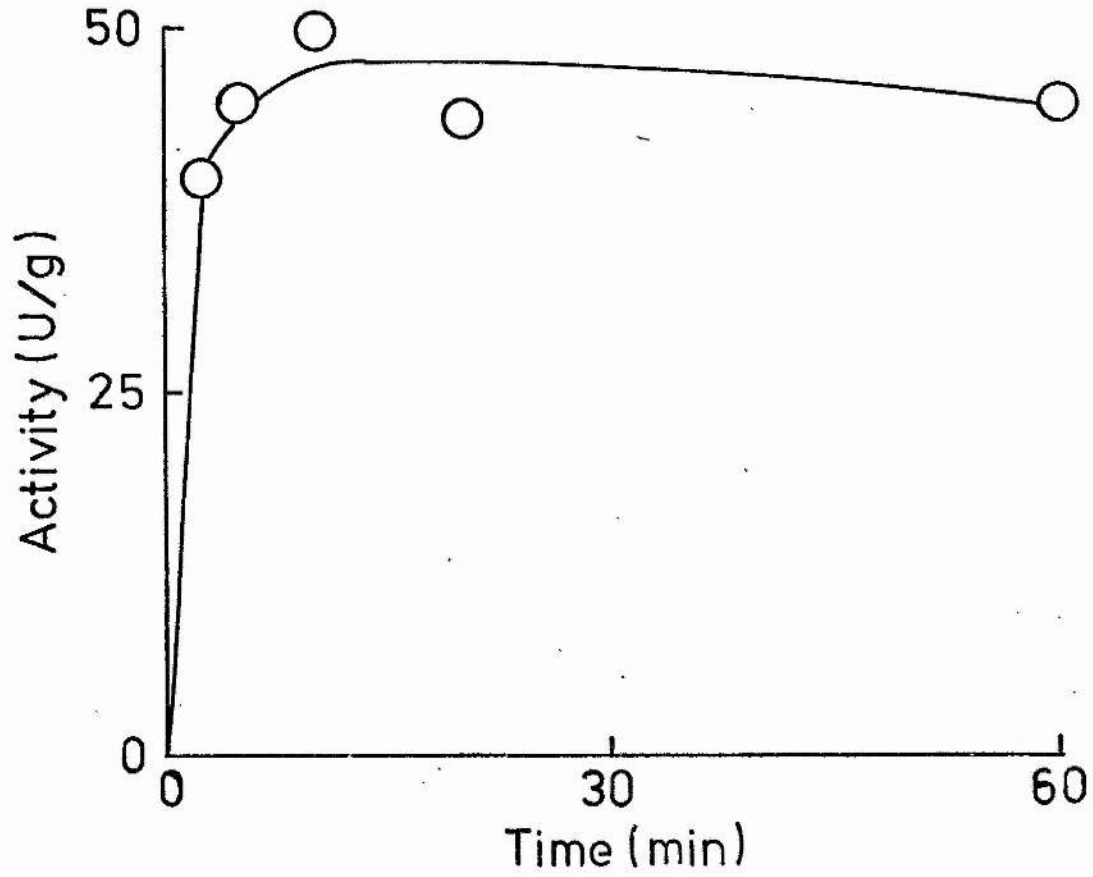
Fig. 13.

Effect of varying the glutaraldehyde concentration in the activation procedure.

Fig. 14.

Effect of varying the glutaraldehyde activation time of Cellex-AE.


60
30
0
Activity (U/g)
0
3
6
% Glutaraldehyde


50
25
0
Activity (U/g)
0
30
60
Time (min)

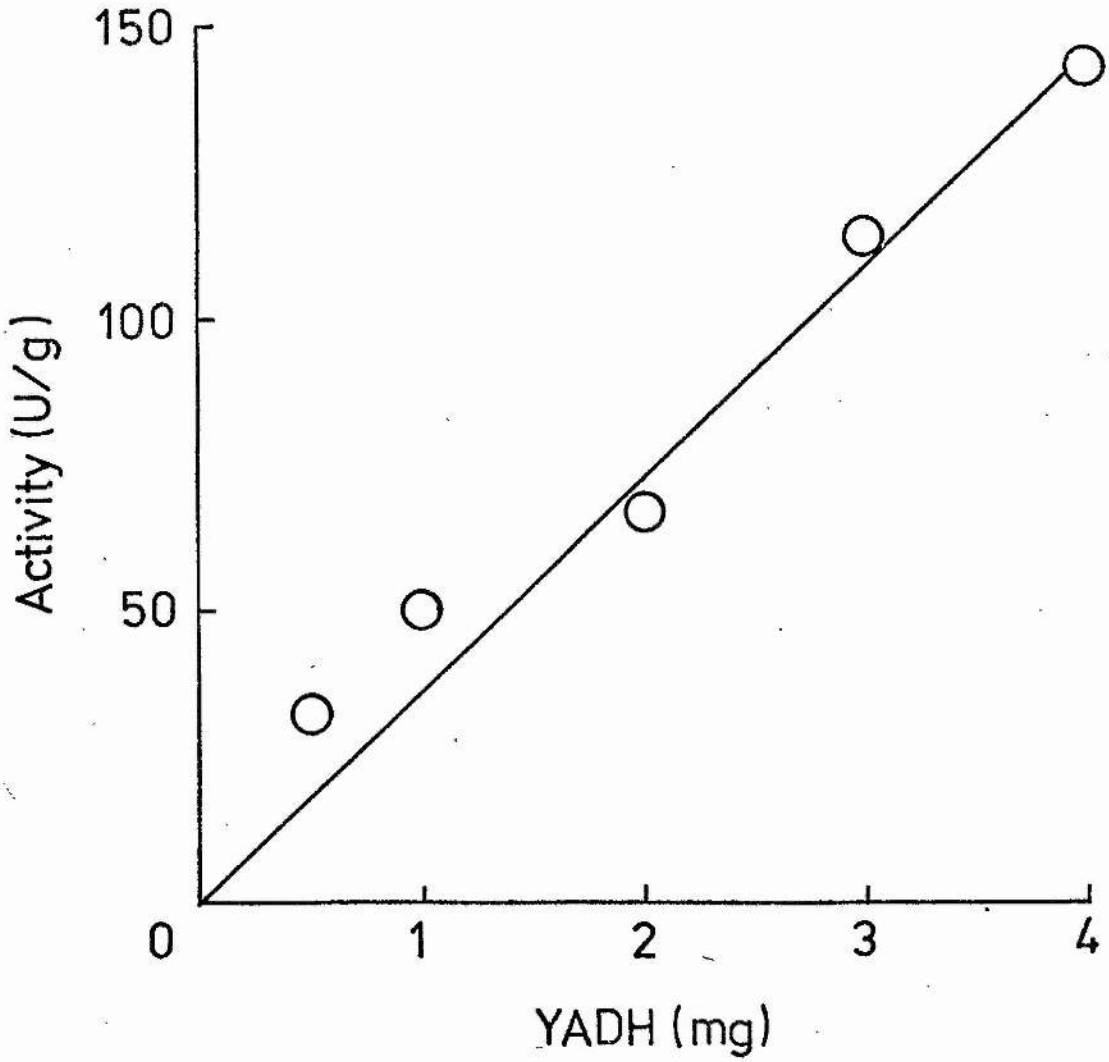


Fig. 15.

Effect of enzyme concentration on the immobilisation of YADH to Cellex-AE.

4.2.7. The Effect of Enzyme Concentration on the Immobilisation of YADH to Cellex-AE

Activated Cellex-AE was resuspended in phosphate buffer (pH7.4,I0.1) containing different concentrations of YADH. The coupling procedure was as described in Section 2.18.2.1. The results presented in Fig. 15 show that the support specific activity increased linearly with mg YADH present in the coupling mixture up to a concentration of 4mg YADH. The amount of YADH which was actually immobilised was not calculated but from later experiments (see Section 4.2.9), there is not a proportional decrease in the percentage of initial YADH left in the supernatant as the suspension activity increases. A possible explanation of this will be discussed later.

4.2.8. General Conclusions

The experiments described in Sections 4.2.1 to 4.2.7 provided the following optimal conditions for the coupling of YADH to Cellex-AE using the bifunctional reagent glutaraldehyde: 250mg Cellex-AE, suspended in phosphate buffer (pH7.4,I0.1), is activated for 3min at 25^{0} with a 2% (w/w) solution of glutaraldehyde. After washing, the activated Cellex-AE is resuspended in phosphate buffer (pH7.4,I0.1) containing 2mg YADH and stirred on an ice-bath for 3h.

Table 5..

Derivative	mg protein in coupling	% Initial Protein Coupled	mg YADH per g support	Activity (U/g)	Activity (U/mg immobilised YADH)
"200µg"	0.2	73	0.6	49	82
"Reduced"	1	42	1.8	59	33
"Unreduced"	1	43	1.8	106	59
"5mg"	5	65	13	205	16

This coupling procedure gives an immobilised enzyme derivative with a support specific activity of about 75U/g support. For general convenience however, an immobilised preparation of YADH on Cellex-AE was prepared as described in Sections 2.14.2.1 and 2.18.2.1.

4.2.9. Immobilised YADH Derivatives for Kinetic and Other Analysis

Using the information gleaned from Sections 4.2.1 to 4.2.7, four different immobilised YADH derivatives were prepared, and used subsequently for kinetic, equilibrium and stability studies. The first of these derivatives is known as the "200µg" preparation; this preparation contained 200µg YADH in the initial coupling procedure. The next two derivatives contained exactly the same initial YADH concentration (1mg in 5ml), in the coupling procedure but one of these was reduced with sodium borohydride as described in Section 2.18.2.1; these are known as the "reduced" and "unreduced" derivatives. The final derivative contained 5mg YADH in the initial coupling procedure and is known as the "5mg" preparation; this preparation was reduced with sodium borohydride. Table 5 gives a summary of the support specific activity, the per cent initial YADH coupled, and the number of mg YADH coupled per g of Cellex-AE for each of the preparations.

The following points may be deduced from the table. When the concentration of YADH is low in the coupling suspension, then a large percentage of it is immobilised. When the YADH concentration is increased five-fold, the percentage amount of initial enzyme protein immobilised decreases although the total amount of YADH which is immobilised under these conditions increases three-fold. When a large initial concentration of enzyme protein is present, the percentage of protein coupled increases. This is probably due to each enzyme molecule having fewer glutaraldehyde residues to react with. It should also be noted that although the amount of YADH bound per g support increases in the ratio 1:3:3:22, the activities of the supports increase in the ratio 1:1.2:2.2:4.2. Also the ratio of support activity per mg YADH coupled is in the ratio 5:2:4:1. This could be due to overcrowding of the support and hence decreased availability of the active centres for binding and reaction with the substrates. Similar conclusions were derived when ficin was bound to CM-cellulose (Lilly et al, 1966). Also noteworthy is the lower support activity of the derivative which has been reduced with sodium borohydride; this has been previously commented on in Section 4.1.1.

4.3. Attachment of YADH to DEAE-cellulose

4.3.1. Variation of Coupling Time of modified Cyanuric Chloride to DEAE-cellulose

The coupling time of the modified cyanuric chloride derivative to DEAE-cellulose was varied to see if a longer coupling time was required than for chymotrypsin (Kay and Lilly, 1970). The results are presented in Table 6.

The results show that far higher activities were attained than using glutaraldehyde as the bifunctional reagent. DEAE-cellulose however, has at least three times as many functional groups capable of reaction with the modified cyanuric chloride as Cellex-AE does with glutaraldehyde; this could account for the difference. One might argue that "AE-cellulose should give a similar value to the DEAE-cellulose but glutaraldehyde is known to exist in dimers and trimers as well as monomers (Korn, 1972); hence one glutaraldehyde "molecule" could modify two or three amino groups on the Cellex-AE whereas one modified cyanuric chloride molecule will react with only one sugar hydroxyl group on the DEAE-cellulose.

4.3.2. The Effect of varying the Time of YADH coupling to activated DEAE-cellulose

1g DEAE-cellulose was activated with modified cyanuric chloride for 15min as described in

Table 6.

Precoupling Time (min)	Activity (U/g)
15	506
30	434

Table 7.

Coupling Time (h)	Activity (U/g)
1	519
4	455
10	369

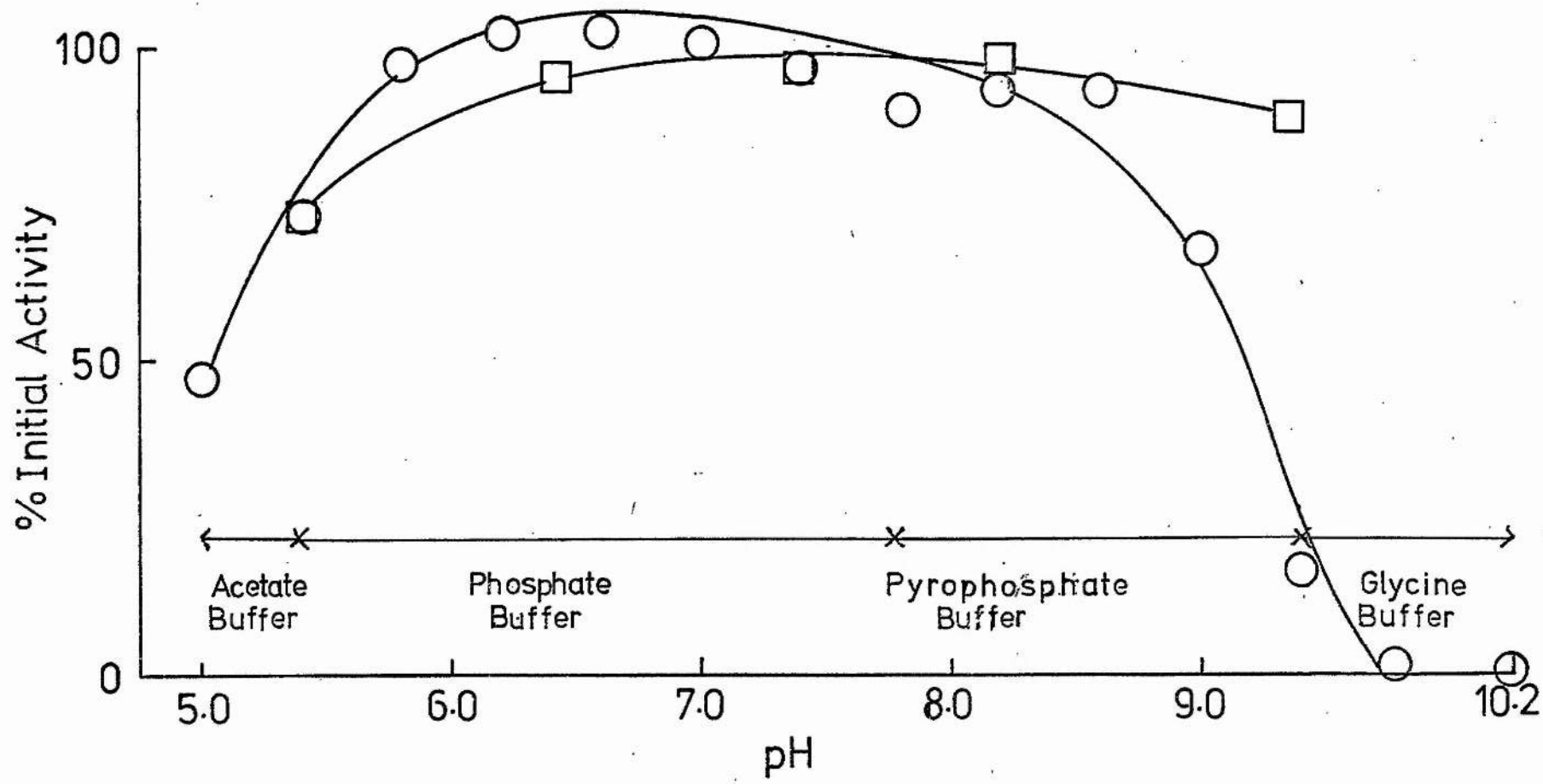


Fig. 16.

Effect of pH on the short term stability at 4°C of soluble YADH (○) and Cellex-AE-YADH (□).

Section 2.15. The activated DEAE-cellulose was suspended in 25ml phosphate buffer (pH7.4,I0.1) containing 10mg YADH and aliquots were withdrawn at intervals, washed and assayed for activity. The results of these experiments are presented in Table 7.

It can be seen that 60min gives a preparation of a very high activity (519U/g enzyme support).

4.4. Stability of Soluble and Immobilised YADH

4.4.1. Effect of pH on the short term stability of soluble YADH

The stability of soluble YADH was studied in the pH range 5 to 10 by incubating the enzyme in the appropriate buffer for 2h at $4^{o}C$ as described in Section 2.13.1. Assays were carried out after 2h as described in Sections 2.8.2.1.1, 2.8.3.1.1, and 2.8.4. The results are presented in Fig. 16 and show that YADH is most stable in the pH range 6.2 to 7.8. When used for kinetic studies, soluble YADH solutions were always diluted with phosphate buffer (pH7.4,I0.1) containing 0.1% (w/w) bovine serum albumin.

4.4.2. Effect of pH on the long term stability of soluble and immobilised YADH

The stability at $4^{o}C$ of soluble and immobilised YADH was studied over a period of seven weeks by incubating the soluble or immobilised enzyme

in various buffers. The immobilised preparation used was the "5mg" preparation described in Section 4.2.9. The results, presented in Figs. 17 and 18, show that there is very little difference between the stabilities of soluble and immobilised YADH preparations. At the lower pH values the immobilised derivatives tend to be slightly more stable. Notable also is that the residual activity of both soluble and immobilised YADH is always tending to a constant value.

4.4.3. Stability of reduced and unreduced Cellex-AE-YADH derivatives

The stability at 4°C of reduced and unreduced derivatives of YADH on Cellex-AE was studied over a period of two months. The results are presented in Fig. 19 and show that the unreduced derivative is slightly less stable at 4°C than the reduced derivative.

4.4.4. Comparison of the stability of reduced Cellex-AE-YADH and DEAE-Cellulose-YADH

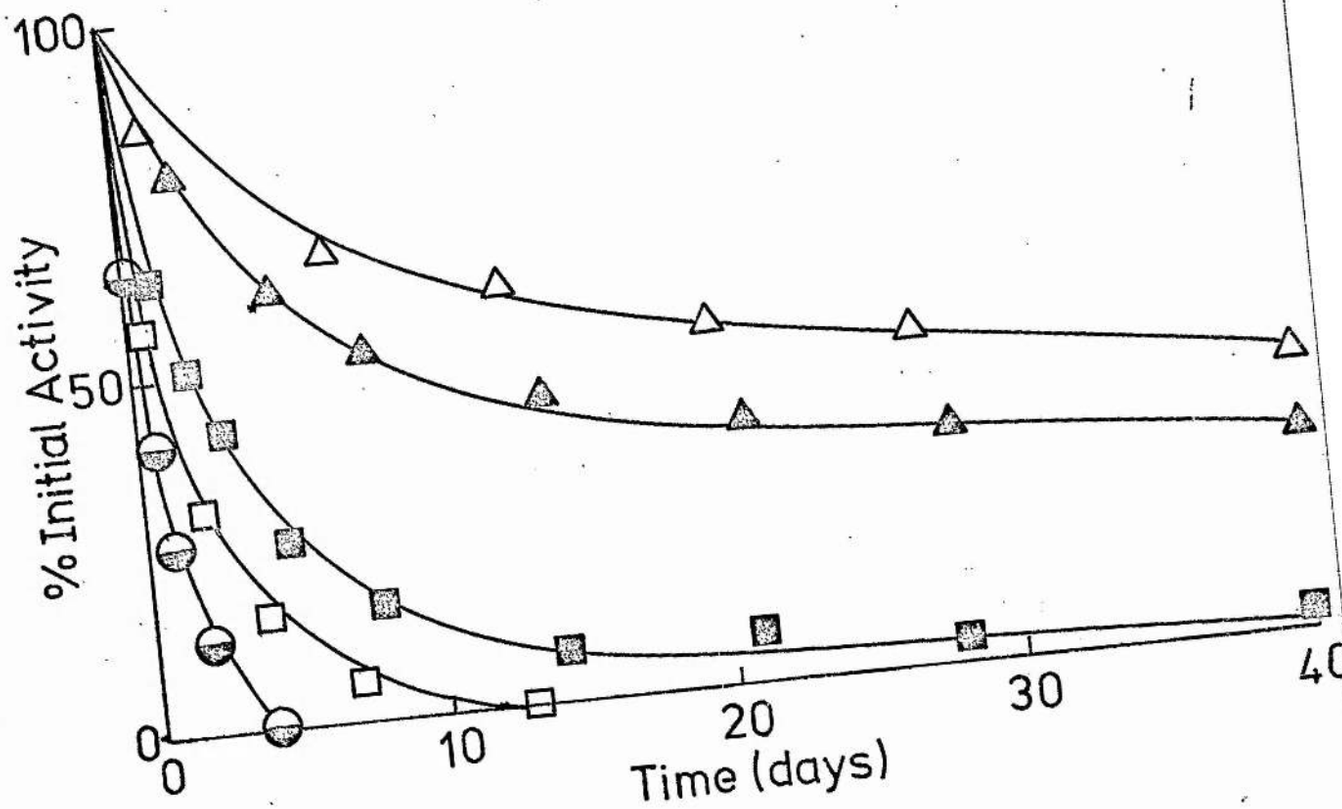
Fig. 19(b) compares the long term stability of YADH attached to Cellex-AE and to DEAE-Cellulose on storage at 4°C. After twelve days, the Cellex derivative has retained 37% of its original activity, whereas the DEAE derivative has retained only 10% of its original activity.

Fig. 17.

Comparison of the long term stability at 4°C of soluble YADH (unfilled symbols) and Cellex-AE-YADH (filled symbols) at pH5.4 (◯), at pH6.4 (□) and at pH7.4 (△).

Fig. 18.

Comparison of the long term stability at 4°C of soluble YADH (unfilled symbols) and Cellex-AE-YADH (filled symbols) at pH8.4 (□) and at pH9.0 (◯).


100
50
0
% Initial Activity
0
10
20
30
40
Time (days)

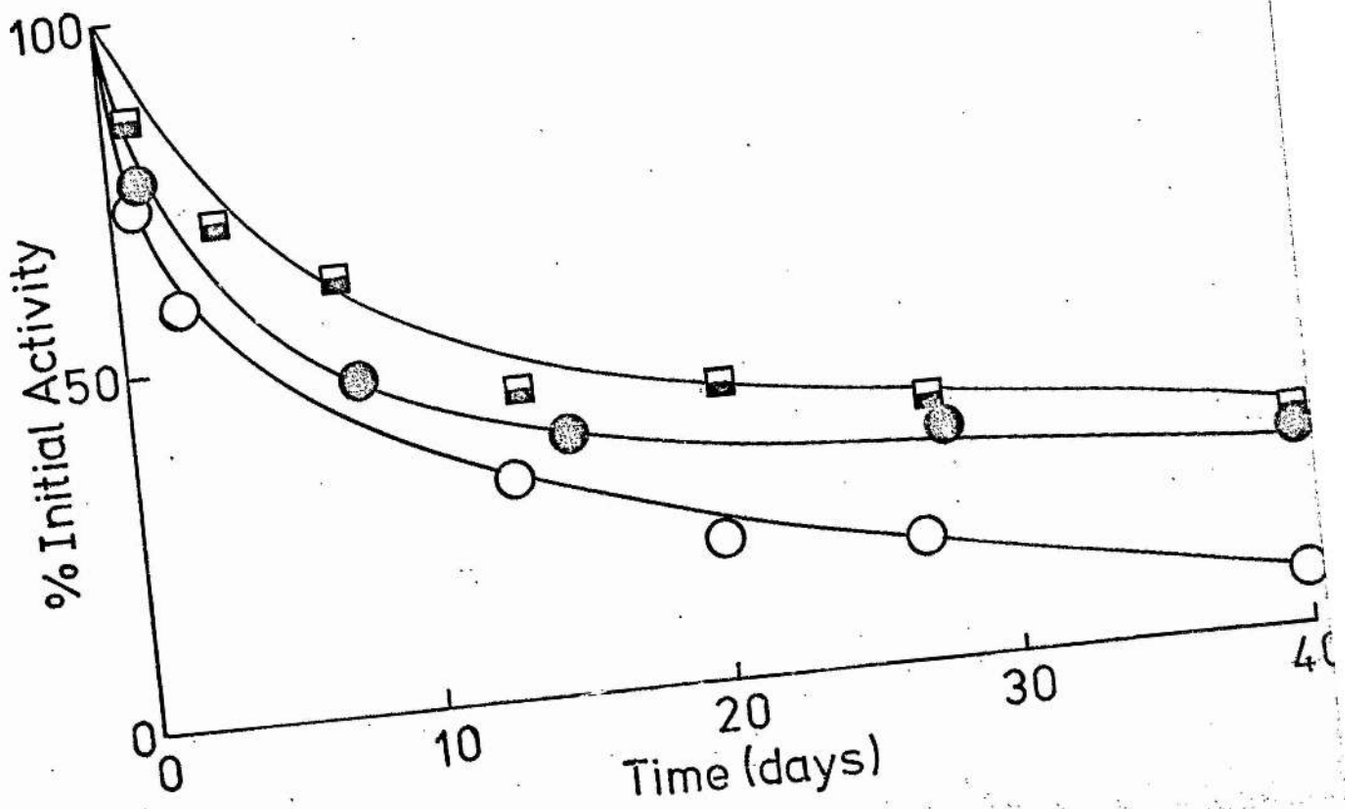

100
50
0
% Initial Activity
0
10
20
30
40
Time (days)

Fig. 19.

Comparison of the stability at 4°C and at pH7.4 of soluble YADH (△), and the "reduced" (○) and "unreduced" (□) preparations described in Section 4.4.3.

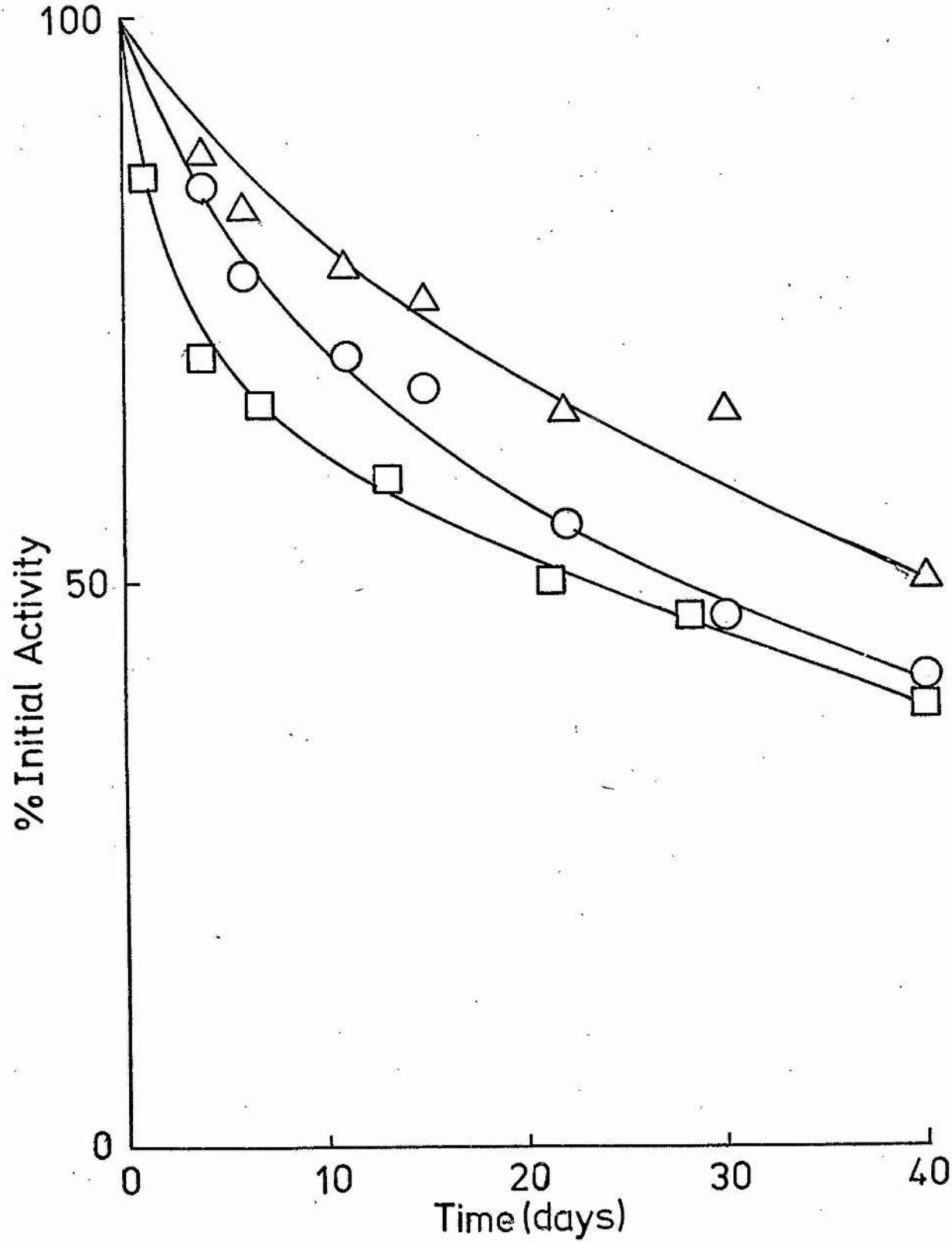
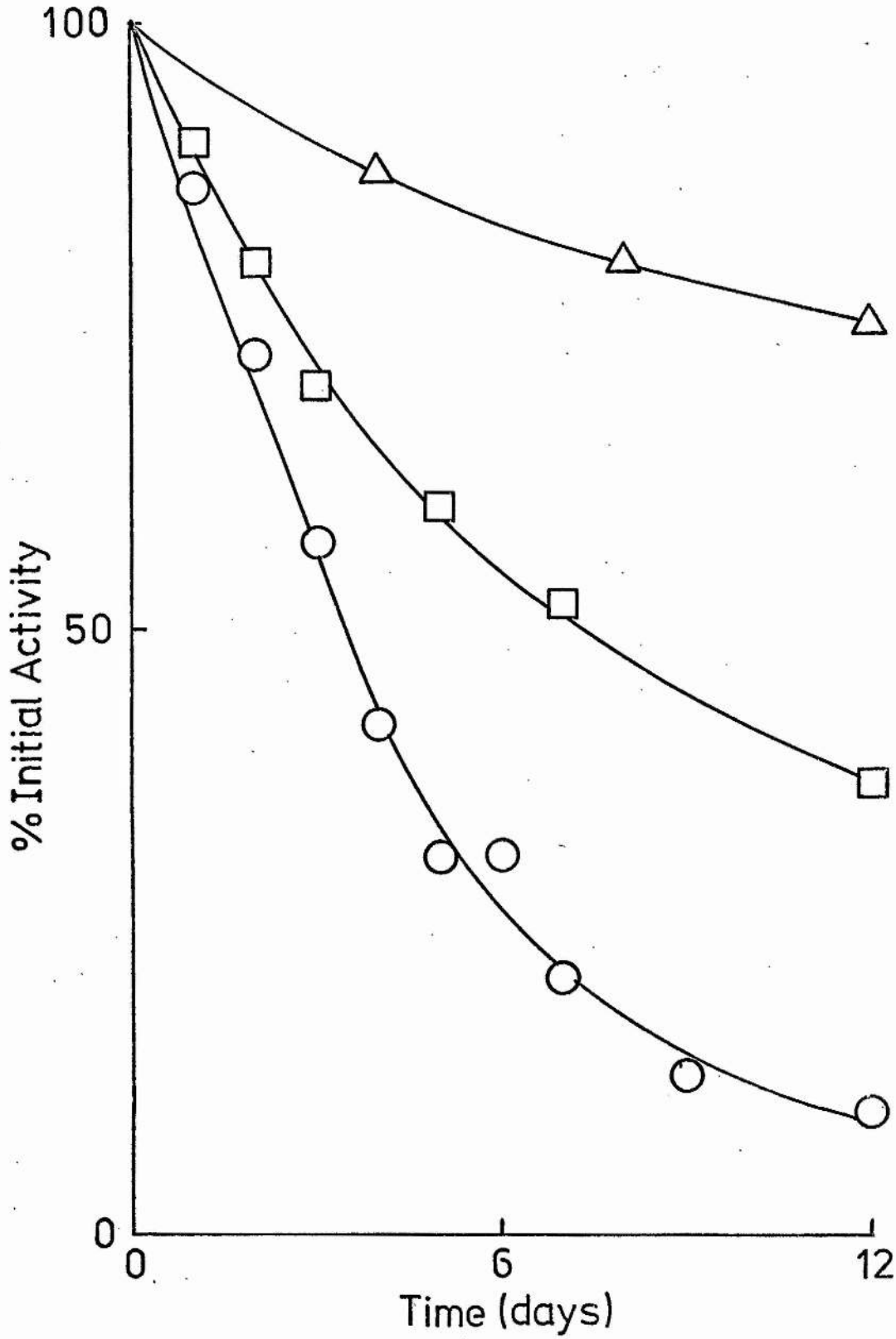

100
50
0
% Initial Activity
0
10
20
30
40
Time (days)

Fig. 19(b).

Comparison of the stability at 4°C of soluble YADH (△), Cellex-AE-YADH (□) and DEAE-cellulose-YADH (○) at pH7.4.


100
50
0
% Initial Activity
0
6
12
Time (days)

4.5. Michaelis parameters of soluble and immobilised YADH

Kinetic experiments were carried out on soluble YADH and the derivatives described in Sections 4.2.9. The experiments were undertaken in order to determine the variation of Michaelis parameters in both the forward and reverse directions for soluble YADH and for DEAE-cellulose-YADH. The Michaelis parameters for all four substrates at pH7.4 were determined for the derivatives prepared in Section 4.2.9 and the results were plotted according to the method of Lineweaver and Burk, (1934).

4.5.1. The Effect of pH on the Michaelis Parameters

The Michaelis parameters for all four substrates were determined for soluble and DEAE-cellulose-YADH in the pH range 6 to 9. For each determination, one of the substrates was varied whilst the other was held constant at a sufficiently saturating concentration. The K_m's obtained by double-reciprocal plots are not those at an infinite concentration of one of the substrates and are therefore known as apparent K_m's. Typical double-reciprocal plots obtained are presented in Figs. 20, 21, 22, and 23.

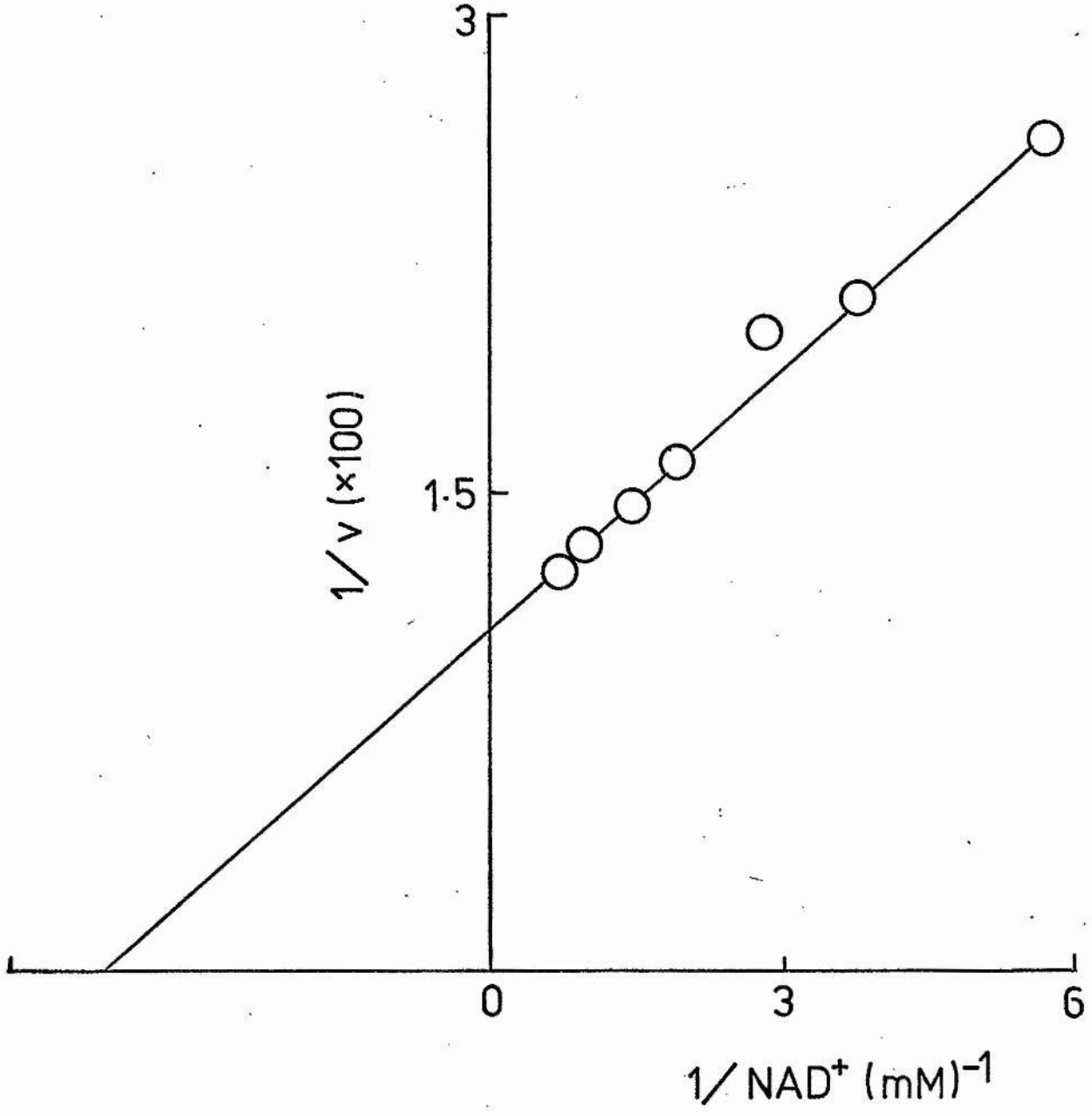


Fig. 20.

Primary reciprocal plot of initial velocity data for soluble YADH at pH6.0. The ethanol concentration was held constant at 1M. Temperatures of assays were 25°C. Units of 'v' are micromoles per ml per min per mg YADH.

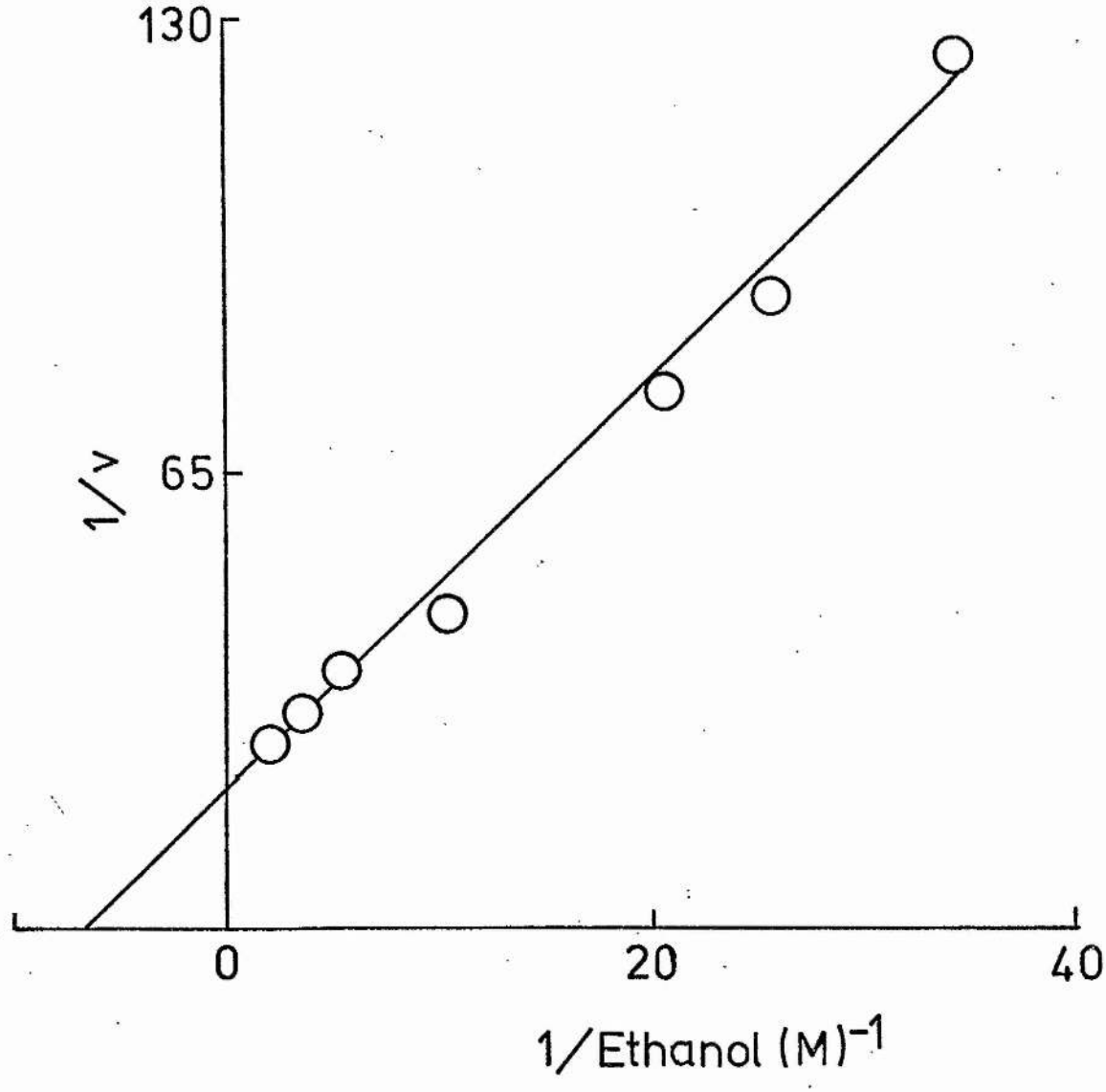


Fig. 21.

Primary reciprocal plot of initial velocity data for DEAE-cellulose-YADH at pH6.0. NAD^+ concentration was held constant at 2mM. Temperatures of assays were 25°C. Units of 'v' are micromoles per ml per min per mg enzyme-support.

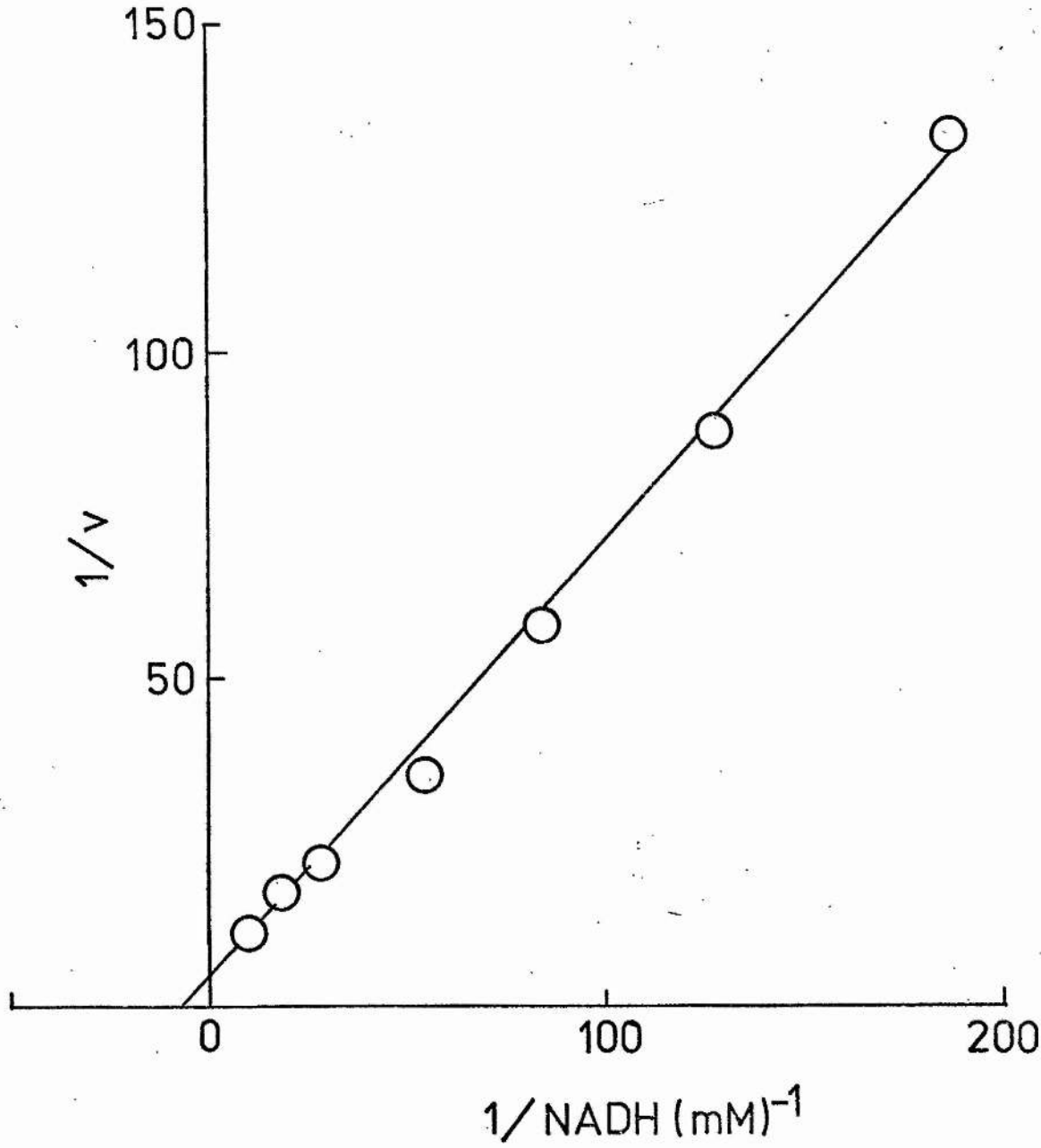


Fig. 22.

Primary reciprocal plot of initial velocity data for DEAE-cellulose-YADH at pH8.2. Acetaldehyde concentration was held constant at 5mM. Temperatures of assays were 25°C. Units of 'v' are micromoles per ml per min per mg enzyme-support.

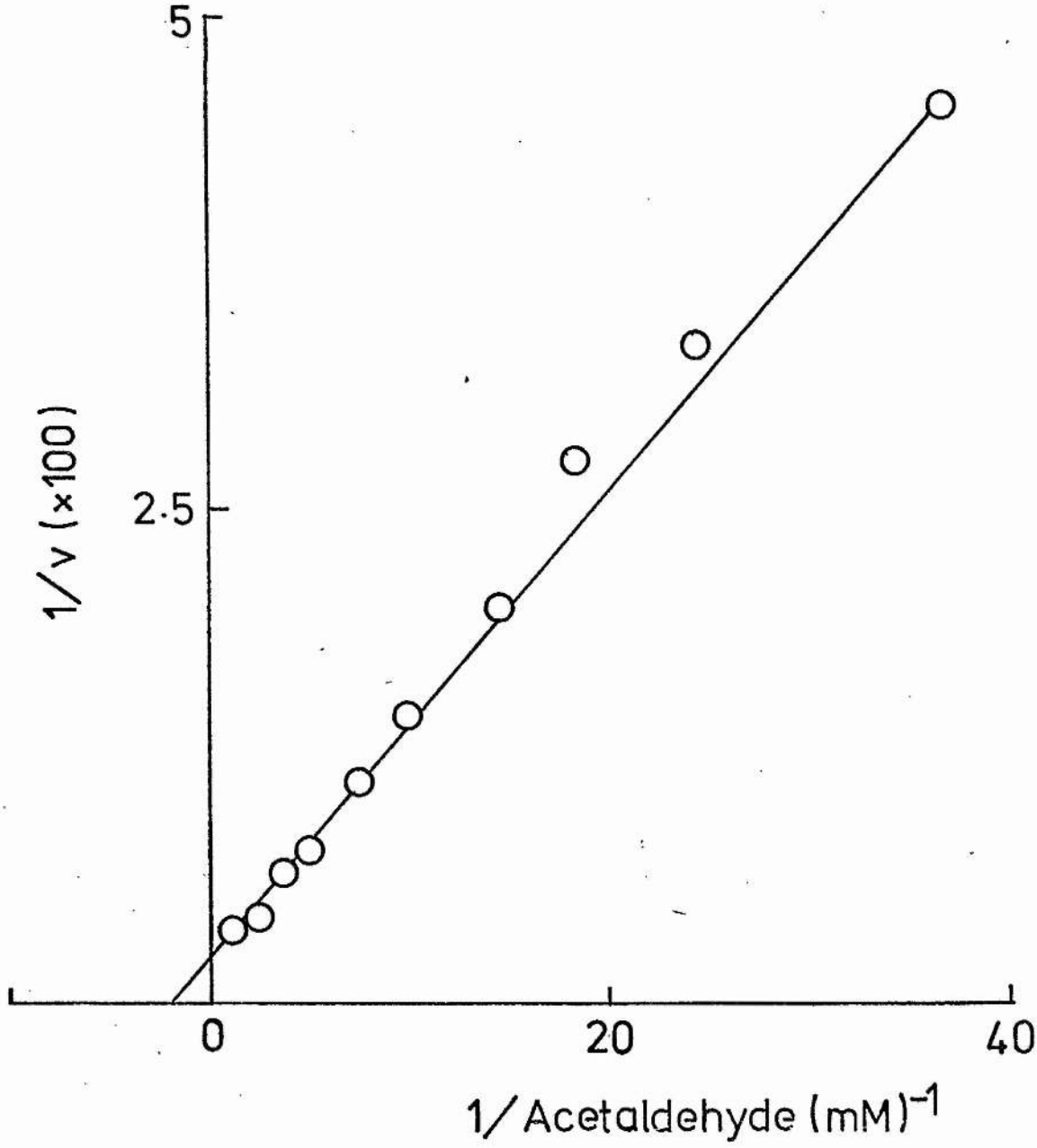


Fig. 23.

Primary reciprocal plot of initial velocity data for soluble YADH at pH8.4. NADH concentration was held constant at 150μM. Temperatures of assays were 25°C. Units of 'v' are micromoles per ml per min per mg YADH.

Fig. 24 shows the variation of apparent K_m (K_m') of NAD^+ for soluble and DEAE-cellulose-YADH with pH. There is little difference between the values obtained although the minimum value of K_m' for NAD^+ (110μM at pH7.0) for the soluble enzyme has moved to pH7.4 when YADH is immobilised on DEAE-cellulose. The K_m' values obtained for NAD^+ for the immobilised enzyme are slightly higher than those for the soluble enzyme. Both effects could be due to the charge on the support and also the presence of the aromatic triazine ring.

Fig. 25 shows the variation of K_m' with pH for ethanol for both soluble and immobilised YADH. There is virtually no difference in the values obtained and this was expected since K_m' for ethanol is millimolar and not micromolar as are acetaldehyde, NADH, and NAD^+. The saturating effect of such a concentration, in addition to ethanol being a neutral molecule, would mask any charge effect of the support.

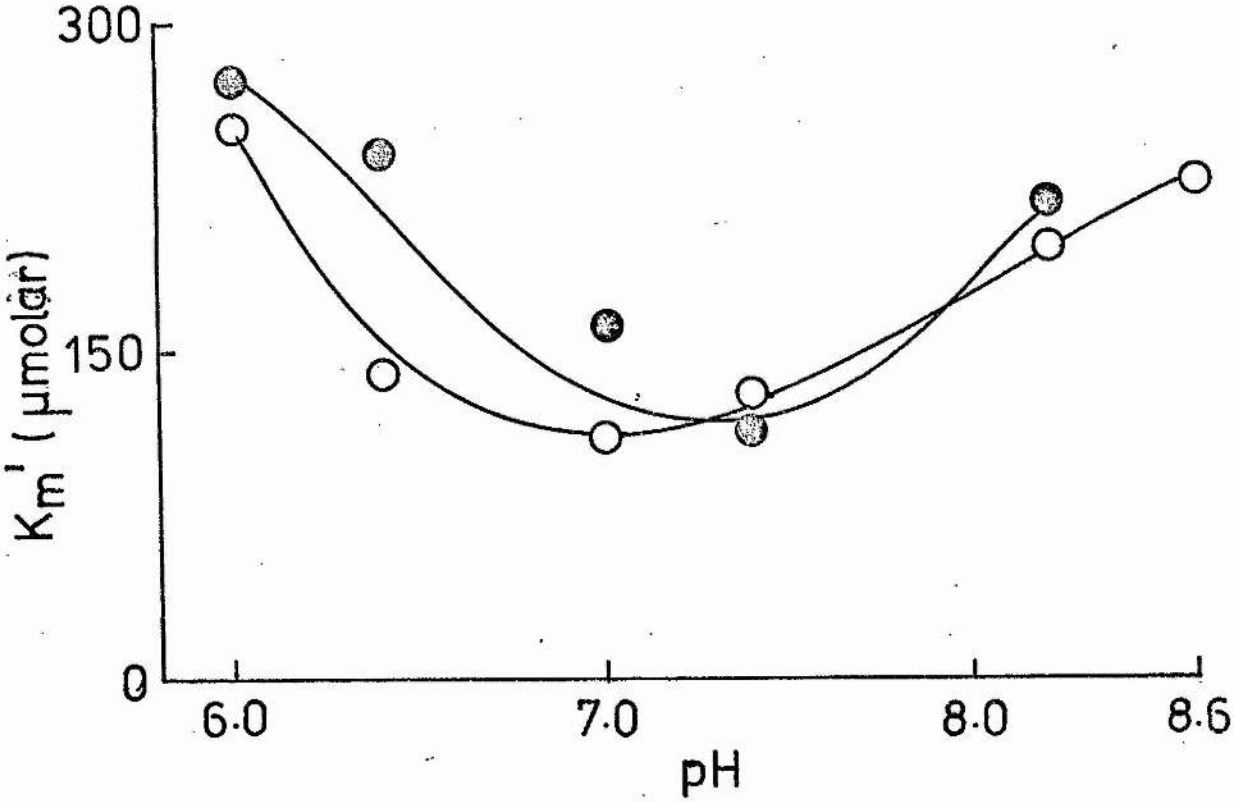
Fig. 26 shows the variation of K_m' with pH for NADH for soluble and immobilised YADH. As for NAD^+, very little change in the values was obtained, and where a small change does occur, this is probably due to the charge on the support and to the nature of the bifunctional linking compound.

Fig. 24.

Variation of K_m' of NAD^+ with pH for soluble YADH (○) and DEAE-cellulose-YADH (●).

Fig. 25.

Variation of K_m' of ethanol with pH for soluble YADH (□) and DEAE-cellulose-YADH (■).


300
150
0
K_m' (μmolar)
6·0
7·0
8·0
8·6
pH

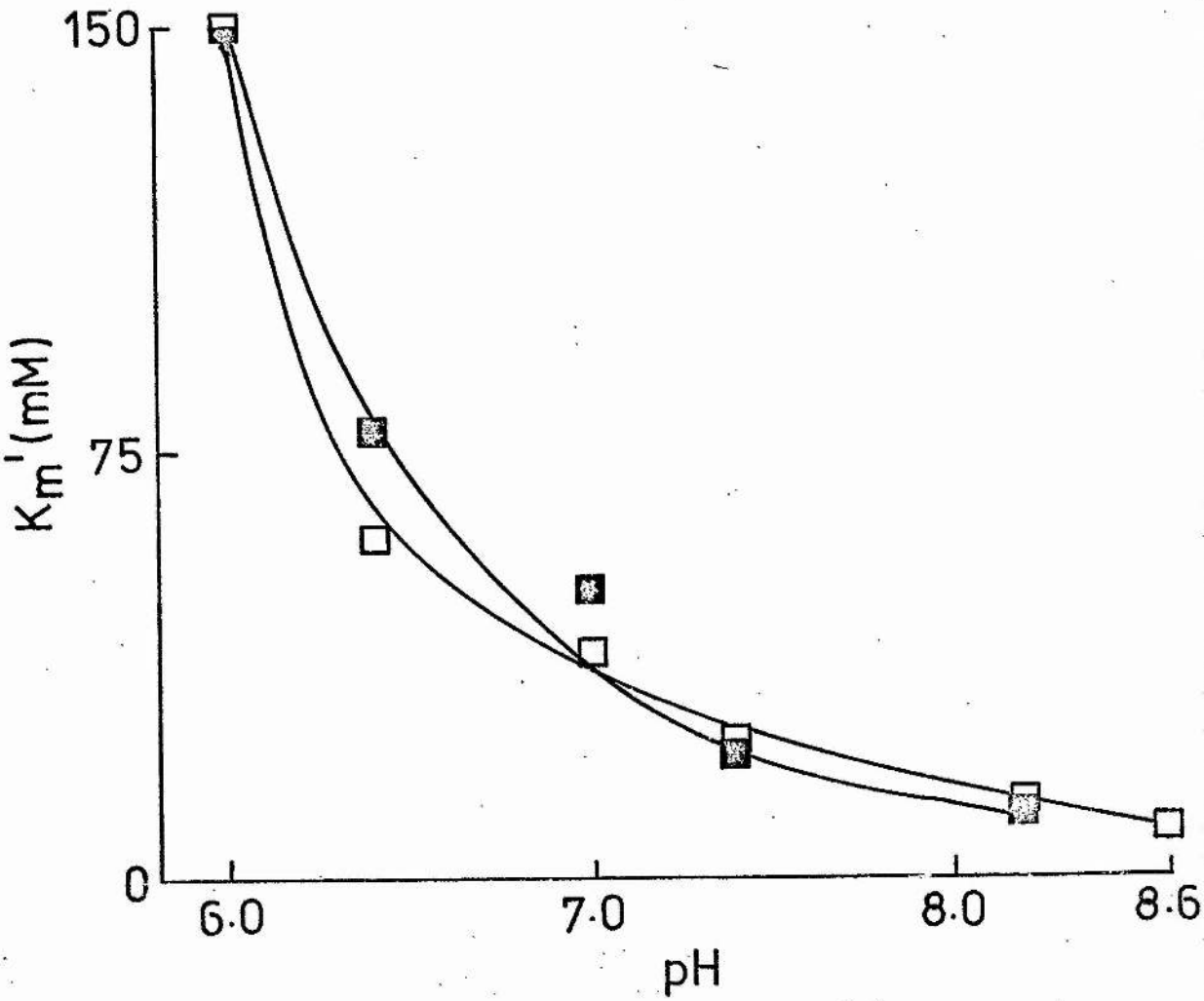
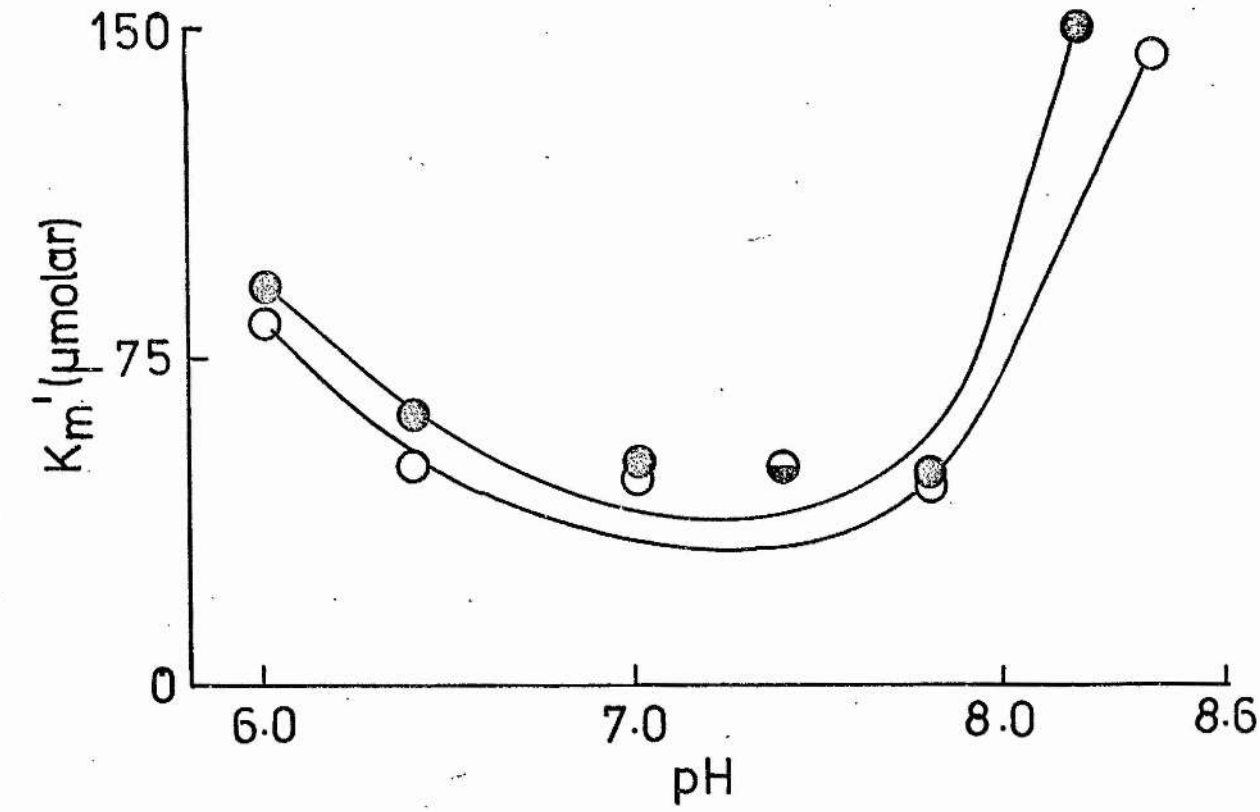

150
75
0
K_m'(mM)
6·0
7·0
8·0
8·6
pH

Fig. 26.

Variation of K_m' of NADH with pH for soluble YADH (○) and DEAE-cellulose-YADH (●).

Fig. 27.

Variation of K_m' of acetaldehyde with pH for soluble YADH (□) and DEAE-cellulose-YADH (■).


150
75
0
K_m' (μmolar)
6·0
7·0
8·0
8·6
pH

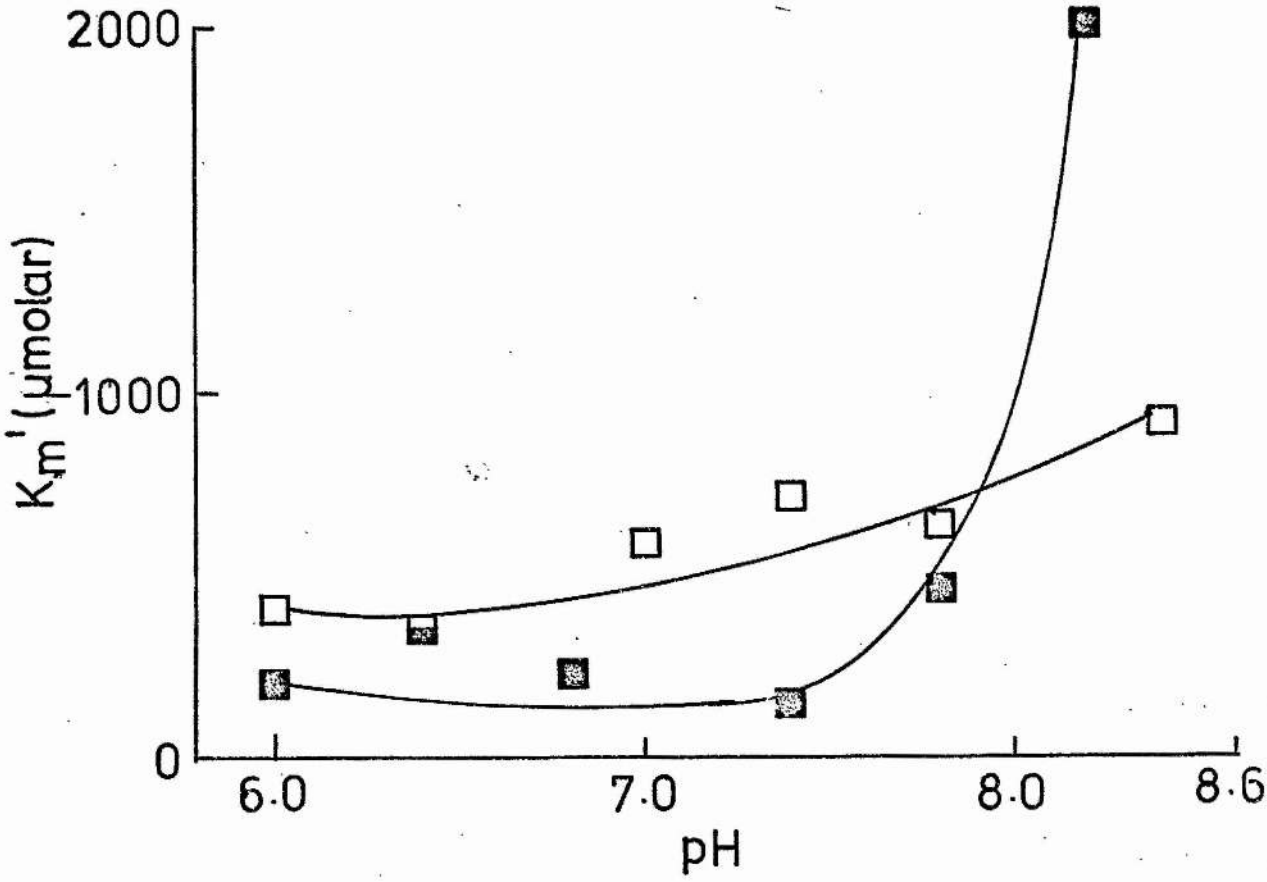

2000
1000
0
K_m' (μmolar)
6·0
7·0
8·0
8·6
pH

Fig. 28.

Variation of V_{max}' with pH for the forward (□) and the reverse (○) directions of the YADH catalysed reactions. Unfilled symbols represent the soluble enzyme; filled symbols represent the DEAE-cellulose immobilised enzyme.

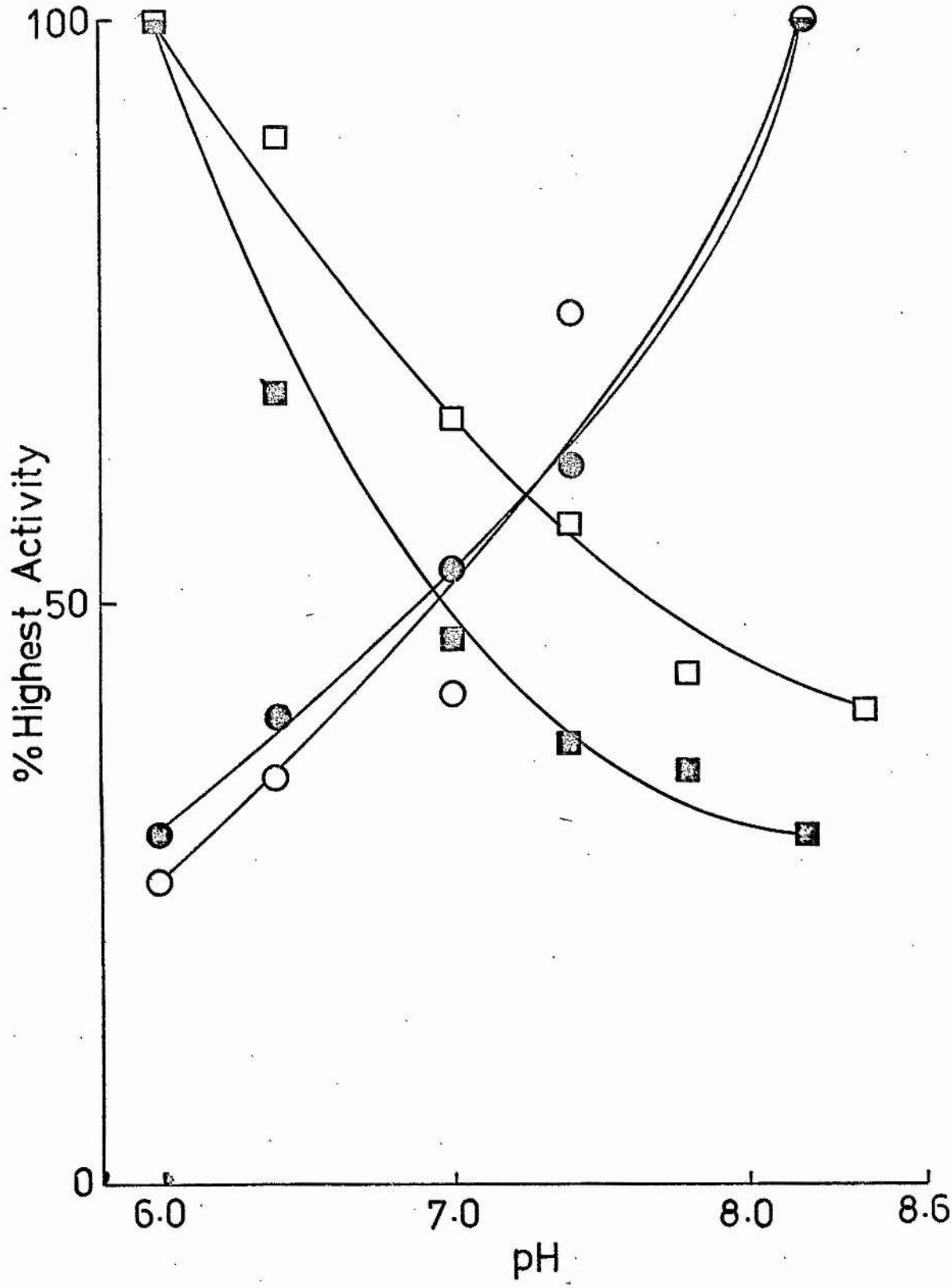

100
50
0
% Highest Activity
6·0
7·0
8·0
8·6
pH

Table 8.

Derivative	pH	V_{max}' [1] (forward)	V_{max}' [2] (reverse)	Ratio
Soluble	6.0	154	1300	1 : 8.5
	6.4	207	1100	1 : 5.5
	7.0	250	990	1 : 4
	7.4	452	830	1 : 1.8
	8.4	600	625	1 : 1.05
	9.0	950	430	1 : 0.45
DEAE	6.0	0.0410	0.730	1 : 18
	6.4	0.0630	0.500	1 : 8
	7.0	0.0750	0.345	1 : 4.5
	7.4	0.0920	0.270	1 : 3
	7.8		0.240	
	8.2	0.1680	0.218	1 : 1.3

[1]Units of micromoles NADH formed per ml per min per mg enzyme or per mg enzyme-support.

[2]Units of micromoles NADH oxidised per ml per min per mg enzyme or per mg enzyme-support.

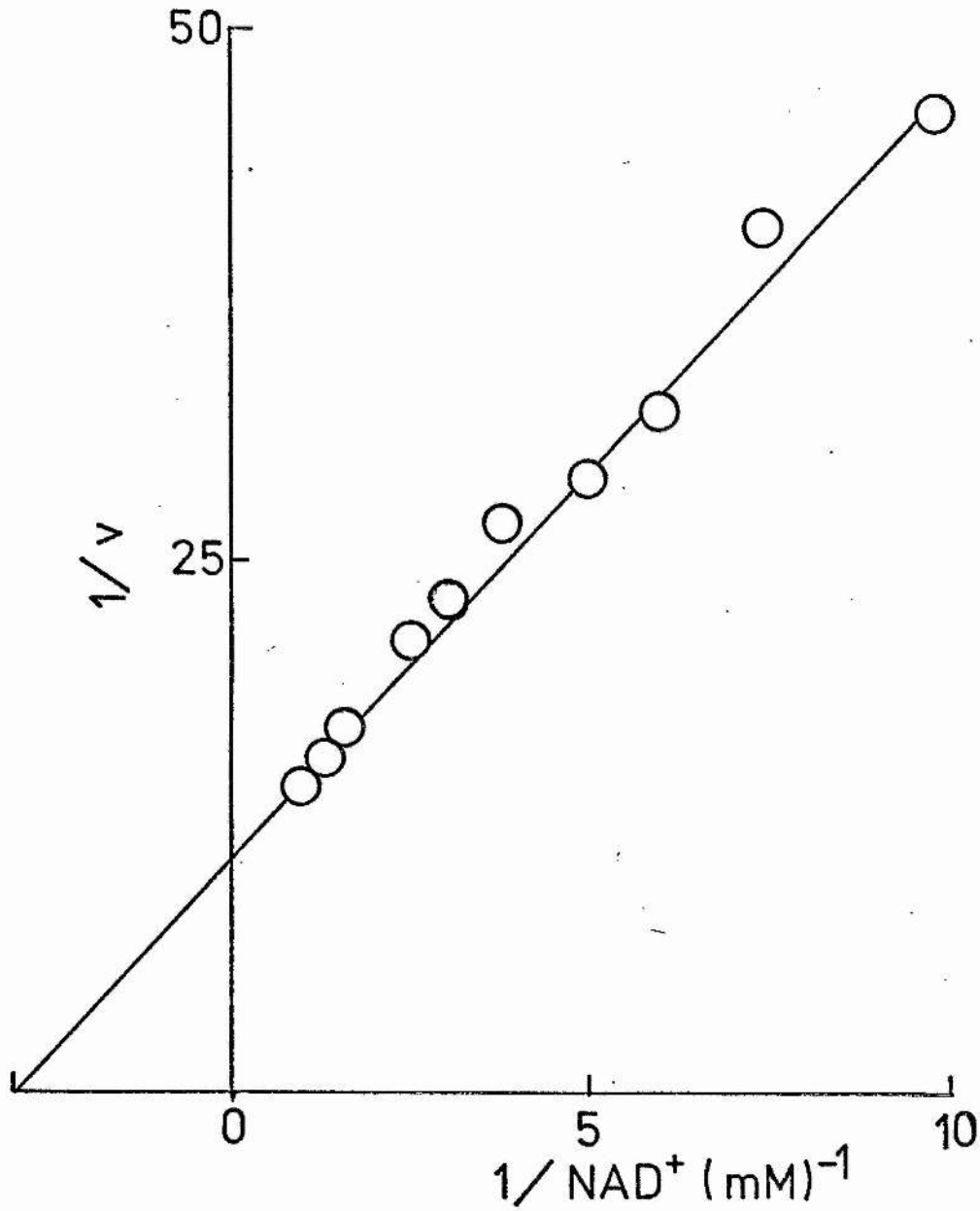


Fig. 29.

Primary reciprocal plot of initial velocity data for "5mg" preparation at pH7.4. Ethanol concentration was held constant at 500mM. Temperatures of assays were 25°C. Units of 'v' are micromoles per ml per min per mg enzyme-support.

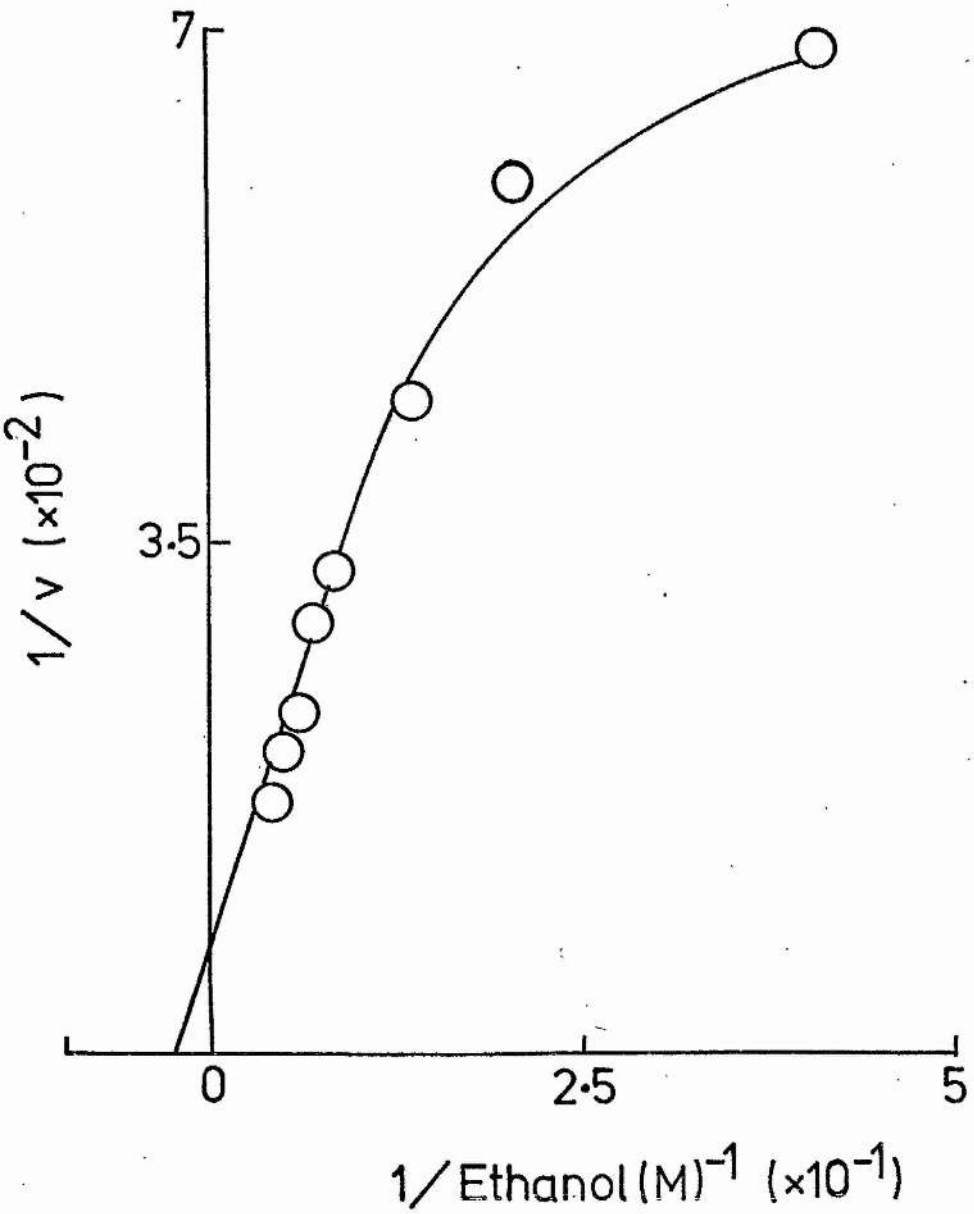


Fig. 30.

Primary reciprocal plot of initial velocity data for "200µg" preparation at pH7.4. NAD^+ concentration was held constant at 600µM. Temperatures of assays were 25°C. Units of 'v' are micromoles per ml per min per mg enzyme-support.

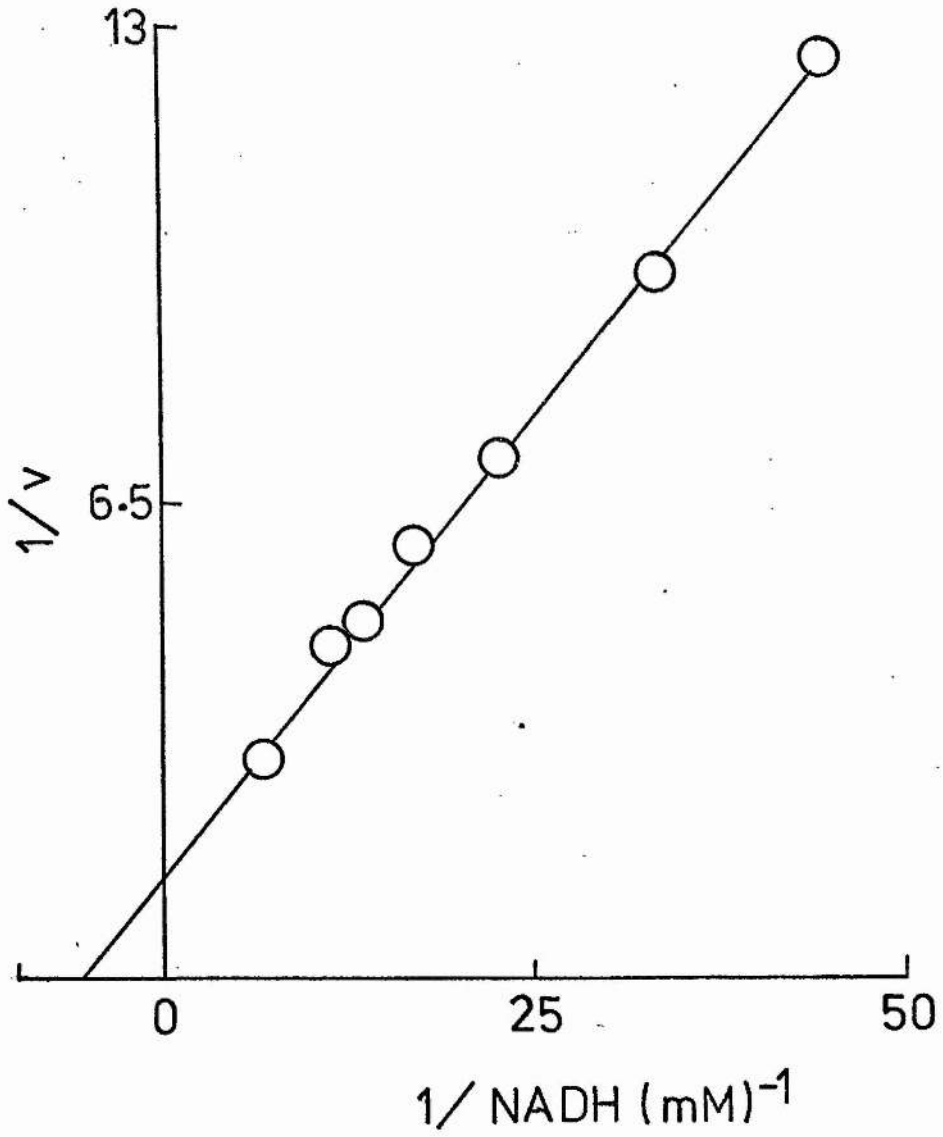


Fig. 31.

Primary reciprocal plot of initial velocity data for "unreduced" preparation at pH7.4. Acetaldehyde concentration was held constant at 600μM. Temperatures of assays were 25°C. Units of 'v' are micromoles per ml per min per mg enzyme-support.

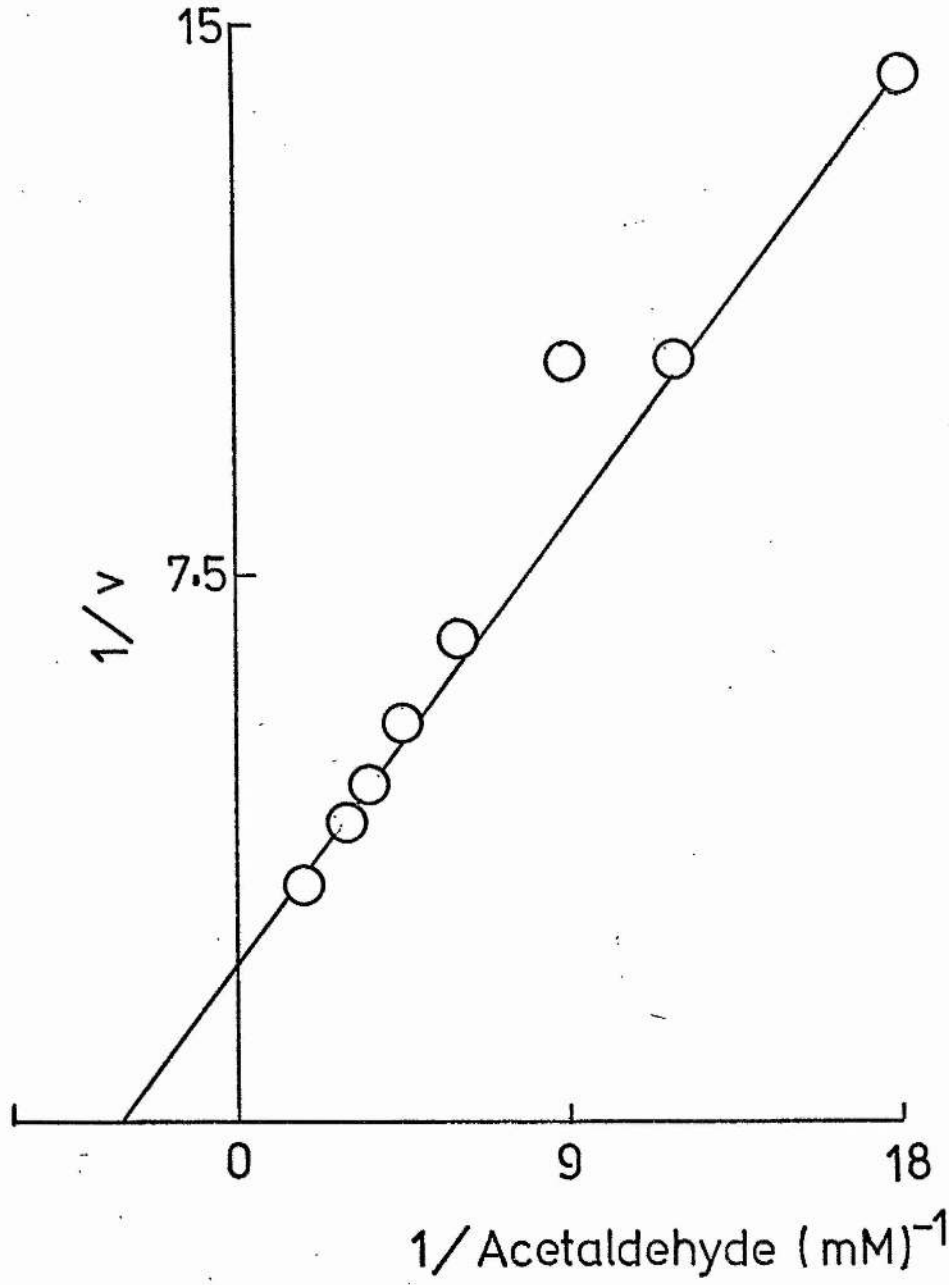


Fig. 32.

Primary reciprocal plot of initial velocity data for "reduced" preparation at pH7.4. NADH concentration was held constant at 150μM. Temperatures of assays were 25°C. Units of 'v' are micromoles per ml per min per mg enzyme-support.

Fig. 27 shows the variation of K_m' with pH for acetaldehyde for soluble and immobilised YADH. The values obtained for the soluble enzyme are slightly higher than those for the immobilised enzyme with a slight increase towards the alkaline side. The K_m' values for the immobilised enzyme, however, tend to increase gradually up to alkaline pH values where a rapid increase is observed.

The effect of pH on the apparent V_{max} of soluble and immobilised enzymes is shown in Fig. 28. No pH optimum, in the pH range studied, was obtained in the forward and reverse directions. Table 8 shows the ratio of the velocities of the forward reactions to those of the reverse directions for both the soluble and the immobilised enzymes.

4.5.2. Michaelis parameters for YADH immobilised on Cellex-AE

The kinetics of the immobilised YADH derivatives described in Section 4.2.9 were studied at pH7.4. As in Section 4.5.1 one substrate was varied whilst the other was held constant at a sufficiently saturating concentration. Typical double-reciprocal plots are presented in Figs. 29, 30, 31, and 32. Apparent K_m's and V_{max}'s for all four substrates were calculated and the results are presented in

Table 9.

The Michaelis constants for soluble YADH have been calculated at pH7.9 (Hayes and Velik, 1953), pH7.15 and pH7.05 (Nygaard and Theorell, 1955), and at pH7.05 (Dickinson and Monger, 1973). By reference to Figs. 24, 25, 26, and 27 which show the variation of Michaelis constants with pH, the values obtained at pH7.4 in these experiments are in reasonable agreement with the values obtained by the authors mentioned.

Table 9 indicates a number of features. The K_m' for ethanol has increased in all immobilised derivatives. The high values obtained for NAD^+ and ethanol for the "200µg" preparation are only estimated values since exact values could not be obtained as the double-reciprocal plots were curved. The K_m' values for NAD^+ have also increased approximately three-fold on immobilisation, the anomalous result again being the K_m' for the "200µg" preparation. For NADH, the K_m' values of the immobilised preparations have doubled relative to the value for the soluble enzyme. With the exception of the "reduced" preparation, the K_m' values for acetaldehyde all decrease on immobilisation.

Table 9.

Derivative [1]	Substrate	K_m' [2]	V_{max}' [3]
Soluble	Ethanol	22	452
	NAD^+	130	452
	Acetaldehyde	700	775
	NADH	50	872
"200µg"	Ethanol	'1000'	0.0131
	NAD^+	'3300'	0.0148
	Acetaldehyde	529	0.141
	NADH	83	0.165
"Reduced"	Ethanol	91	0.0188
	NAD^+	333	0.0204
	Acetaldehyde	333	0.440
	NADH	143	0.333
"Unreduced"	Ethanol	130	0.0315
	NAD^+	455	0.0250
	Acetaldehyde	715	0.476
	NADH	143	0.242
"5mg"	Ethanol	62.5	0.0415
	NAD^+	370	0.0660
	Acetaldehyde	238	0.496
	NADH	119	0.484

[1] "200µg", "Reduced", "Unreduced" and "5mg" have the same significance as in Section 4.2.9.

[2] K_m' : units of micromolar for acetaldehyde, NADH and NAD^+. Units of millimolar for ethanol.

[3] V_{max}' : units as in Table 8.

Table 10.

Enzyme	Derivative	Relative Forward Rate	Relative Reverse Rate
YADH	soluble	1	1.8
	"200µg"	1	10.7
	"Reduced"	1	19
	"Unreduced"	1	15
	"5mg"	1	9

The V_{max}' values obtained for the immobilised derivatives all increase as the amount of protein immobilised increases. Table 10 shows the ratio of the velocities of the forward to the reverse reactions for the soluble and immobilised preparations.

4.6. The Effect of immobilisation of YADH on the equilibrium constant for the YADH-catalysed reaction

The equilibrium constant for soluble and immobilised derivatives of YADH (Section 4.2.9) was determined using the method described in Section 2.11.1 and the results calculated according to the method of Hakala et al. (1956). What these authors did was to take one lactate concentration, a range of NAD^+ concentrations, add to this LDH and measure the increase in absorbancy at 340nm owing to the formation of NADH. Since they knew the initial concentrations of lactate and NAD^+ and the final concentration of NADH, they were able to calculate the equilibrium concentration of all four products. A correction factor for a small amount of pyruvate (1.93×10^{-2}%) impurity in the lactate was also applied. If we consider the equilibrium reaction

$$A + B \overset{K_{eq}}{\rightleftharpoons} C + D$$

then

$$K_{eq} = \frac{(C)(D)}{(A)(B)}$$

A plot of (C)(D) against (A)(B) should give a straight line with the slope equal to the equilibrium constant.

This is the basis of the calculation of the equilibrium constant for LDH by Hakala et al.(1956). LDH catalyses the reaction

$$\text{Lactate} + \text{NAD}^+ \overset{K_{eq}}{\rightleftharpoons} \text{Pyruvate} + \text{NADH} + \text{H}^+$$

and

$$K_{eq} = \frac{(\text{Pyruvate})(\text{NADH})(\text{H}^+)}{(\text{Lactate})(\text{NAD}^+)}$$

A plot of (Pyruvate)(NADH) against (Lactate)(NAD^+) will give a straight line, the slope of which, when multiplied by the hydrogen ion concentration, will give the equilibrium constant.

This is the basis of the calculation of the equilibrium constant. Instead of plotting the product of the equilibrium concentration of pyruvate and NADH against the product of the equilibrium concentrations of lactate and NAD^+, the product of the equilibrium concentrations of acetaldehyde and NADH were plotted against the product of the corresponding equilibrium concentrations of ethanol and NAD^+. Typical plots obtained in this way are presented in Figs. 33 and 34. Table 11 summarises these and other data for the soluble and immobilised derivatives.

The value of the equilibrium constant obtained for the soluble enzyme is in good agreement with that reported by Bäcklin (1958), by Nygaard and Theorell

Fig. 33.

Typical plot for the determination of the equilibrium constant for soluble YADH. Initial concentration of ethanol was 40.754mM; initial concentrations of NAD^+ were 736μM, 552μM, 368μM and 184μM. Other experimental conditions were as described in Section 2.11.1.

O : NAD^+.

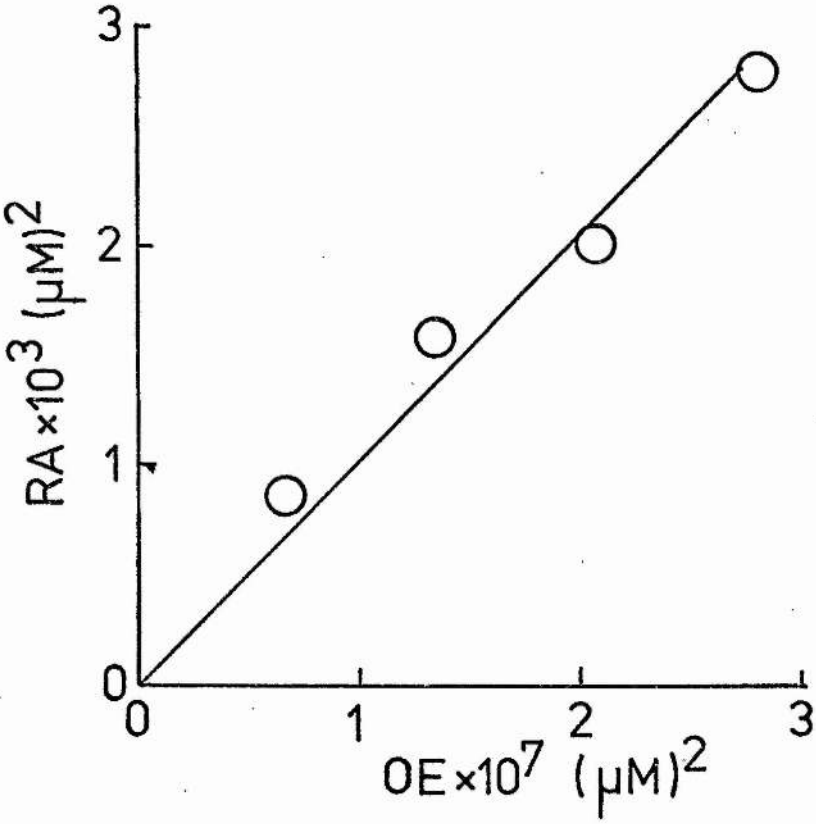
E : Ethanol.

R : NADH.

A : Acetaldehyde.

Fig. 34.

Typical plot for the determination of the equilibrium constant for the "unreduced" derivative. Initial concentration of ethanol was 30.547mM; initial concentrations of NAD^+ were 688μM, 481μM and 241μM. Other experimental conditions were as described in Section 2.11.1. O, E, R and A have the same significance as for Fig. 33.


3
2
1
0
0
1
2
3
RA ×10³ (μM)²
OE ×10⁷ (μM)²

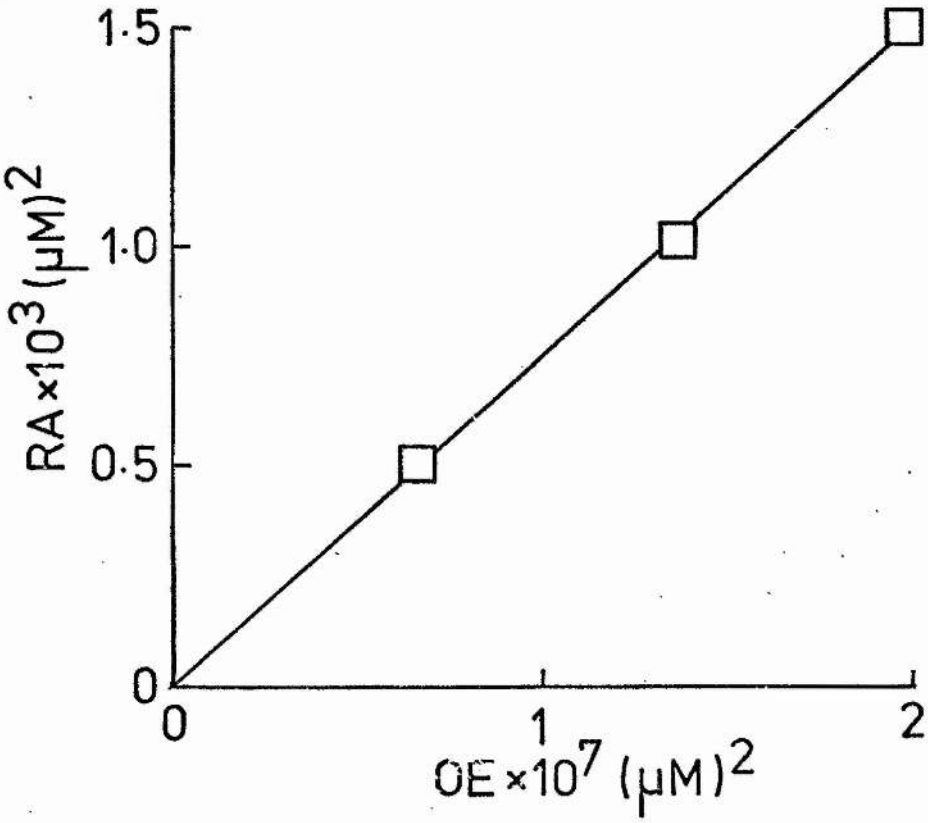

1·5
1·0
0·5
0
0
1
2
RA ×10³ (μM)²
OE ×10⁷ (μM)²

Table 11.

Derivative	$K_{eq}(M) \times 10^{11}$
Soluble	1.105
"200µg"	0.765
"Reduced"	0.693
"Unreduced"	0.780
"5mg"	0.820

Fig. 35.

Effect of incubation at 40°C on the stability of soluble YADH (○), "unreduced" derivative (△), "reduced" derivative (■) and "200µg" derivative (□). The trace obtained for the "5mg" derivative is omitted for clarity. It is identical to that obtained for the "reduced" derivative.

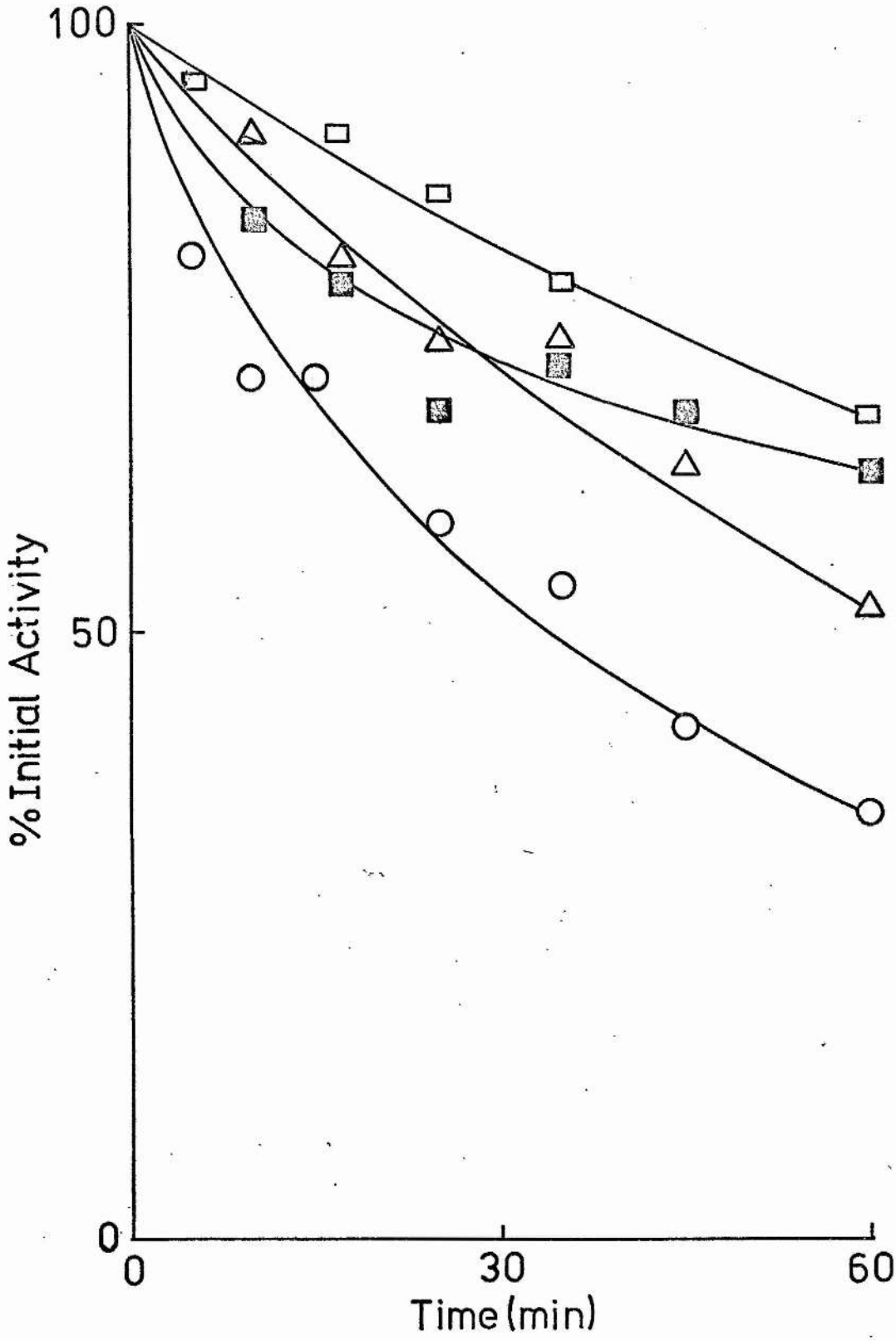
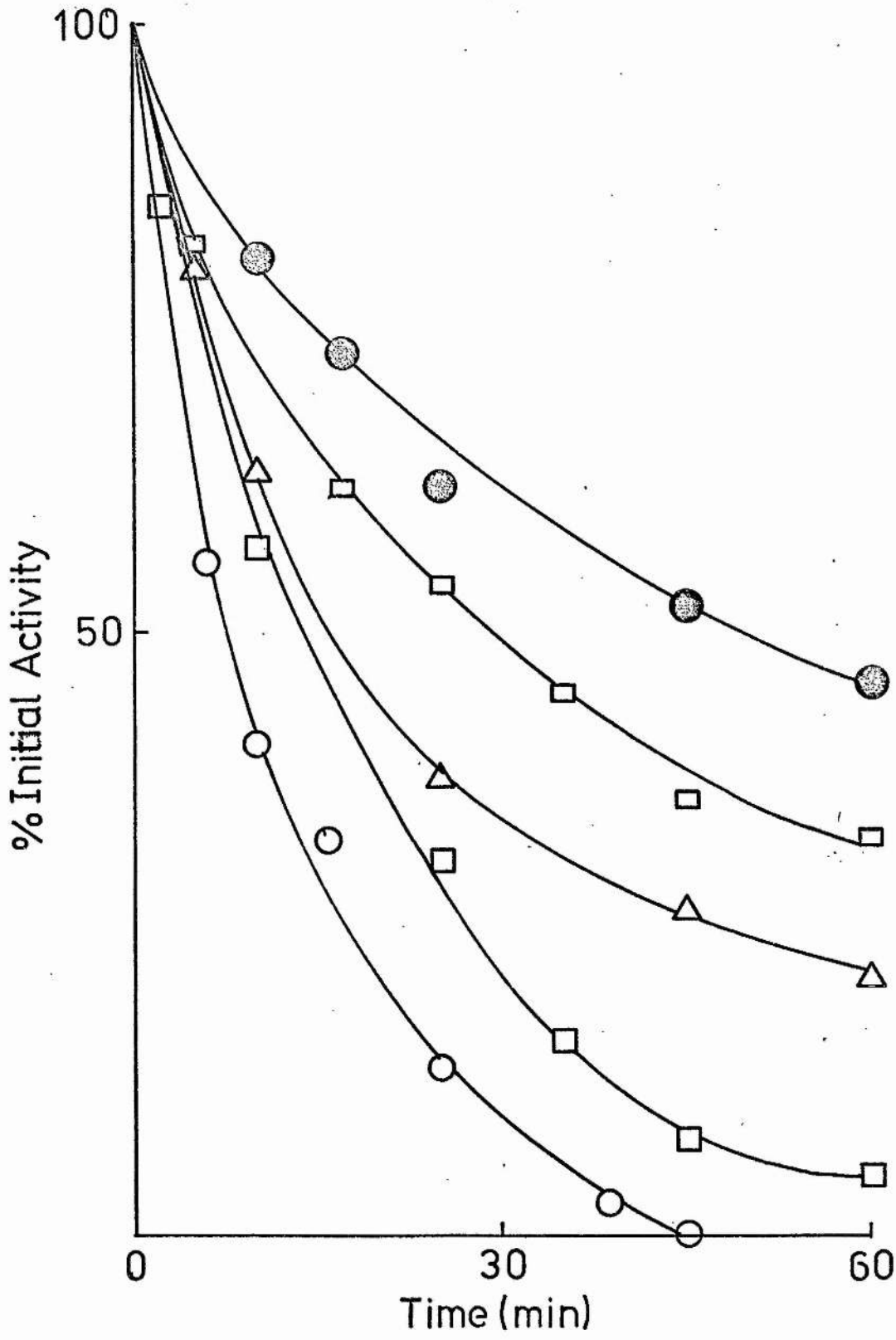

100
50
0
% Initial Activity
0
30
60
Time (min)

Fig. 36.

Effect of incubation at 50°C on the stability of soluble YADH (○), "unreduced" derivative (□), "5mg" derivative (△), "200µg" derivative (▭) and "reduced" derivative (◉).


100
50
% Initial Activity
0
30
60
Time (min)

(1955) and by Hayes and Velick (1953), but is lower than that reported by Dickinson and Monger (1973) who used kinetic coefficients for the calculation of the equilibrium constant. Table 11 shows that the equilibrium constant is lowered for all four derivatives and is lowest for the "reduced" derivative.

4.7. Effect of Temperature on the stability of soluble and immobilised YADH

The stability of soluble YADH and the derivatives described in Section 4.2.9 was studied at 40°C and 50°C. The results are presented in Figs. 35 and 36. These show that the soluble enzyme is stabilised towards raised temperature immobilisation to Cellex-AE. At 50°C, however, there is a greater variation in stability and Fig. 36 shows that the "reduced" derivative is the most stable. After 30min at 50°C, the soluble enzyme derivative has retained 60% of its original activity. At 40°C, there is little difference in the stabilities of the four immobilised derivatives, but the soluble enzyme is less stable than any of them. After 45min the soluble enzyme retained 43% of its original activity, whereas the "reduced" derivative retained 65% of its original activity.

Lactate Dehydrogenase

Chapter 5

5.1. Attachment of LDH to macromolecular supports

LDH was immobilised on activated macromolecular supports as described in Sections 2.20.1 to 2.20.3.3. Several derivatives were prepared and their properties described in the subsequent sections.

5.2.1. Effect of pH on the coupling of LDH to Cellex-AE

The pH of the coupling buffer for the attachment of LDH to activated Cellex-AE was varied. 150mg freshly activated Cellex-AE was resuspended in 5.0ml of the appropriate buffer and 0.2ml LDH solution (1mg/ml) was added. Coupling and washing was as described in Section 2.20.1. The results are presented in Fig. 37 and show that a maximum support specific activity of 65U/g was attained at pH7.4.

5.2.2. Immobilisation of LDH on Cellex-AE and PEI

Several immobilised preparations of LDH on Cellex-AE and PEI were prepared as described below in Sections 5.2.2.1 to 5.2.2.5. Table 12 summarises their activities and protein content.

5.2.2.1 Attachment of LDH to Cellex-AE followed by reduction

Two derivatives were prepared, using the coupling procedure described in Section 2.20.1.

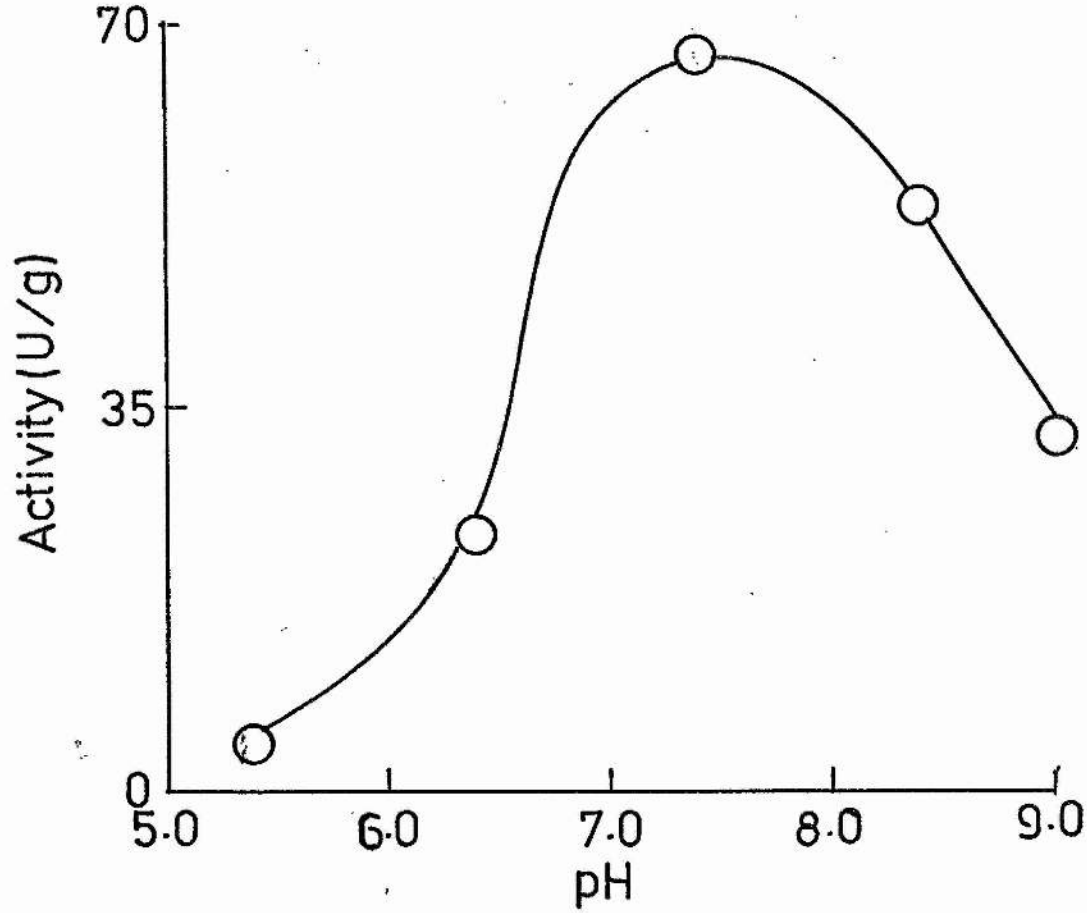


Fig. 37.

Effect of pH on the coupling of LDH to Cellex-AE.

In one case, the coupling suspension contained 1.5mg LDH, in the other case 1.0mg LDH. These two borohydride reduced preparations are known as the "1.5mg" derivative and the "reduced" derivative.

5.2.2.2. Attachment of LDH to Cellex-AE

Two derivatives were prepared, following the coupling procedure described in Section 2.20.1. Neither preparation was reduced with sodium borohydride. In one case the coupling suspension contained 2.5mg LDH and in the other case 1.0mgLDH. These two preparations are known as the "2.5mg" and the "unreduced" derivatives.

5.2.2.3. Attachment of LDH to PEI

LDH was attached to PEI as described in Section 2.20.3.1 and separated on a Sephadex G-200 column as described in Section 2.20.3.1. The derivative was not reduced with sodium borohydride. Two derivatives were prepared. In one case, the initial coupling suspension contained 80µg LDH (giving derivative I) and in the other case 300µg LDH (giving derivative II).

5.2.2.4. Attachment of PEI-LDH to Cellex-AE

The PEI-LDH derivative prepared in Section 2.20.3.1 was coupled to 100mg activated

Table 12.

Derivative	mg protein in coupling	% Initial Protein coupled	mg LDH per g support	Activity (U/g)	Activity (U/mg immobilised LDH)
"2.5mg"	2.5	52	3.2	137	43
"1.5mg"	1.5	48	2.9	88	30
PEI-LDH (I)	0.1	35	200	5000	25
PEI-LDH (II)	0.3	70	70	1700	24
"Reduced"	1	40	1.6	65	40
"Unreduced"	1	40	1.6	64	40
AEC-PEI-LDH [1]	0.75	99.5	2.9	100	34
AEC-(PEI-LDH) [1]	-	92	-	21.4	-

[1] AEC : Cellex-AE

Cellex-AE as described in Section 2.20.3.2. The derivative was not reduced.

5.2.2.5. Attachment of LDH to Cellex-AE-PEI

LDH was attached to an adduct of PEI and Cellex-AE prepared as described in Section 2.20.3.3. The derivative was not reduced.

The data presented in Table 12 show that the support specific activities attained by the Cellex-AE derivatives are very much lower than those attained by the PEI derivatives. This would be expected since, when LDH is attached to PEI, the support-enzyme product remains soluble in water whereas on immobilisation of LDH to Cellex-AE, the LDH changes phase and becomes "insolubilised".

Comparison of the "reduced" and "unreduced" derivatives prepared as described in Sections 5.2.2.1 and 5.2.2.1, shows that sodium borohydride reduction has no effect on the initial support specific activity. LDH contains no disulphide bridges (Levi and Kaplan, 1971) and hence no change in activity would be expected on reduction, whereas YADH shows a fall in activity on treatment with sodium borohydride (see Section 4.1.1).

The results obtained for the "2.5mg", "1.5mg" and

"reduced" preparations are very similar to those for YADH (see Section 4.2.9) in that increasing the enzyme concentration in the coupling suspension results in a reasonable increase in support specific activity without a corresponding increase in the protein content of the support. For instance, comparing the "2.5mg" and "reduced" derivatives, the 250% increase in initial coupling protein resulted in a two-fold increase in mg LDH immobilised per g Cellex-AE and also a two-fold increase in the activity of the support, but the percentage protein coupled only increased by 30%.

5.3. Attachment of LDH to NP/3 Nylon Powder

Four immobilised preparations of LDH on NP/3 Nylon Powder were prepared as described in Section 2.20.2. A control coupling in which glutaraldehyde had been omitted was also carried out to ascertain how much LDH is adsorbed on the support. All couplings contained 300µg LDH initially. Table 13 shows the amounts of protein bound, both covalently and physically, and the activities of the various derivatives. The activities obtained are very low compared to those of the Cellex-AE derivatives. For instance, the most active nylon derivative prepared had a support specific activity

Table 13.

Derivative	mg protein in coupling	% Initial Protein coupled	mg LDH per g support	Activity (U/g)	Activity (U/mg immobilised LDH)
NP/3 (unactivated)	0.3	30	0.4	6.1	15
NP/3 (Prep. I)	0.3	35	0.56	12.3	22
NP/3 (Prep. II)	0.3	35	0.54	8.8	16
NP/3 (Prep. III)	0.3	36	0.57	6.4	11
NP/3 (Prep. IV)	0.3	33	0.52	8.6	16

of 12.3U/g whereas the most active Cellex-AE derivative had a support specific activity of 137U/g. The NP/3 nylon powder has about 0.083mequiv of amino groups per g whereas the Cellex-AE derivative has about 0.3mequiv of amino groups per g. This means the Cellex-AE has a four-fold excess of reactive centres. The initial amount of protein used in the coupling to NP/3 was very small compared to the amounts used in the Cellex-AE couplings. Also notable is the high percentage of adsorbed protein on the NP/3 Nylon Powder whereas a control coupling on the Cellex-AE, in which the glutaraldehyde activating step was omitted, resulted in a derivative with no enzymic activity, indicating that no protein had been adsorbed.

5.4. Stability of LDH and Its Immobilised Derivatives

5.4.1. Effect of pH on the stability of soluble LDH

The stability of soluble LDH was studied by incubating the enzyme in the appropriate buffer for 2h at 4°C in the pH range 5.0 to 10.2. Results are presented (Fig. 38) for both pig heart LDH and for rabbit muscle LDH. The pig heart enzyme is unstable between pH5.0 and pH7.0, but is stable

above pH9.0. The rabbit muscle enzyme is stable between pH5.0 and pH7.0 and is unstable above pH9.0. The results presented in this figure also show that, under experimental coupling conditions, there will be no decrease in soluble enzyme activity.

5.4.2. Effect of sodium borohydride reduction on the stability of LDH attached to Cellex-AE

The derivatives prepared as described in Sections 5.2.2.1 and 5.2.2.2 were stored at 4°C and assayed intermittently over a period of three months. The results are presented in Fig. 39 and show that over the three month period, the immobilised preparations lost only about 35% of their initial activity, whereas the soluble enzyme lost 54% of its activity. There is a difference of about 5% in the stabilities of the "reduced" and "unreduced" preparations over this period. It should also be noted that the loss of activity curves are hyperbolic, an effect which was also noted in Section 4.4.2.

5.4.3. Effect of pH on the stability of soluble and immobilised LDH

The stability of soluble and immobilised LDH at 4°C was studied in the pH range 5.0 to 9.0 over a period of seven weeks. The results are presented in Figs. 40 and 41. They show that at

pH5.0, after 35 days, the soluble enzyme has lost 98% of its activity whereas the immobilised derivative lost only 41%. A similar result is obtained at pH9.0 where the soluble enzyme has lost 90% of its activity but the immobilised preparation only 40%. At pH6.4, 7.4 and 8.4 the differences are less marked. At pH6.4, the soluble enzyme has lost 35%, the immobilised enzyme has lost 21%, of its activity; at pH7.4, the soluble enzyme has lost 40., the immobilised enzyme 22%, of its activity; and at pH8.4, both soluble and immobilised enzymes have lost 40% of their activity.

5.5. Michaelis parameters of soluble and immobilised LDH

Kinetic studies were carried out on soluble LDH and NP/3-LDH over the pH range 6.0 to 9.0. The soluble enzyme reaction was studied in both directions but, owing to the low activity of the NP/3-LDH, the immobilised enzyme was studied only in the reverse direction i.e. in the direction of NAD^+ formation. Kinetic studies in both forward and reverse directions were also carried out at pH7.4 on the Cellex-AE and PEI derivatives described in Sections 5.2.2.1 to 5.2.2.5.

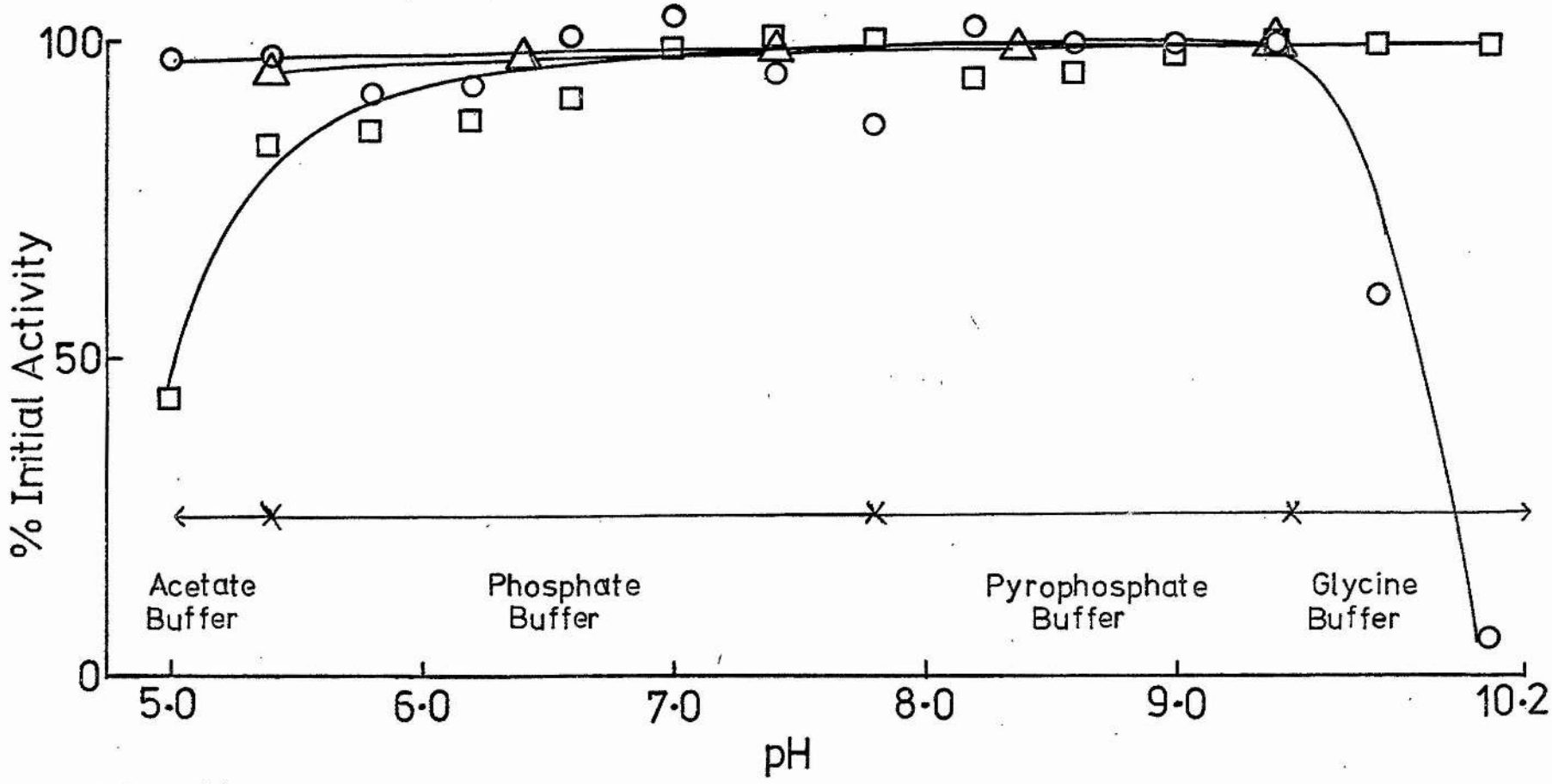


Fig. 38.

Effect of pH on the short term stability at 4°C of soluble LDH (○), soluble pig heart LDH (□) and Cellex-AE-LDH (△).

Fig. 39.

Comparison of the stability at 4°C of soluble LDH (△), and the "reduced" (○) and "unreduced" (□) preparations described in Section 5.4.2.

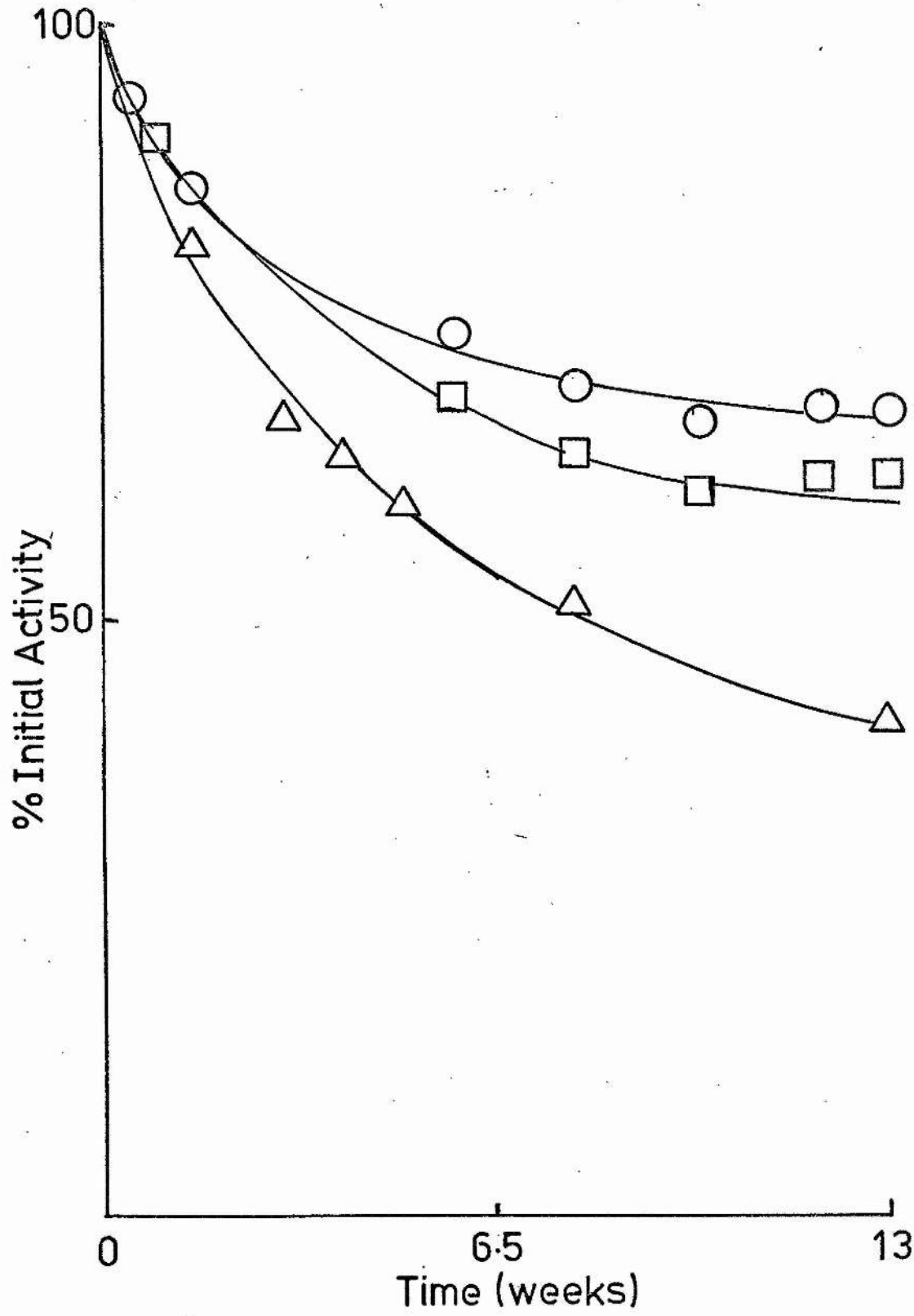
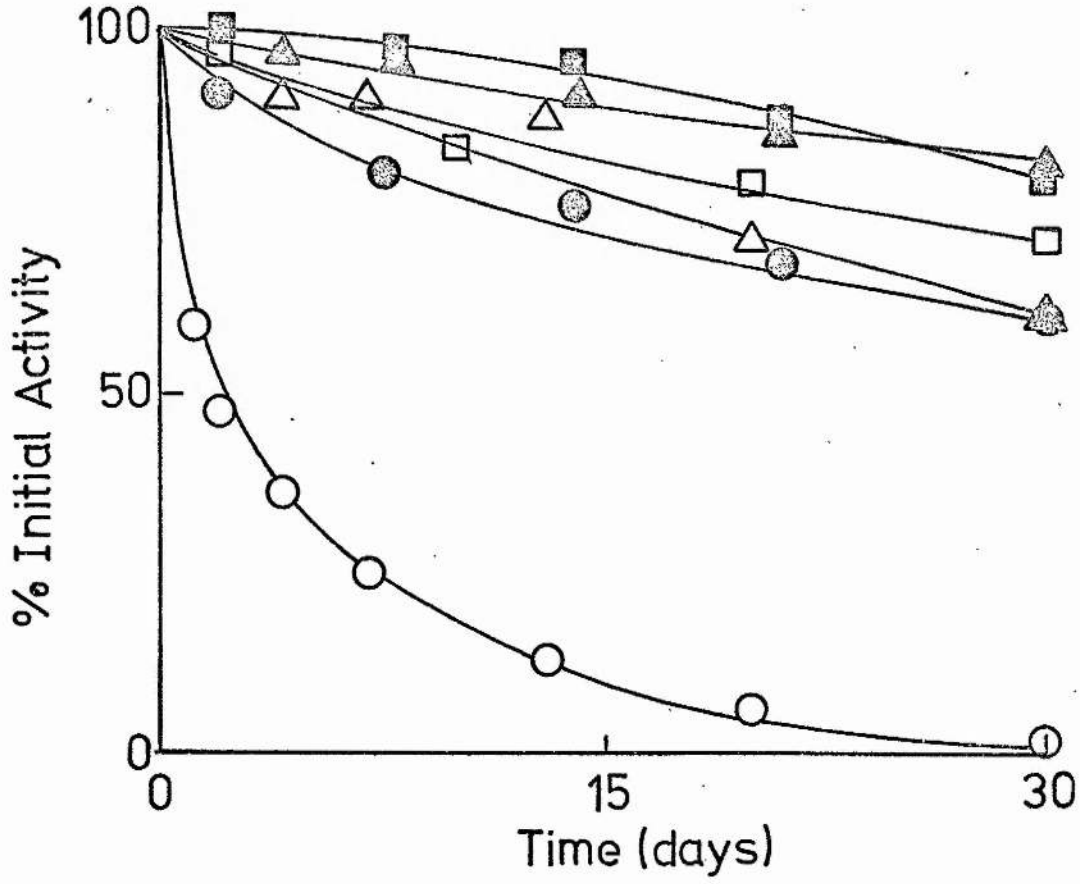

100
50
% Initial Activity
0
6·5
13
Time (weeks)

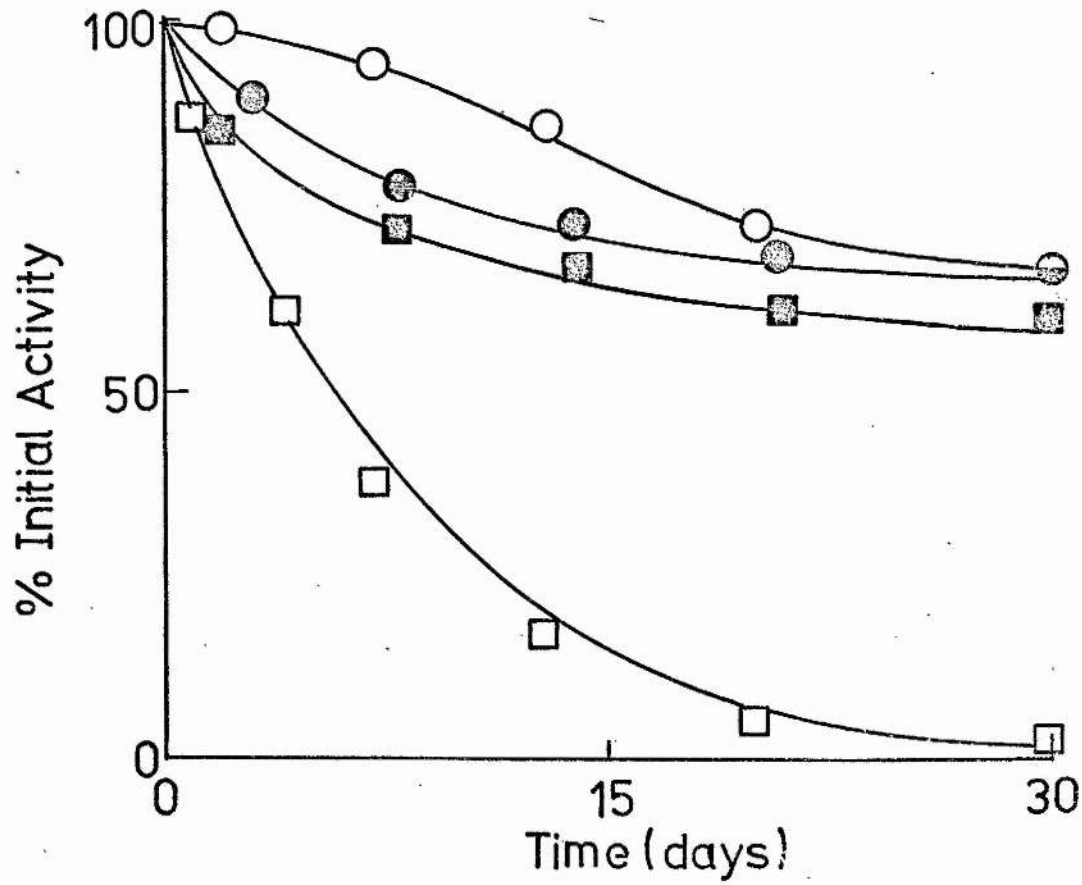
Fig. 40.

Comparison of the long term stability at 4°C of soluble LDH (unfilled symbols) and Cellex-AE-LDH (filled symbols) at pH5.4 (○), at pH6.4 (□) and at pH7.4 (△).

Fig. 41.

Comparison of the long term stability at 4°C of soluble LDH (unfilled symbols) and Cellex-AE-LDH (filled symbols) at pH8.4 (○) and at pH9.0 (□).


100
50
0
% Initial Activity
0
15
30
Time (days)


100
50
0
% Initial Activity
0
15
30
Time (days)

5.5.1. Kinetics of soluble LDH and NP/3-LDH

Kinetic studies over a range of pH values were carried out on both soluble LDH and LDH attached to NP/3. Four derivatives of LDH, immobilised on NP/3, were prepared and were designated preparations I, II, III, IV respectively (see Section 2.20.2). Preparation I had no arm attached; preparation II had N,N-dimethylaminopropylamine attached; preparation III had n-butylamine attached; preparation IV had γ-aminobutyric acid attached. As for YADH kinetic studies, one substrate was varied while the other was kept constant at a sufficiently saturating concentration. Double reciprocal plots were used for the determination of the Michaelis parameters. These were linear except at more alkaline pH values where curved plots were obtained, making a precise measurement of K_m' and V_{max}' difficult. Typical double reciprocal plots, are presented in Figs. 42, 43, 44 and 45. Figs. 46,47, 48 and 49 show the variation of the Michaelis parameters with pH for the soluble enzyme. The K_m' of pyruvate increases with pH, the effect being more dramatic at higher pH values. The curve describing the pH dependence of K_m' for NADH shows a minimum at pH7.4. At this pH, K_m' is 3μM. Similar curves describing the pH dependence of K_m' were obtained for lactate and NAD^+. The former

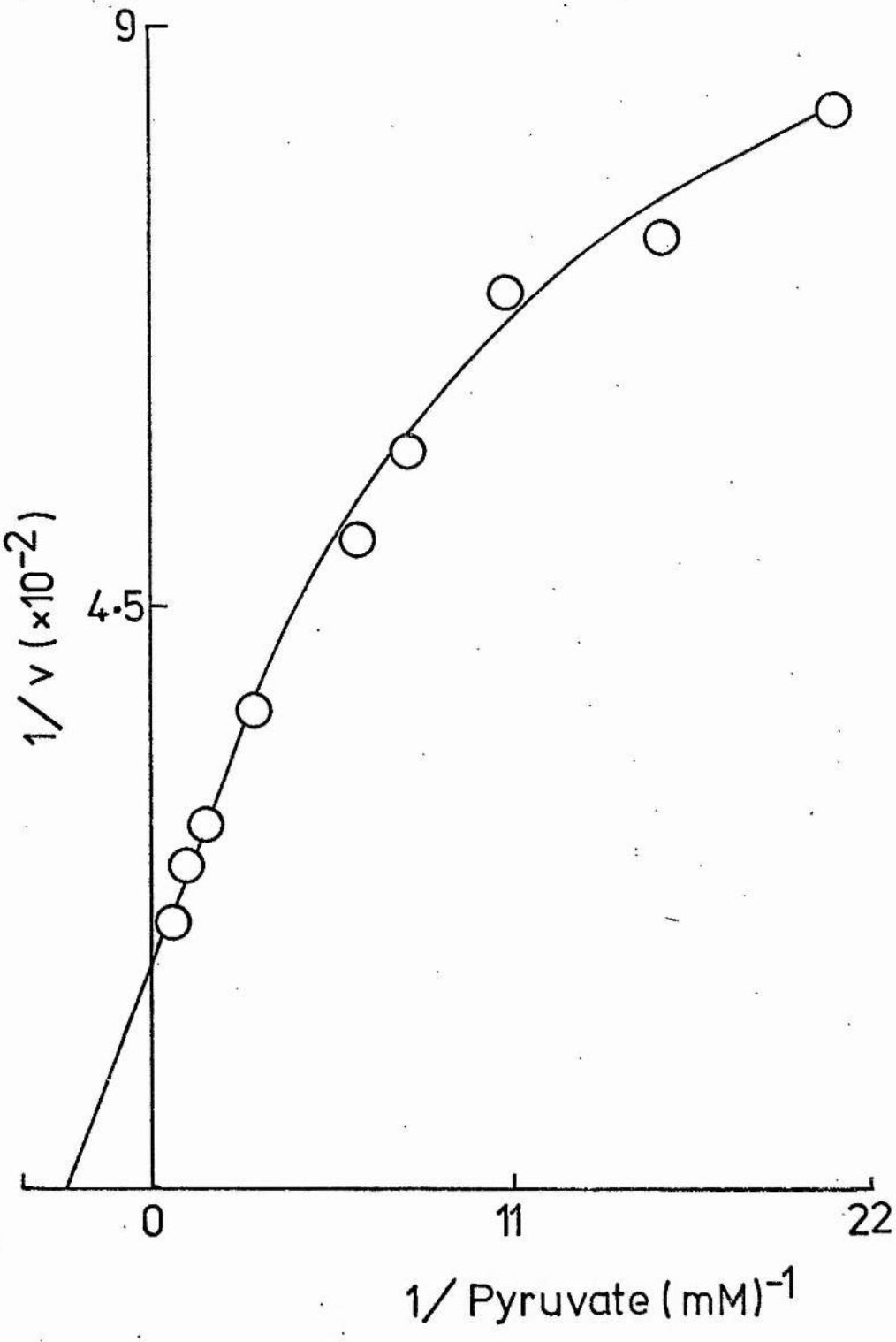


Fig. 42.

Primary reciprocal plot of initial velocity data for Preparation II at pH7.8. NADH concentration was held constant at 150μM. Temperatures of assays were 25°C. Units of 'v' are micromoles per ml per min per mg enzyme-support.

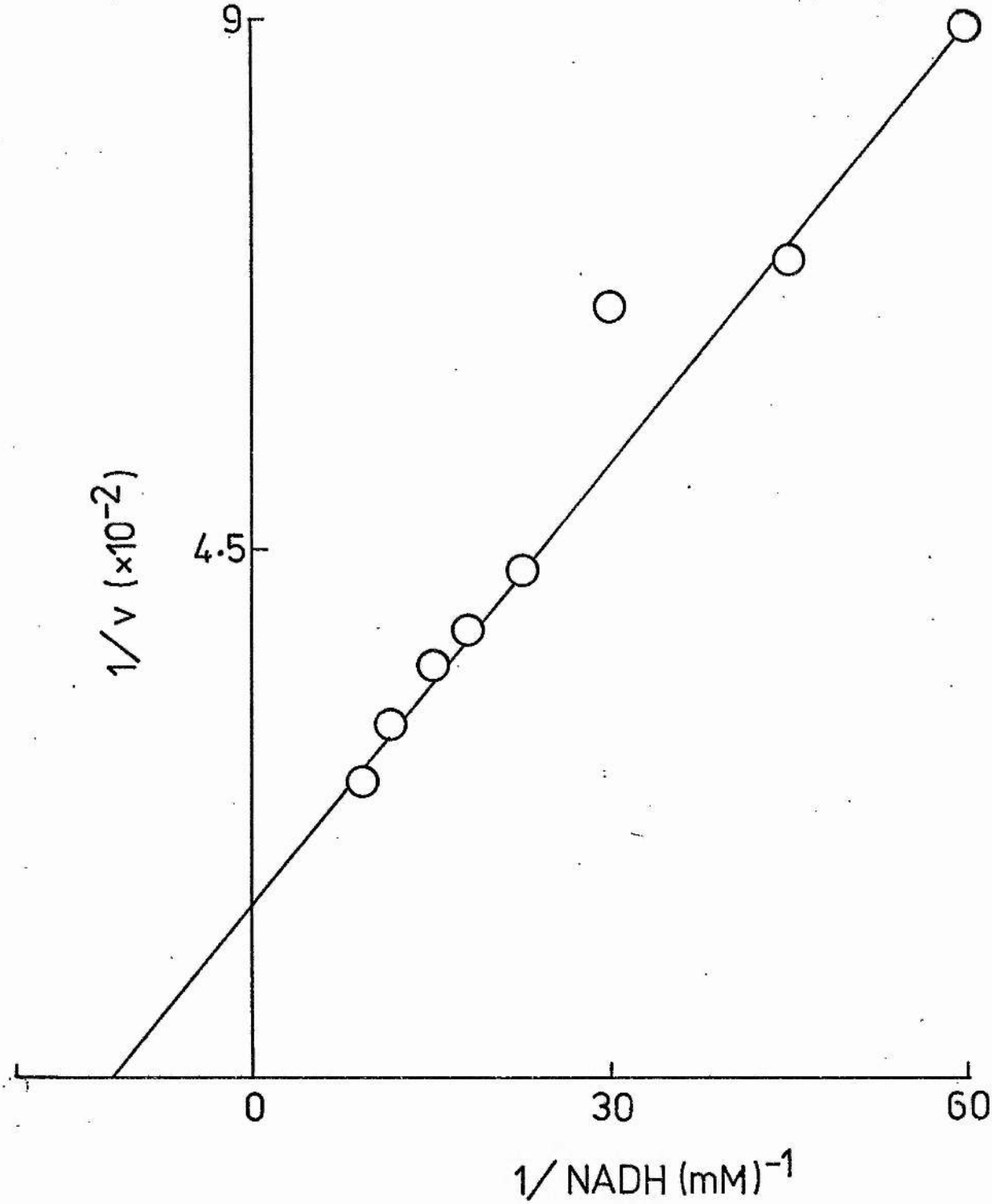


Fig. 43.

Primary reciprocal plot of initial velocity data for Preparation I at pH9.0. Pyruvate concentration was held constant at 2.2mM. Temperatures of assays were 25°C. Units of 'v' are micromoles per ml per min per mg enzyme-support.

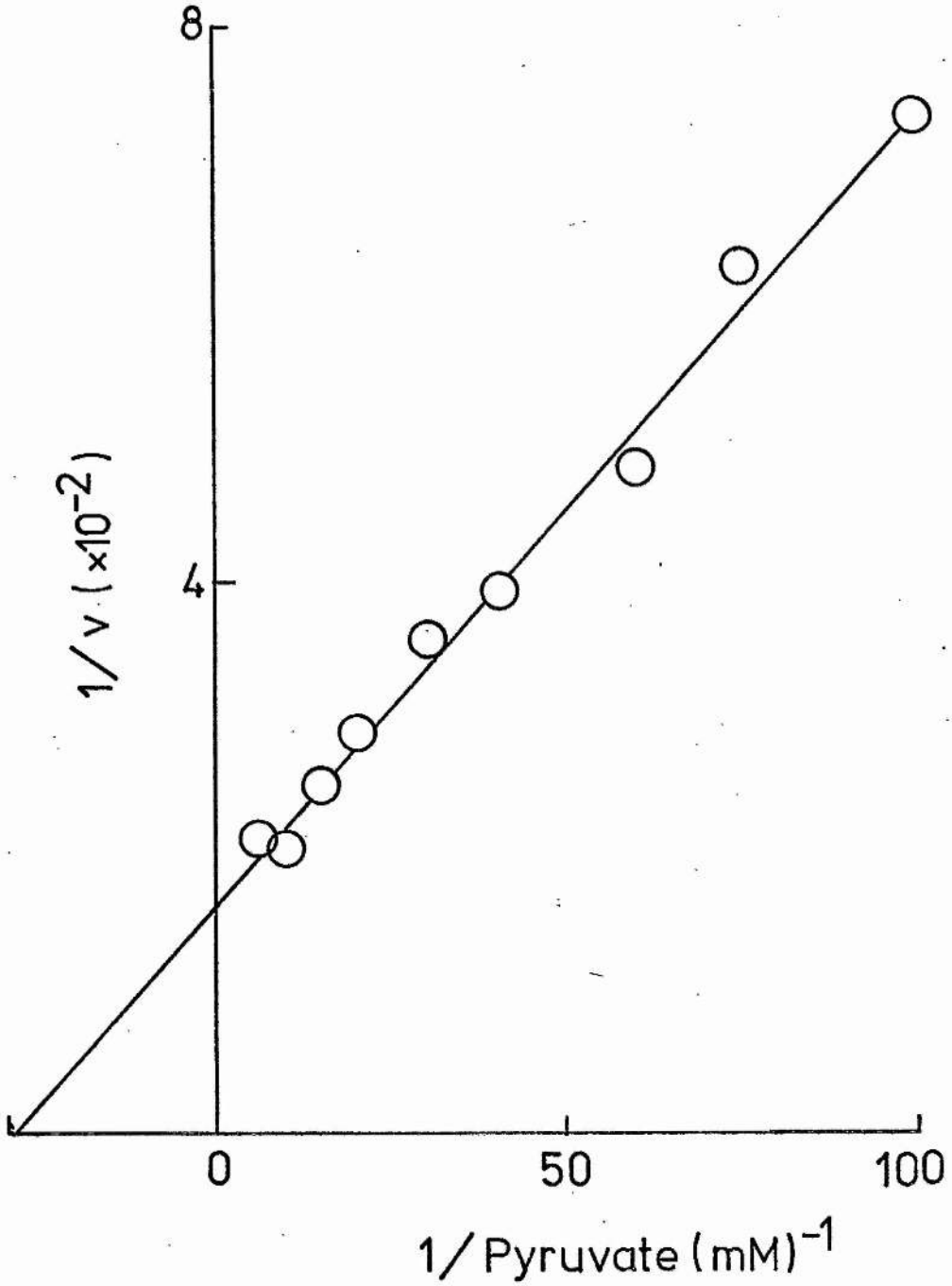


Fig. 44.

Primary reciprocal plot of initial velocity data for Preparation IV at pH6.0. NADH concentration was held constant at 150μM. Temperatures of assays were 25°C. Units of 'v' are micromoles per ml per min per mg enzyme-support.

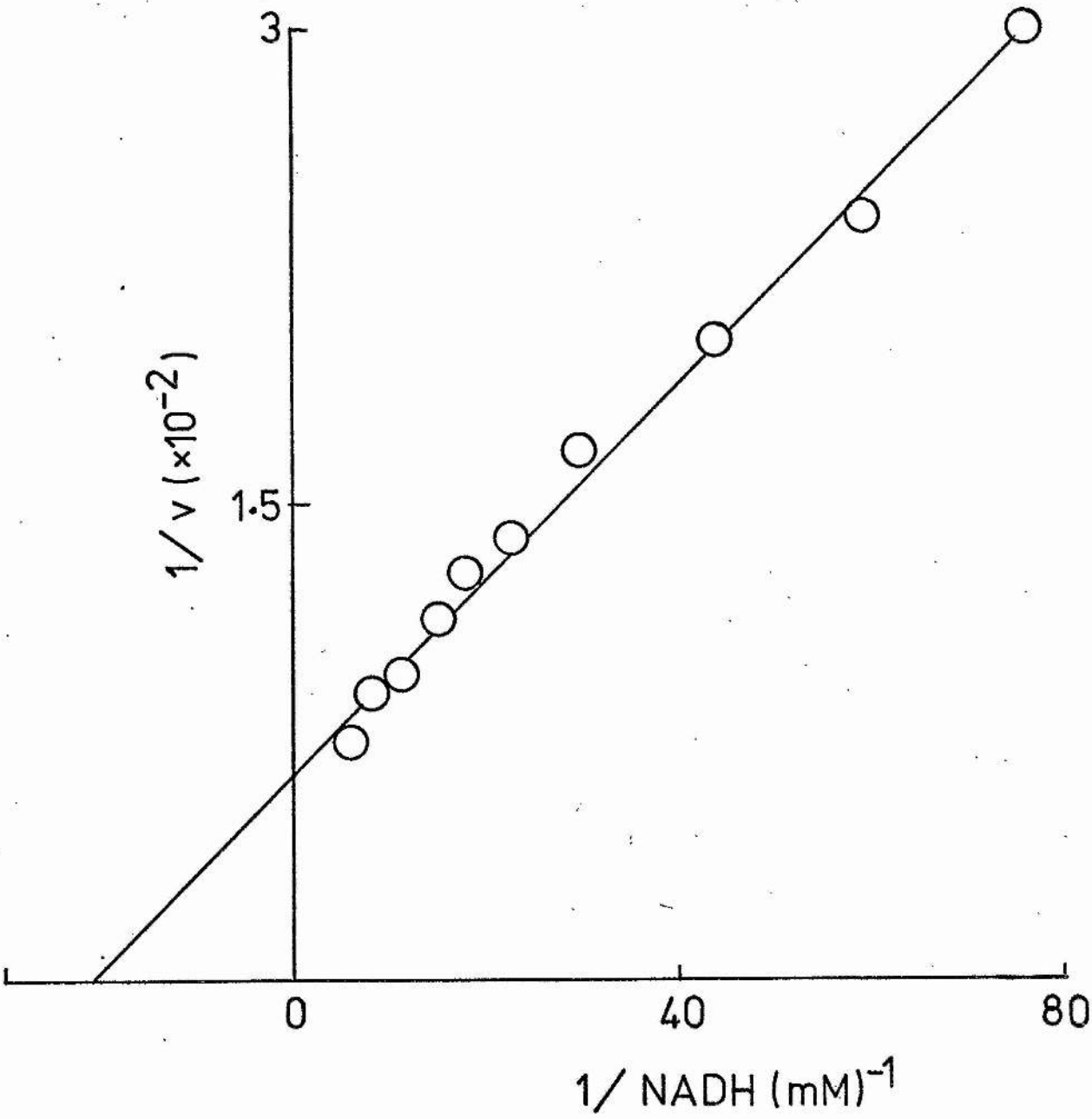


Fig. 45.

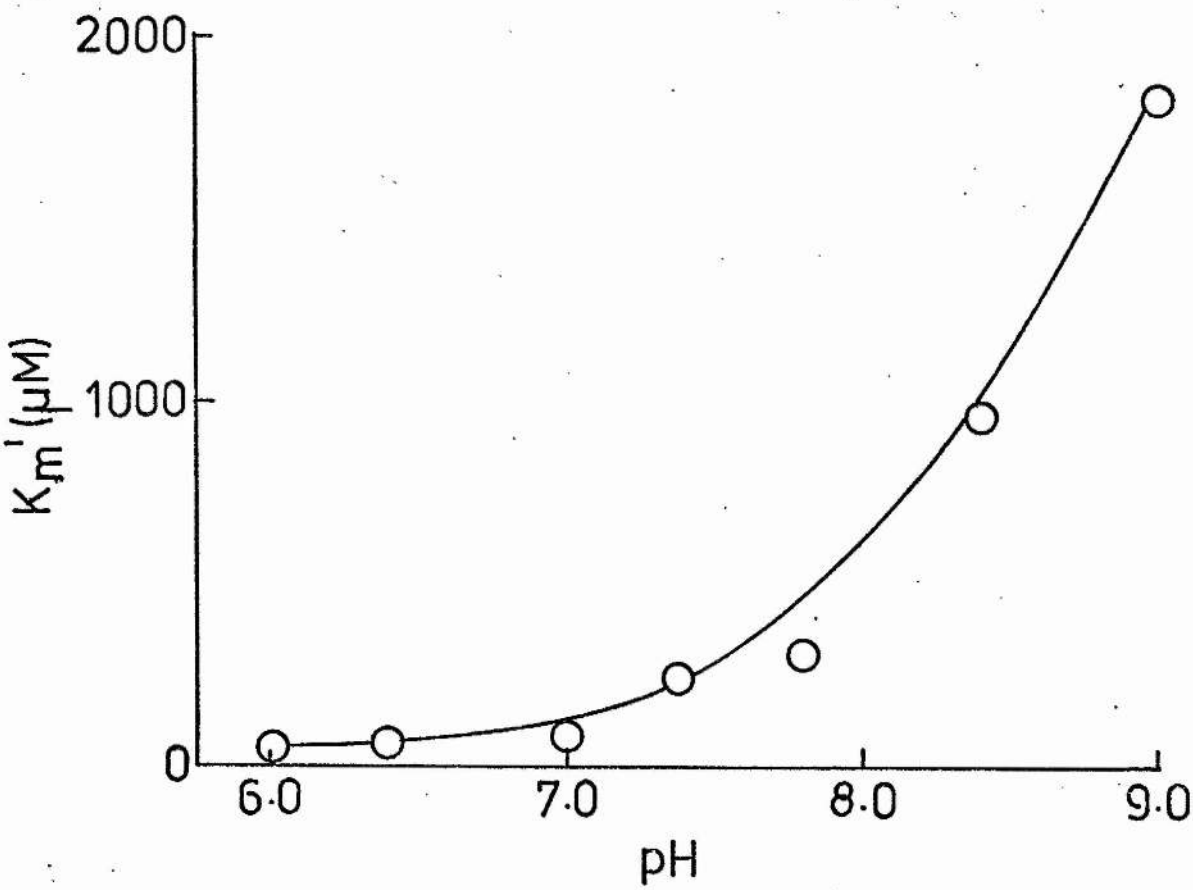
Primary reciprocal plot of initial velocity data for Preparation III at pH7.8. Pyruvate concentration was held constant at 2.2mM. Temperatures of assays were 25°C. Units of 'v' are micromoles per ml per min per mg enzyme-support.

Fig. 46.

Variation of K_m' of pyruvate with pH for soluble LDH.

Fig. 47.

Variation of K_m' of NADH with pH for soluble LDH.


2000
1000
0
K_m' (μM)
6·0
7·0
8·0
9·0
pH

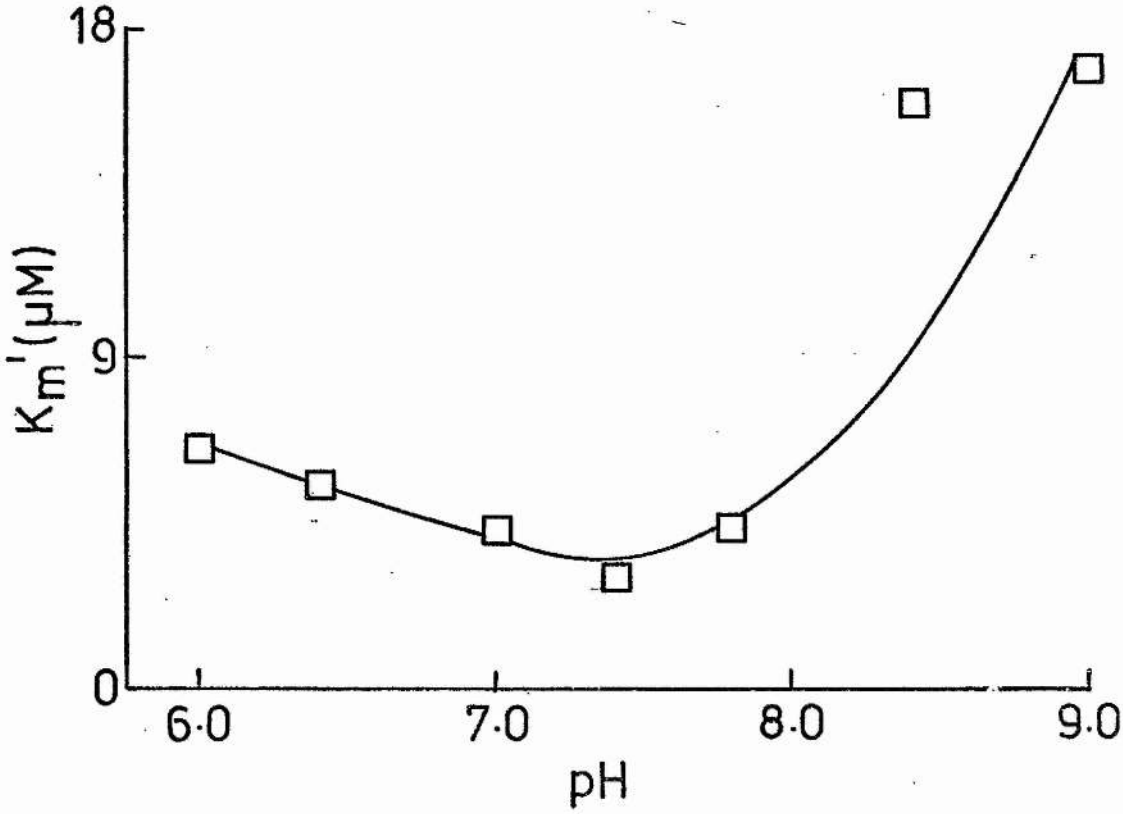
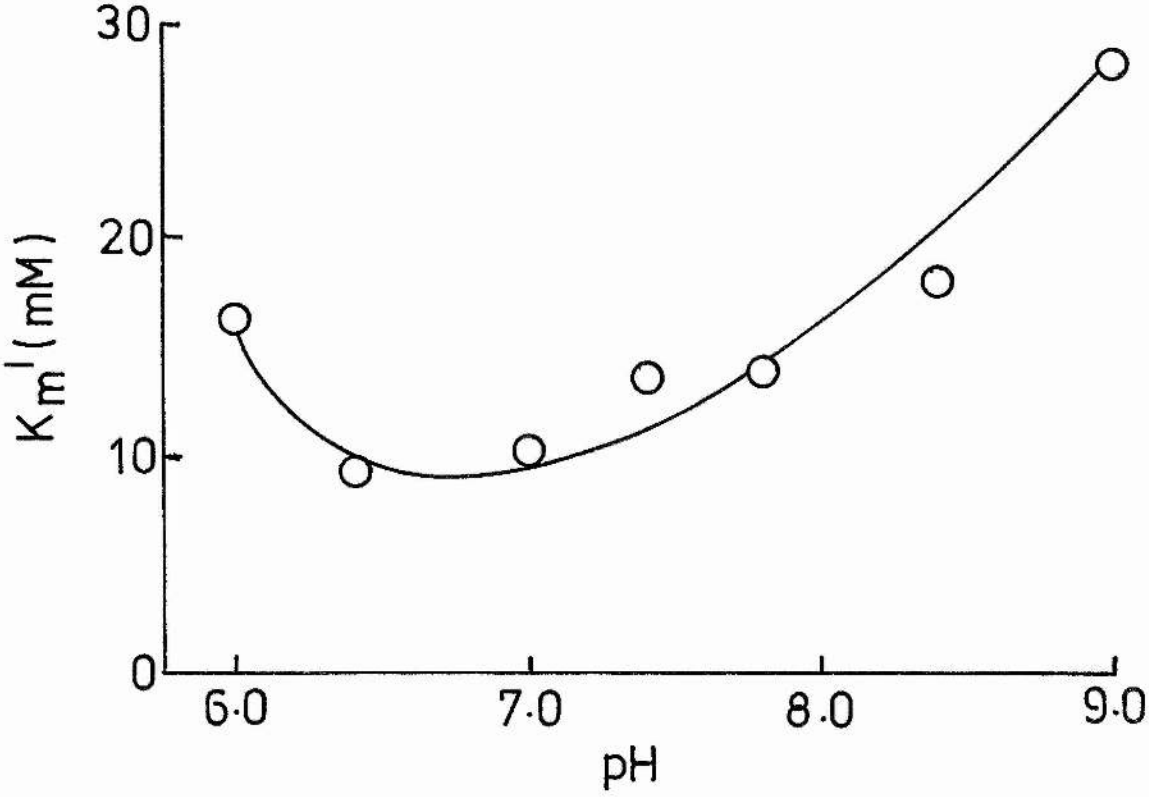

18
9
0
K_m' (μM)
6·0
7·0
8·0
9·0
pH

Fig. 48.

Variation of K_m' of lactate with pH for soluble LDH.

Fig. 49.

Variation of K_m' of NAD^+ with pH for soluble LDH.


30
20
10
0
K_m' (mM)
6·0
7·0
8·0
9·0
pH

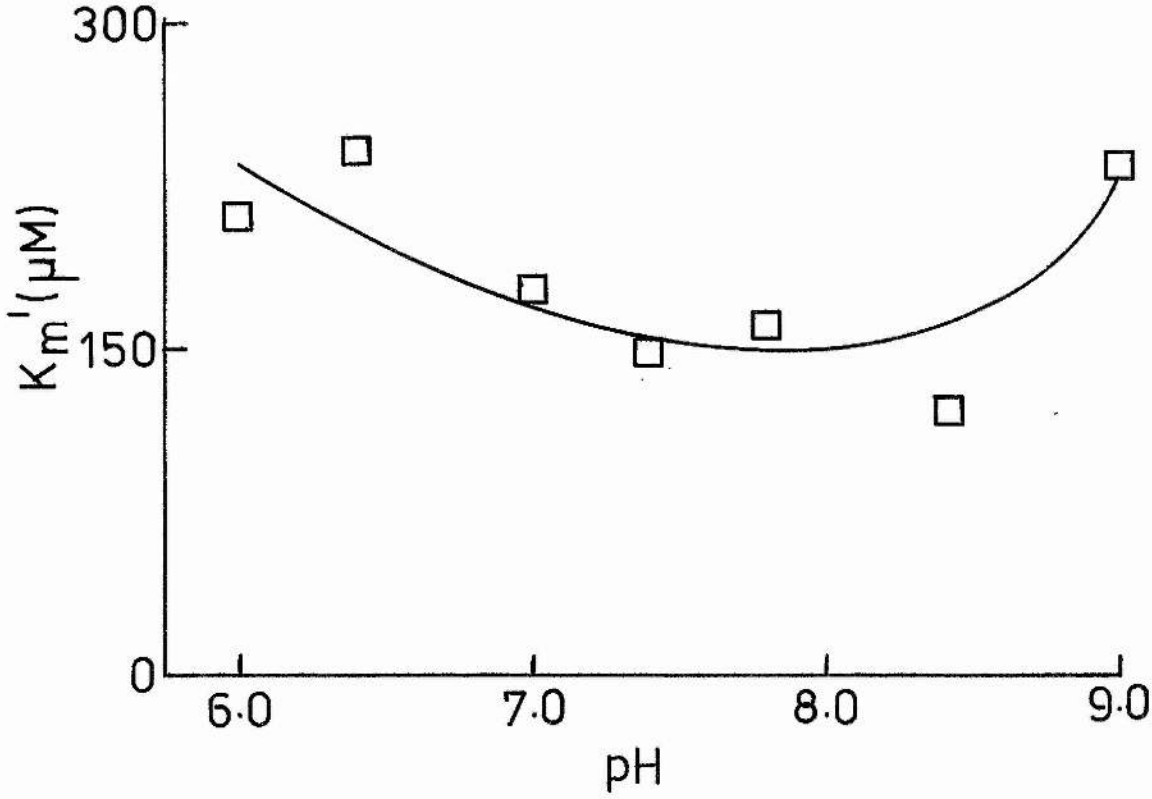

300
150
0
K_m' (μM)
6·0
7·0
8·0
9·0
pH

Fig. 50.

Variation of V_{max}' with pH for the forward (□) and the reverse (○) directions of the LDH catalysed reaction.

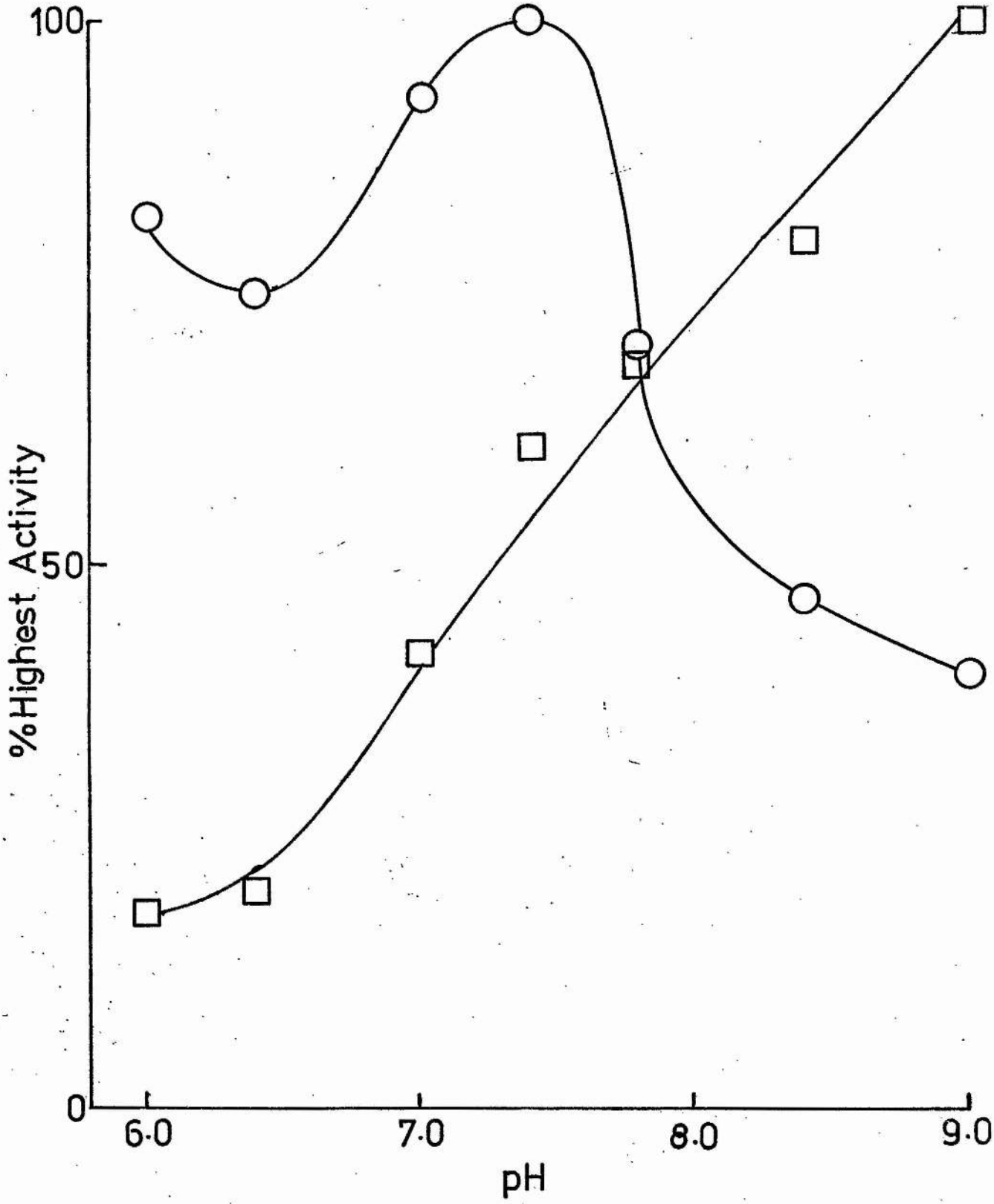

100
50
0
% Highest Activity
6·0
7·0
8·0
9·0
pH

Fig. 51

Dependence of V_{max}' on pH for both soluble and immobilised preparations of LDH. H.A. is the abbreviation for highest activity.

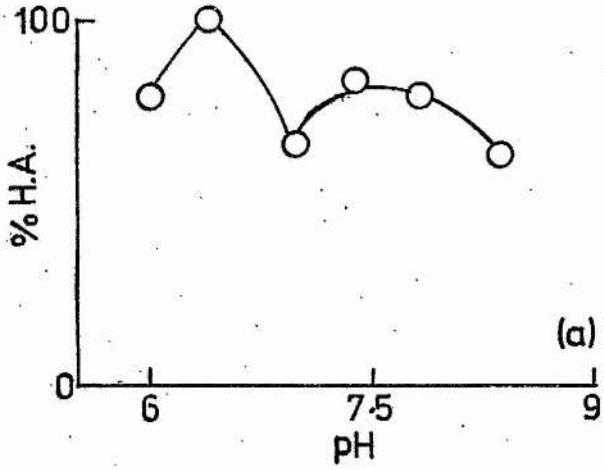
(a) Preparation I.

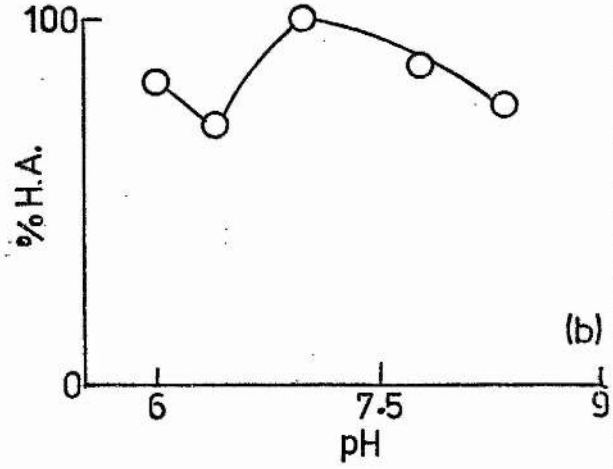
(b) Preparation II.

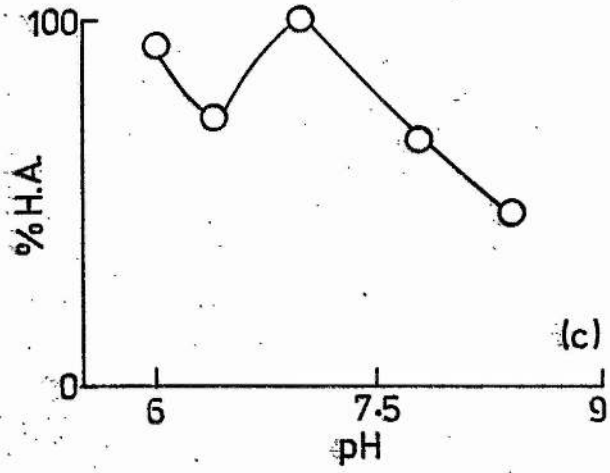
(c) Preparation III.

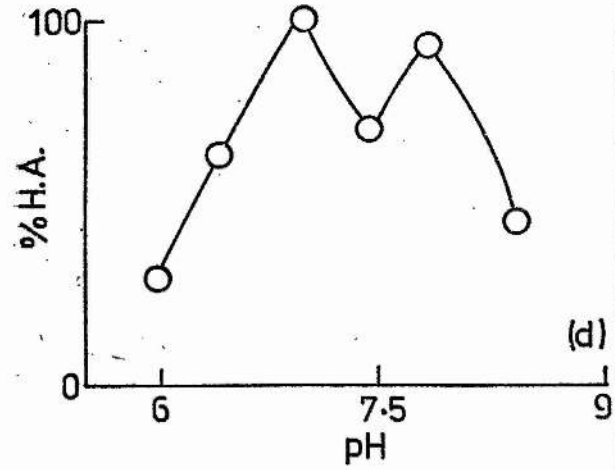
(d) Preparation IV.

(e) Soluble enzyme.


100
% H.A.
0
6
7·5
9
pH
(a)


100
% H.A.
0
6
7·5
9
pH
(b)


100
% H.A.
0
6
7·5
9
pH
(c)


100
% H.A.
0
6
7·5
9
pH
(d)

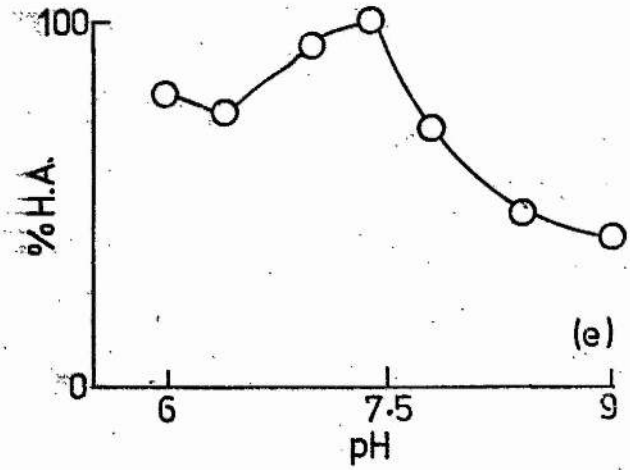

100
% H.A.
0
6
7·5
9
pH
(e)

Fig. 52.

Dependence of K_m' of pyruvate on pH for both soluble and immobilised preparations of LDH.

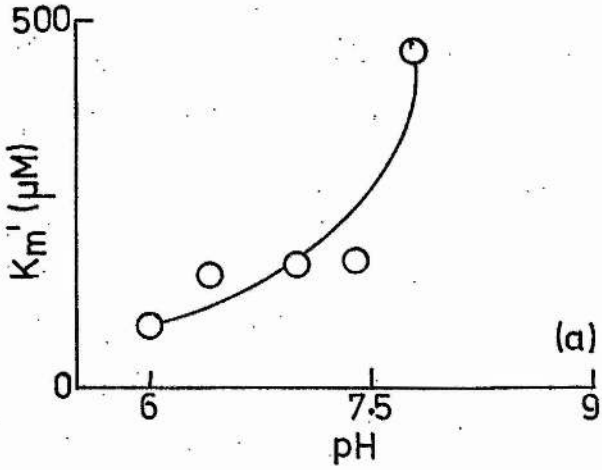
(a) Preparation I.

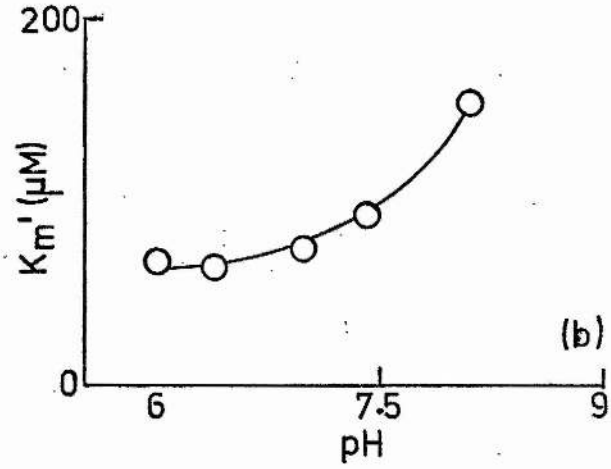
(b) Preparation II.

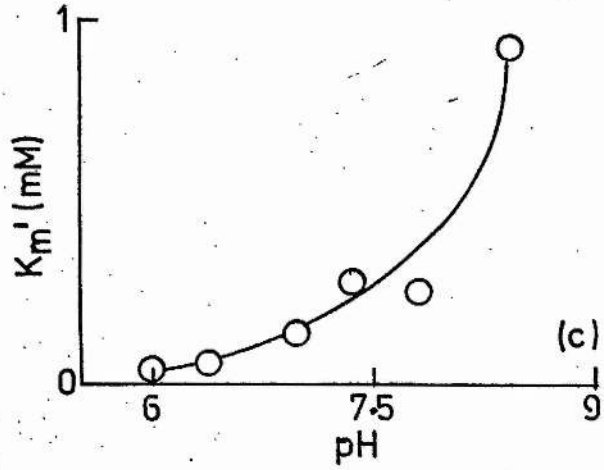
(c) Preparation III.

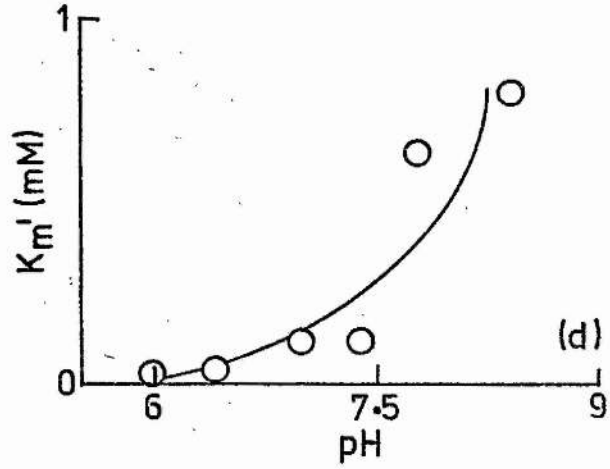
(d) Preparation IV.

(e) Soluble enzyme.


500
0
K_m' (μM)
(a)
6
7·5
9
pH


200
0
K_m' (μM)
(b)
6
7·5
9
pH


1
0
K_m' (mM)
(c)
6
7·5
9
pH


1
0
K_m' (mM)
(d)
6
7·5
9
pH

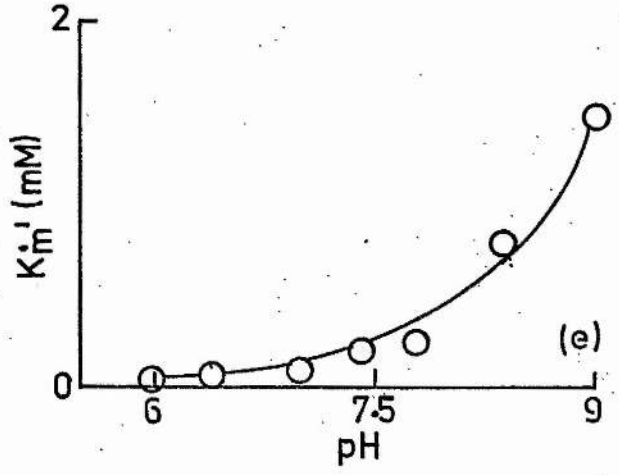

2
0
K_m' (mM)
(e)
6
7·5
9
pH

Fig. 53.

Dependence of K_m' of NADH on pH for both soluble and immobilised preparations of LDH.

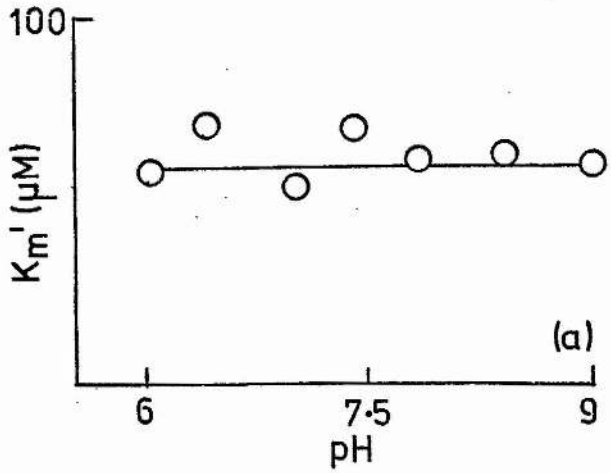
(a) Preparation I.

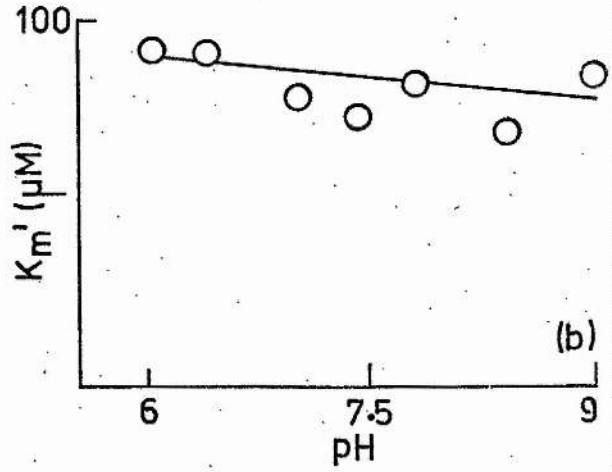
(b) Preparation II.

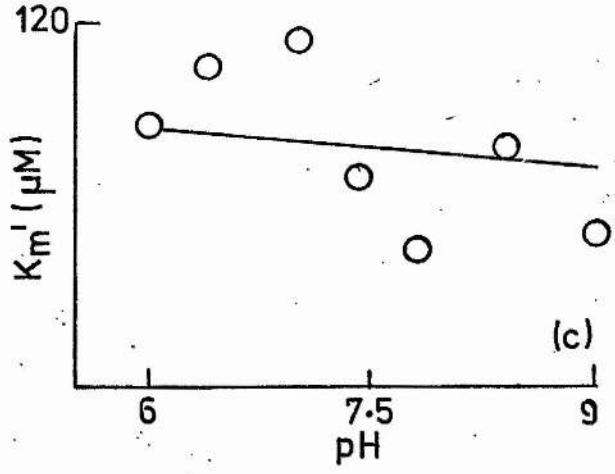
(c) Preparation III.

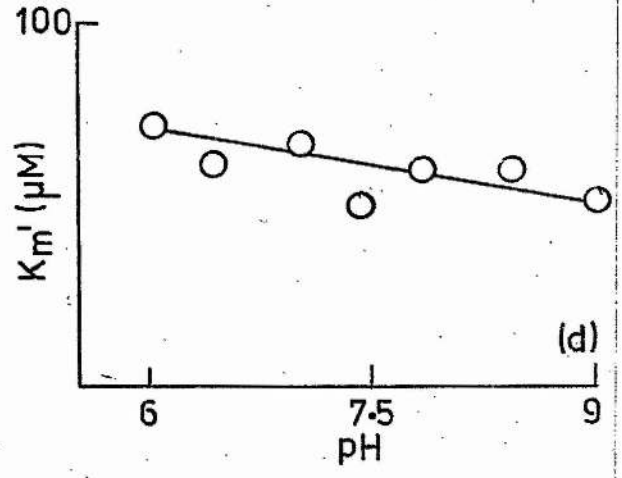
(d) Preparation IV.

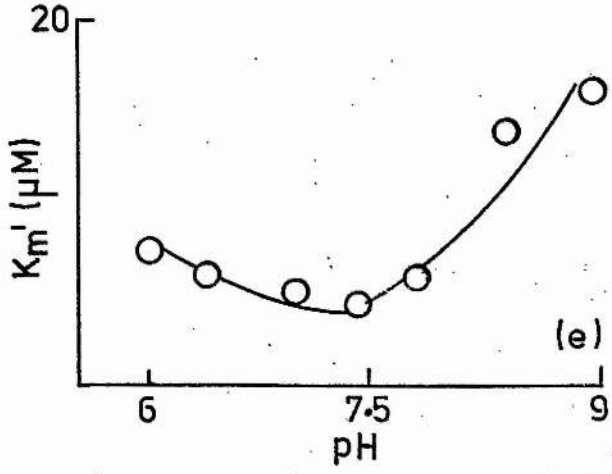
(e) Soluble enzyme.


100
K_m' (μM)
(a)
6
7·5
9
pH


100
K_m' (μM)
(b)
6
7·5
9
pH


120
K_m' (μM)
(c)
6
7·5
9
pH


100
K_m' (μM)
(d)
6
7·5
9
pH


20
K_m' (μM)
(e)
6
7·5
9
pH

a minimum at pH6.4 with a K_m' of 9mM; the latter shows a minimum at pH8.4 with a K_m' of 120μM.

Fig. 50 shows the variation of V_{max}' with pH for the soluble enzyme in both the forward and reverse directions. This is similar, in the pH range studied, to that obtained by Winer and Schwert (1963) for beef heart LDH.

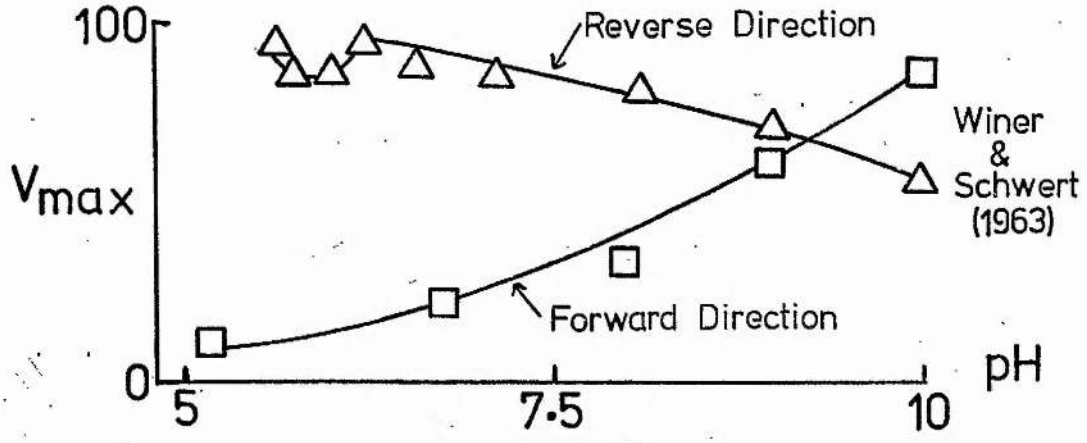


Fig. 51 shows the dependence of V_{max}' on pH for both soluble and immobilised preparations of LDH. Curves (a), (b), (c) and (d) show the results obtained with preparations I, II, III and IV respectively, and curve (e) the corresponding results for the soluble enzyme. All the results presented in these figures were obtained from straight line double reciprocal plots. In all cases, except for the soluble enzyme, the values of V_{max}', at pH9.0 have been omitted since the double reciprocal plots obtained under these

conditions were curved. Curves (a), (b), (c) and (d) show two maxima as does curve (e) for the soluble enzyme. The soluble enzyme shows a V_{max}' at pH7.4 and this has moved to pH6.4 for preparation I and to pH7.0 for the other three immobilised derivatives.

Fig. 52 shows the pH dependence of K_m' for pyruvate for both the soluble and NP/3-immobilised LDH. Curves (a), (b), (c) and (d) show the results obtained for the immobilised preparations I, II, III and IV, and (e) shows the results obtained for the soluble enzyme. Because of the curved double reciprocal plots obtained, the values of K_m' at pH9.0 have been omitted in some cases. As for the soluble enzyme, the K_m' increases with pH, the effect becoming more marked at higher pH values. The K_m' values for the immobilised enzymes were always larger than the K_m' values for the soluble enzyme. For instance, at pH7.0, the K_m' for pyruvate has a value of 81μM for the soluble enzyme whereas the K_m' values for preparations I, II, III and IV are 167μM, 90μM, 125μM and 111μM respectively.

Fig. 53 shows the pH dependence of K_m' for NADH for both the soluble LDH and NP/3-LDH. Curves (a), (b), (c) and (d) show the results obtained for preparations

I, II, III and IV, and (e) shows the results obtained for the soluble enzyme. Because of the curved double reciprocal plots obtained, the values of K_m' at pH9.0 have been omitted in some cases. As for the soluble enzyme, the K_m' increases with pH, the effect becoming more marked at higher pH values. The K_m' values for the immobilised enzymes were always larger than the K_m' values for the soluble enzyme, and it is significant that K_m' values for NADH for the soluble enzyme, were always about ten times smaller than those for the immobilised preparations. For instance, at pH7.4, the K_m' for NADH for the soluble enzyme has a value of 3μM whereas the K_m's for NADH for the immobilised preparations I, II, III and IV have values of 91μM, 77μM, 63μM and 63μM respectively. Exceptions to this effect were observed at pH8.4 and pH9.0. At these pH values, the K_m' values for the immobilised enzymes are increased five-fold. For instance, at pH9.0, the K_m' for NADH for the soluble enzyme is 17μM; the immobilised derivatives I, II, III and IV have values of 78μM, 93μM, 53μM and 65μM respectively for NADH.

5.5.2. Michaelis parameters of soluble LDH and Cellex-AE-LDH

Kinetic studies at pH7,4 were carried out on soluble LDH and the derivatives described in

Table 14.

Derivative	Substrate	K_m' [1]	V_{max}' [2]
Soluble	Pyruvate	238	589
	NADH	3	550
	Lactate	13.7	121
	NAD^+	149	118
"2.5mg"	Pyruvate	200	0.145
	NADH	72	0.150
	Lactate	15.2	0.0329
	NAD^+	400	0.0351
"1.5mg"	Pyruvate	190	0.112
	NADH	33	0.0884
	Lactate	13.15	0.0282
	NAD^+	455	0.0314
PEI-LDH (I)	Pyruvate	286	48
	NADH	20	47
	Lactate	11	18.4
	NAD^+	165	17.4
PEI-LDH (II)	Pyruvate	294	20
	NADH	5.6	22
	Lactate	11.1	8.5
	NAD^+	357	8.2
Cellex-AE-(PEI-LDH)	Pyruvate	81	0.0157
	NADH	17.5	0.0169
	Lactate	17.3	0.0031
	NAD^+	349	0.0028
Cellex-AE-PEI-LDH	Pyruvate	256	0.0894
	NADH	33	0.0867
	Lactate	11.5	0.0161
	NAD^+	200	0.0139

[1] K_m' : micromolar for all substrates except lactate (mM).

[2] V_{max}' : micromoles NADH formed or oxidised per ml per min per mg (enzyme or enzyme-support).

Sections 5.2.2.1 to 5.2.2.5. Table 14 gives a comparison of the Michaelis parameters for the soluble and immobilised derivatives.

The immobilisation of LDH on Cellex-AE or on PEI causes very little change in the K_m' for pyruvate. PEI-LDH immobilised on Cellex-AE, however, is anomalous. The K_m' for pyruvate for this derivative is 81μM whereas that for the soluble enzyme is 238μM. In contrast to the slight change in K_m' for pyruvate on immobilisation of LDH, the K_m' for NADH increases at least ten-fold when LDH is immobilised either directly on to Cellex-AE or through PEI. For instance, the K_m' for NADH for the soluble enzyme is 3μM whereas it is 33μM for the "1.5mg" derivative. When, however, LDH is immobilised on PEI alone, the K_m' increases only six-fold to 20μM for derivative I, and only two-fold for derivative II. This is perhaps to be expected since the PEI-LDH is water-soluble whereas the Cellex-AE is insoluble and LDH will change phase on immobilisation. Again, anomalous results were obtained for the derivative prepared by immobilisation of PEI-LDH on Cellex-AE; the K_m' for NADH was not increased.

The immobilisation of LDH to both PEI and Cellex-AE causes an increase in the K_m' for NAD^+ compared to

the soluble enzyme. The increase is small for PEI-LDH, a K_m' value of 165μM being obtained, but is large for Cellex-AE-LDH, a K_m' value of 400μM being obtained compared to the K_m' value of 149μM for the soluble enzyme.

The K_m' for lactate is virtually unchanged upon immobilisation of LDH. Lactate would be expected to show the smallest change in value of K_m' since the K_m' with respect to this substrate (about 11mM) is considerably larger than the K_m' for the other substrates. The minimum concentration of lactate used in kinetic studies was always greater than 1.0mM.

5.6. Equilibrium Constant

The equilibrium constant for soluble LDH and for the immobilised preparations described in Sections 5.2.2.1 to 5.2.2.5 was determined by the method of Hakala et al. (1956). Two typical plots are shown in Figs. 54 and 55 and the complete results summarised in Table 15.

The value of 1.22×10^{-12}M for the equilibrium constant of the soluble enzyme agrees favourably with that obtained by Hakala et al. (1956) who found the equilibrium constant to be 1.05×10^{-12}M at 25°C

and Zewe and Fromm (1965) who found the value to be 4.2×10^{-12}M at 28°C. In all cases, the equilibrium constant for LDH immobilised on a macromolecular support, whether soluble or insoluble, is lower than that for the free enzyme.

5.7. Heat Inactivation Studies

Heat inactivations at 45°C and 50°C were carried out on soluble LDH and on the immobilised preparations described in Sections 5.2.2.1 to 5.2.2.5. The results are presented in Figs. 56 and 57. For both temperatures, immobilisation of LDH on Cellex-AE, either directly by glutaraldehyde or through PEI, results in a more stable derivative. Free LDH and LDH immobilised on a macromolecule such as PEI (which is soluble) are less stable. For instance, after 45min at 50°C, the PEI derivative has lost 89% of its activity, the soluble enzyme 78%, the Cellex-AE-PEI-LDH and the "2.5mg" derivative both 67%, the Cellex-AE-(PEI-LDH) derivative 59% and the "1.5mg" derivative 56% of their activity.

Fig. 54.

Typical plot for the determination of the equilibrium constant for the "2.5mg" preparation. Initial concentration of lactate was 33.433mM; initial concentrations of NAD^+ were 106μM, 79.5μM, 53μM and 26.5μM. Other experimental conditions were as described in Section 2.11.2.

O : NAD^+.

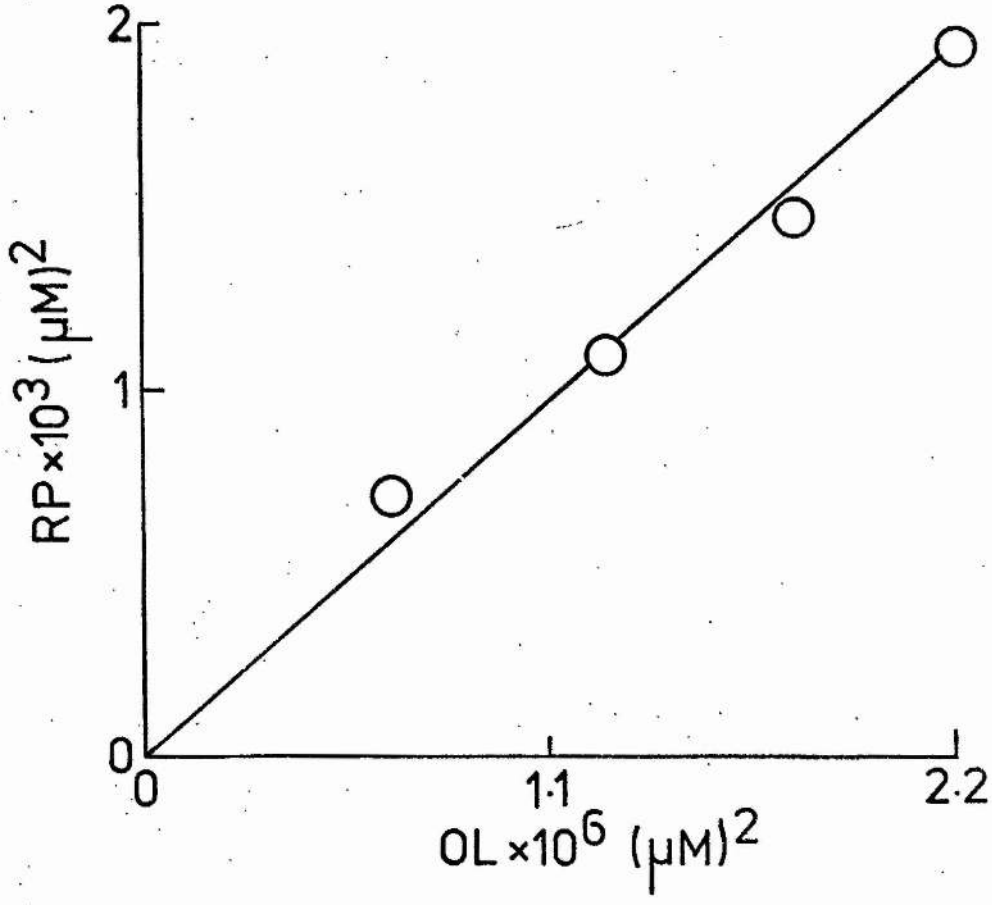
L : Lactate.

R : NADH.

P : Pyruvate.

Fig. 55.

Typical plot for the determination of the equilibrium constant for Cellex-AE-(PEI-LDH). Initial concentration of lactate was 32.333mM; initial concentrations of NAD^+ were 99.0μM, 75.3μM, 49.5μM and 24.8μM. Other experimental conditions were as described in Section 2.11.2. O, L, R and P have the same significance as for Fig. 54.


2
1
0
RP×10³ (μM)²
0
1·1
2·2
OL×10⁶ (μM)²

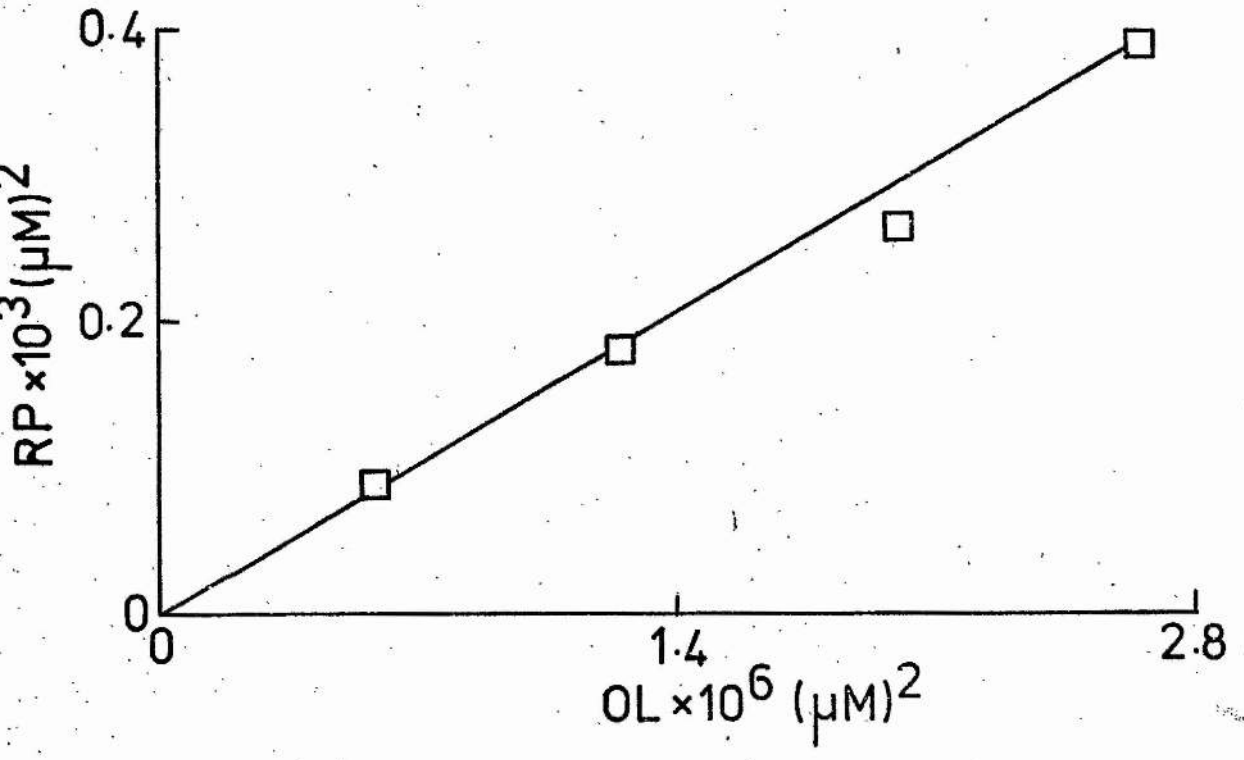

0·4
0·2
0
RP×10³(μM)²
0
1·4
2·8
OL×10⁶ (μM)²

Table 15.

Derivative	$K_{eq}(M) \times 10^{12}$
Soluble	1.220
"2.5mg"	0.956
"1.5mg"	0.820
PEI-LDH (I)	0.653
PEI-LDH (II)	0.930
Cellex-AE-(PEI-LDH)	0.147
Cellex-AE-PEI-LDH	0.600

Fig. 56.

Effect of incubation at 45°C on the stability of soluble LDH (○), PEI-LDH (□), Cellex-AE-(PEI-LDH) (△) and the "1.5mg" derivative (□). The traces obtained for the "2.5mg" and the Cellex-AE-PEI-LDH derivatives are omitted for clarity. They are identical to those obtained for the soluble and the Cellex-AE-(PEI-LDH) derivative respectively.

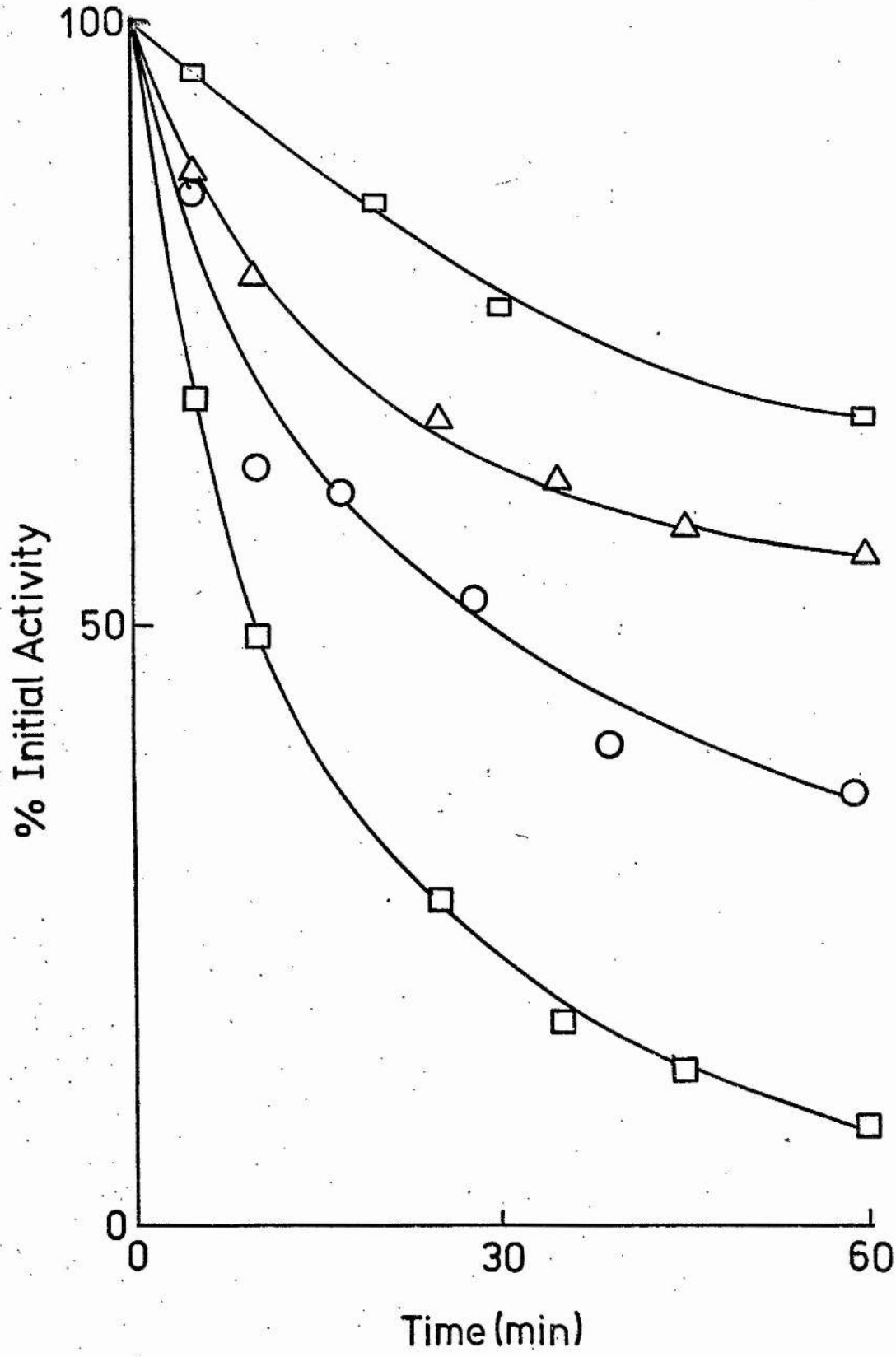
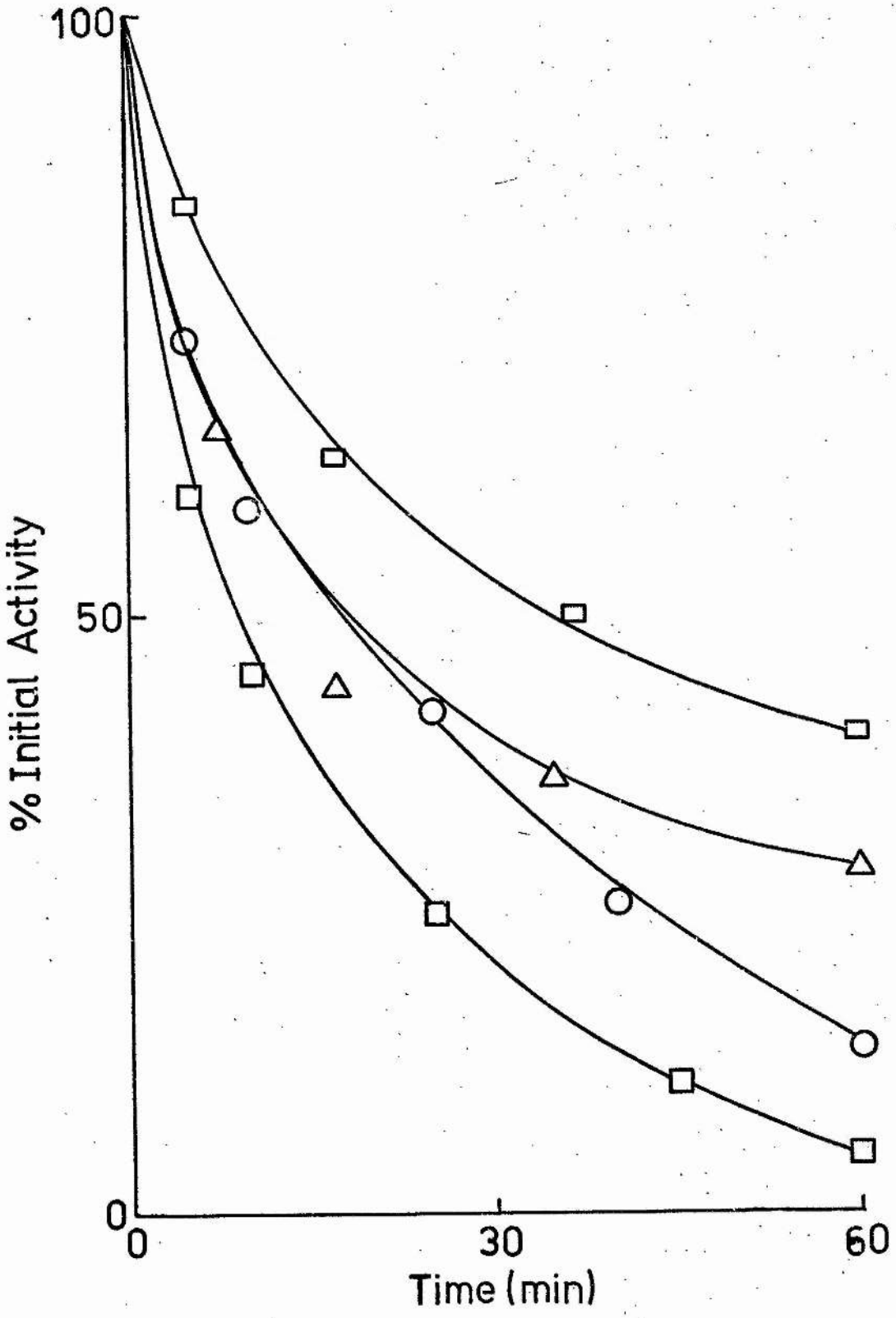

100
50
0
% Initial Activity
0
30
60
Time (min)

Fig. 57.

Effect of incubation at 50°C on the stability of soluble LDH (○), PEI-LDH (□), Cellex-AE-PEI-LDH (△), and the "1.5mg" derivative (▭). The traces obtained for the "2.5mg" and the Cellex-AE-(PEI-LDH) derivatives are omitted for clarity. They are identical to those obtained for the Cellex-AE-PEI-LDH and the "1.5mg" derivatives respectively.


100
50
0
% Initial Activity
0
30
60
Time (min)

Malate Dehydrogenase

Chapter 6

6.1. The Effect of pH on the Coupling of MDH to Cellex-AE

The pH of the coupling step of MDH to activated Cellex-AE was varied. Activated Cellex-AE, prepared as in Section 2.14.2.1 was resuspended in the various coupling buffers (4.8ml). 200µl MDH solution (1mg/ml) was added and coupling allowed to proceed at 4°C for 90min. Washing and resuspension of the derivative was as described in Section 2.21. The results, which are presented in Fig. 58, have been corrected for loss in activity of soluble enzyme at the pH of the coupling buffer. A maximum support specific activity of 33U/g was obtained when the coupling reaction was performed at pH8.5.

Since the support activity reached a maximum at pH8.0 to 9.0, pyrophosphate buffer (pH8.4,I0.1) was used for all further coupling experiments.

6.2. The Effect of Sodium Borohydride Reduction on Cellex-AE--MDH

Two derivatives of MDH coupled to Cellex-AE were prepared. In both cases the initial protein concentration on the coupling suspension was the same. One of the derivatives, however, was reduced with freshly prepared 50mM-sodium borohydride solution before the final washing. The activities and amounts

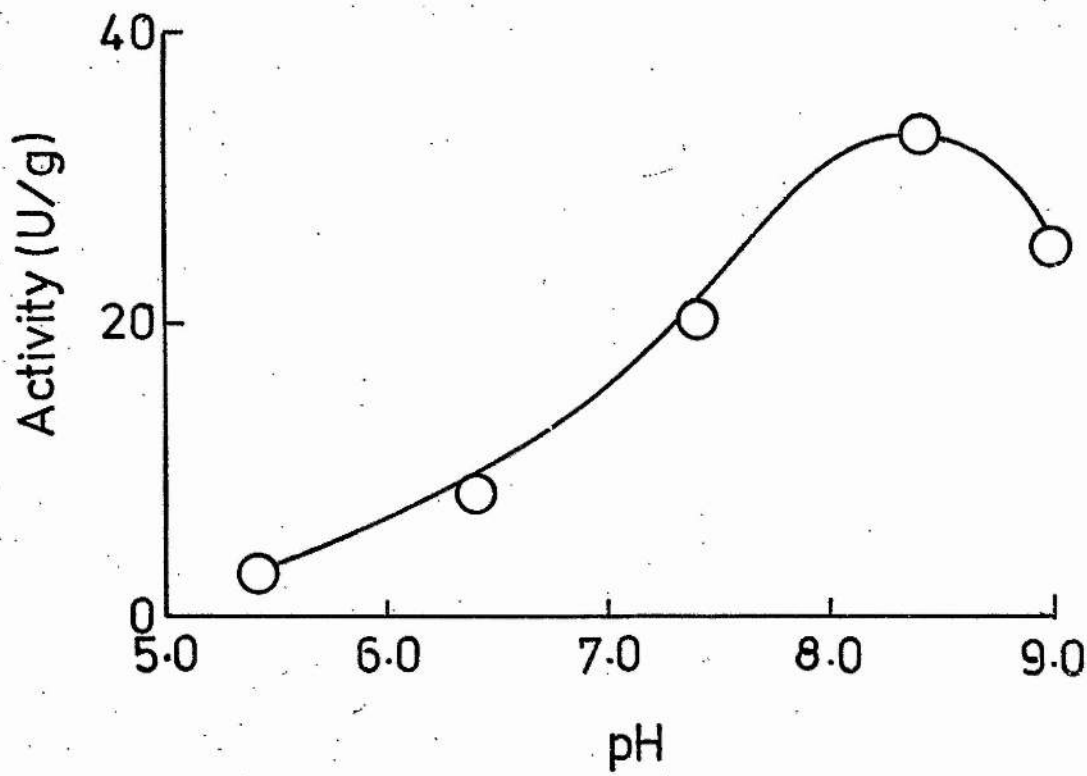


Fig. 58.

Effect of pH on the coupling of MDH to Cellex-AE.

Table 16.

Derivative	mg protein in coupling	% Initial Protein coupled	mg MDH per g support	Activity (U/g)	Activity (U/mg immobilised MDH)
"Unreduced"	0.75	73	2.20	27	12
"Reduced"	0.75	70	2.20	21	9.5

Table 17.

Derivative	mg protein in coupling	% Initial Protein coupled	mg MDH per g support	Activity (U/g)	Activity (U/mg immobilised MDH)
"Unreduced"	0.75	73	2.20	27	12
"3mg"	3	22	2.64	54	20

of protein coupled are presented in Table 16.

Although the amount of protein bound is almost identical, the unreduced preparation is 30% more active than the reduced preparation. Whereas for YADH, this result can be explained in terms of the effect of sodium borohydride on the enzyme, the effect of borohydride reduction on MDH has not been investigated.

6.3. The Effect of Enzyme Concentration on the Immobilisation of MDH to Cellex-AE

MDH was attached to Cellex-AE as described in Section 2.21. The initial protein concentration was 1mg/ml, whereas the initial protein concentration used in Section 6.2 was 250µg/ml. The resulting Cellex-AE-MDH derivative was not reduced with sodium borohydride. Table 17 compares this derivative with the unreduced derivative prepared in Section 6.2 above.

Although the initial protein present in the coupling suspension was quadrupled, the amount of protein coupled to the support has not increased significantly. The support specific activity, however, has doubled.

6.4. The Long Term Stability of reduced and unreduced Cellex-AE-MDH Derivatives

The stability at 4°C of the reduced and unreduced derivatives described in Section 6.2 was studied over a period of one month. Assays were carried out as described in Section 2.8.4. The results, which are presented in Fig. 59. show that, although the initial activity of the reduced suspension is lower, the rate of loss of activity at 4°C is the same as for the unreduced derivative. After one month, the residual activity is 50%. This is consistent with results noted for immobilised YADH and LDH. Figs. 19, 39 and 59, show that sodium borohydride reduction does not affect the stability of the immobilised preparations on storage at 4°C, except in the case of unreduced Cellex-AE-YADH which loses activity slightly more quickly than its corresponding reduced derivative.

6.4.1. Effect of pH on the Short Term Stability of soluble MDH

The stability of soluble MDH was studied in the pH range 5.0 to 10.0. The results, which are presented in Fig. 60, show that MDH is extremely unstable below pH7.0, for example after 2h at pH6.0 90% of the activity was lost. However, on

incubation in buffers at pH values above 7.5, no loss in activity occurred.

6.4.2. Effect of pH on the Long Term Stability of soluble and immobilised MDH

The stability of soluble and immobilised MDH was studied over four weeks at 4°C in various buffers. The immobilised MDH preparation used was that described in Section 6.3. The results are presented in Figs. 61 and 62. Two interesting observations emerge from these results. The first is that at pH values where soluble MDH is unstable, there is virtually no difference in the rate of loss of activity of soluble and immobilised MDH. Secondly, at pH values where MDH is relatively stable, the immobilised derivatives are less stable and over 30days lose about 30% more activity.

6.5. Measurement of the Michaelis parameters of MDH

Kinetic experiments were carried out on soluble MDH and its immobilised derivatives described in Sections 6.2 and 6.3 in pyrophosphate buffer (pH8.4,I0.1) at 25°C. The results were plotted according to the method of Lineweaver and Burk (1934). Typical plots, with details of experimental conditions, are presented in Figs. 63,

64, 65 and 66. Table 18 shows the values of K_m' and V_{max}' at pH8.4 for soluble MDH, and the values of K_m' and V_{max}' for the immobilised derivatives.

The values of the Michaelis parameters obtained for the free enzyme are different from those reported by Raval and Wolfe (1962). The K_m' values for NAD^+, malate and OAA are two-fold larger whereas that for NADH is four-fold larger. These authors used Tris buffer (pH8.5,I0.05), whereas pyrophosphate buffer (pH8.4,I0.1) was used in all the present studies. Ionic strength could account for the differences. Any comparisons of the Michaelis parameters made between the soluble and immobilised derivatives are based on the values obtained for the soluble enzyme under the conditions described in Section 2.10.1. Also noteworthy is that the soluble and "reduced" derivatives have similar K_m' values, as do also the "unreduced" and "3mg" derivatives.

All kinetic studies were carried out by holding the concentration of one substrate constant at a high value whilst varying the other substrate concentration. The different values of V_{max}' for malate and NAD^+ for the "reduced" and "unreduced" derivatives are due to an insufficiently high concentration of the first substrate. A similar explanation applies to the

lower V_{max}' obtained for OAA relative to that for NADH; the concentration of NADH in the cuvette was only twice the apparent K_m.

Considering the V_{max}' values for malate and NADH to be more characteristic of the velocities for the forward and reverse reactions respectively (for the reasons given in the previous paragraph), the ratio of the forward to the reverse reactions can be calculated, and the results are presented in Table 19(b). Raval and Wolfe (1962) obtained a relative velocity ratio of 4 : 1 for the forward to the reverse reactions for soluble MDH.

6.6. The Effect of Temperature on the Stability of soluble and immobilised MDH

The temperature stability of soluble MDH, and of its immobilised derivatives described in Sections 6.2 and 6.3, was studied at 40°C and 50°C. Experimental details are described in Section 2.12 and the results are presented in Figs. 67 and 68.

For both temperatures, the soluble enzyme is very much less stable than any of the immobilised derivatives. After 30min at 40°C, the soluble enzyme has lost 95% of its original activity, whereas the "reduced" derivative has lost 50% and the "unreduced" and the "3mg" have lost 40% of their original activity.

After 30min incubation at 50°C, the soluble enzyme has lost all its activity; the "reduced" and "unreduced" derivatives have lost 90% of their original activity, whereas the "3mg" derivative has lost only 70%.

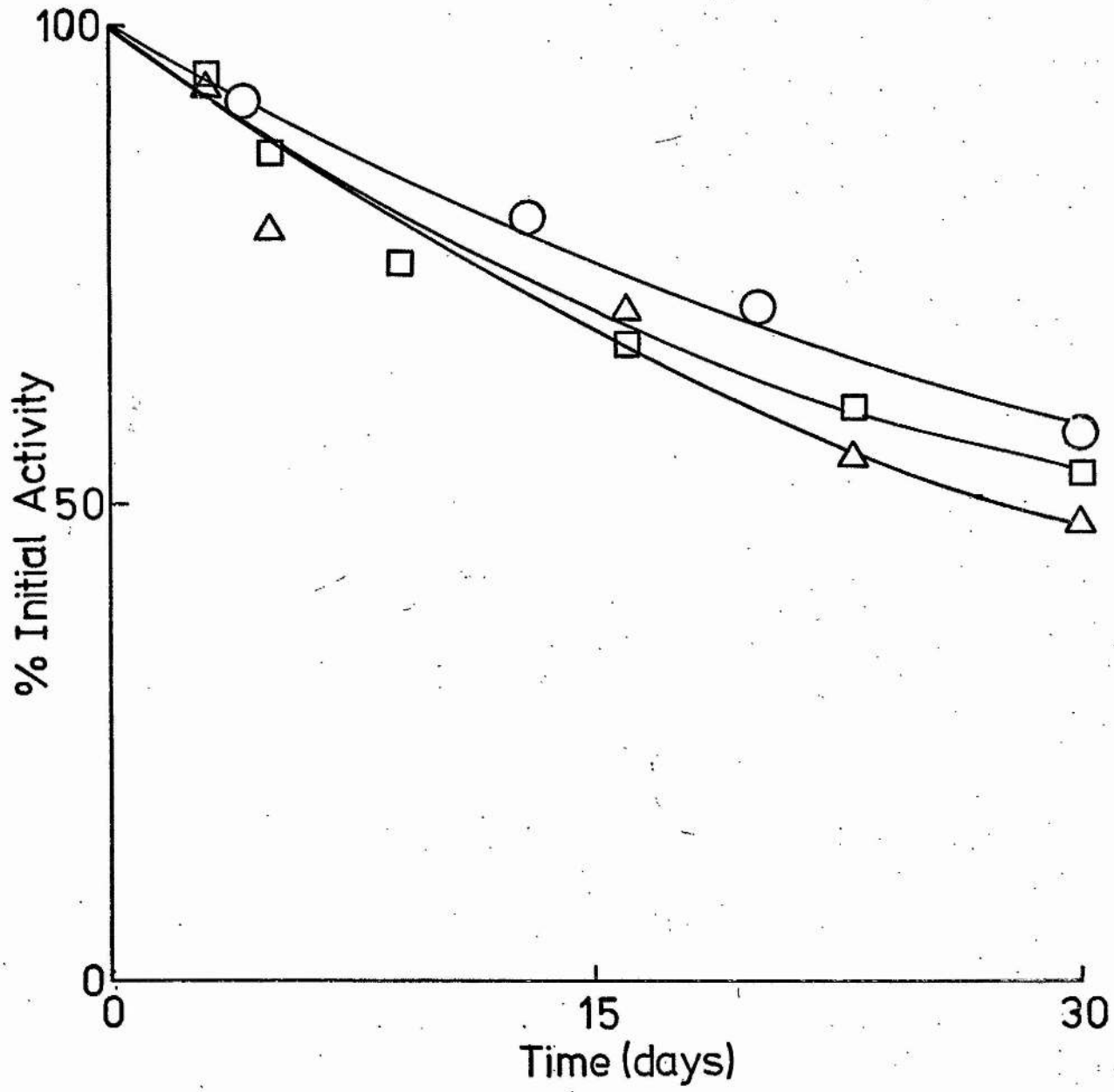


Fig. 59.

Comparison of the long term stability at 4°C of soluble MDH (△), and the "reduced" (□) and the "unreduced" (◯) derivatives described in Section 6.4.

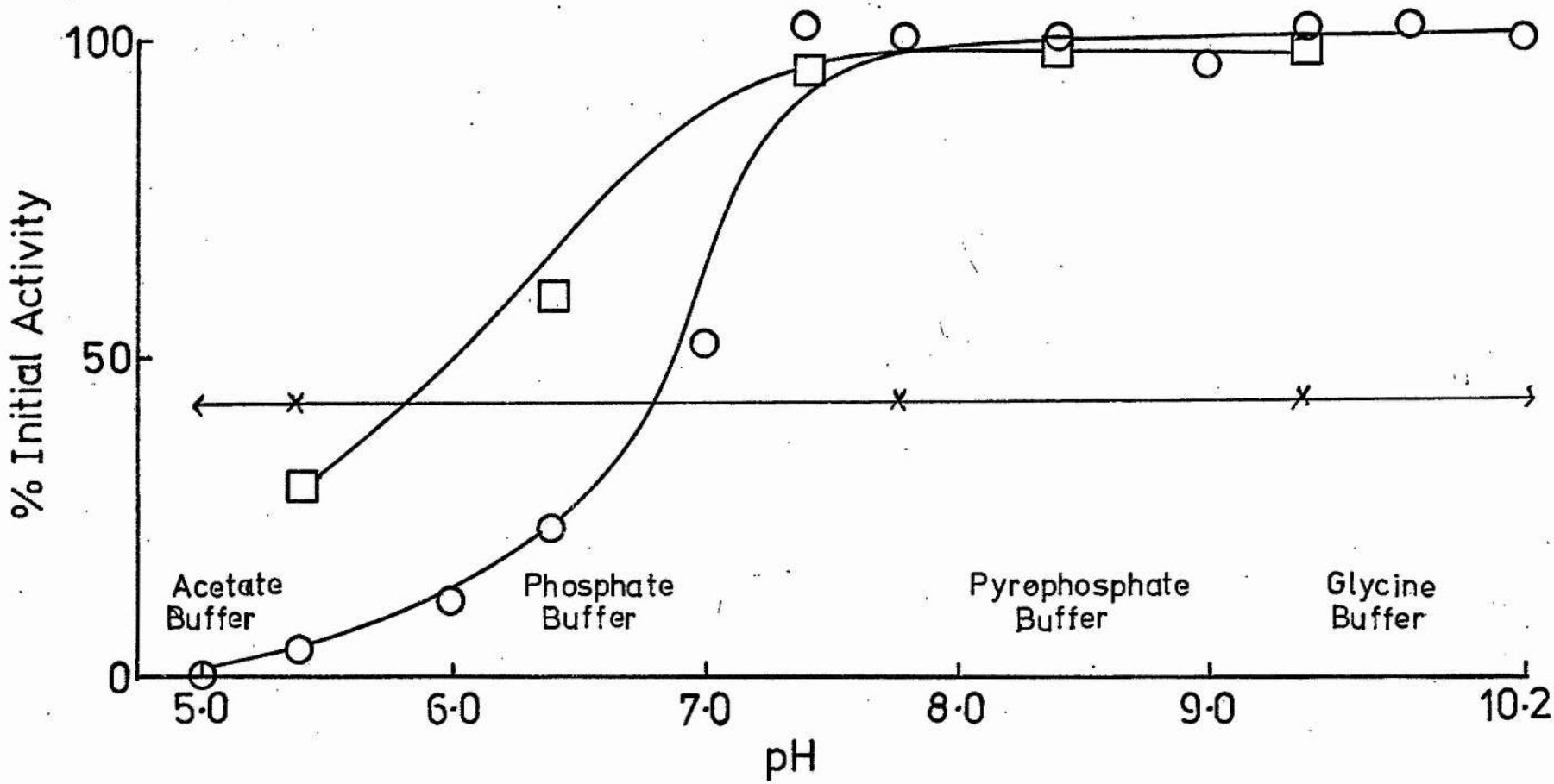


Fig. 60.

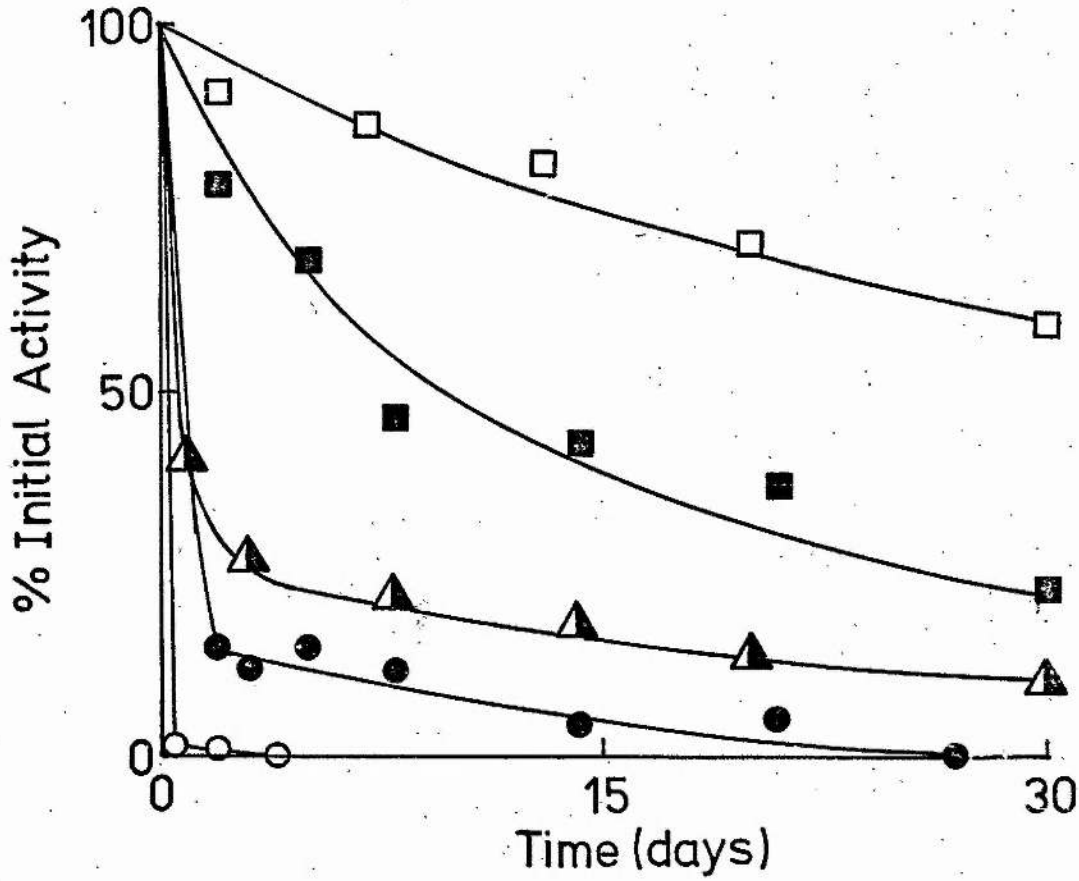
Effect of pH on the short term stability at 4°C of soluble MDH (○) and Cellex-AE-MDH (□).

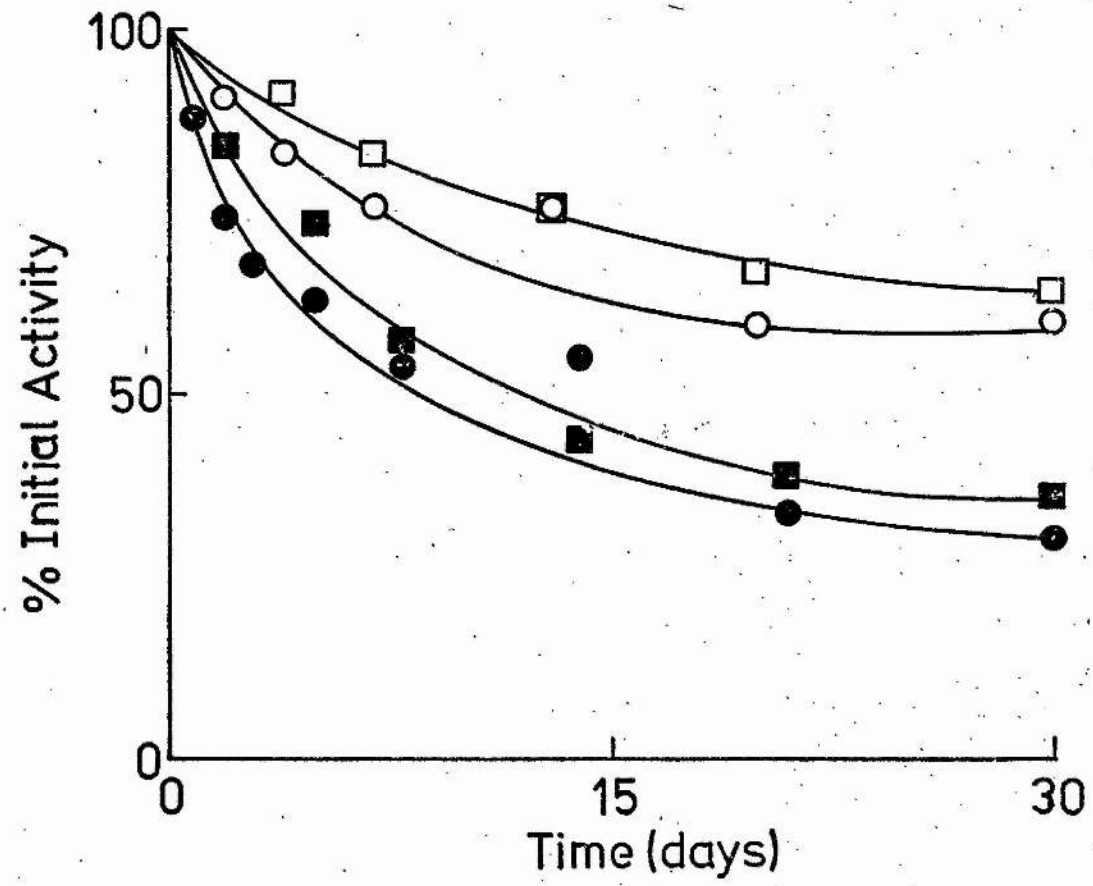
Fig. 61.

Comparison of the long term stability at 4°C of soluble MDH (unfilled symbols) and Cellex-AE-MDH (filled symbols) at pH5.4 (○), at pH6.4 (△) and at pH7.4 (□).

Fig. 62.

Comparison of the long term stability at 4°C of soluble MDH (unfilled symbols) and Cellex-AE-MDH (filled symbols) at pH8.4 (○) and at pH9.0 (□).


100
50
0
% Initial Activity
0
15
30
Time (days)


100
50
0
% Initial Activity
0
15
30
Time (days)

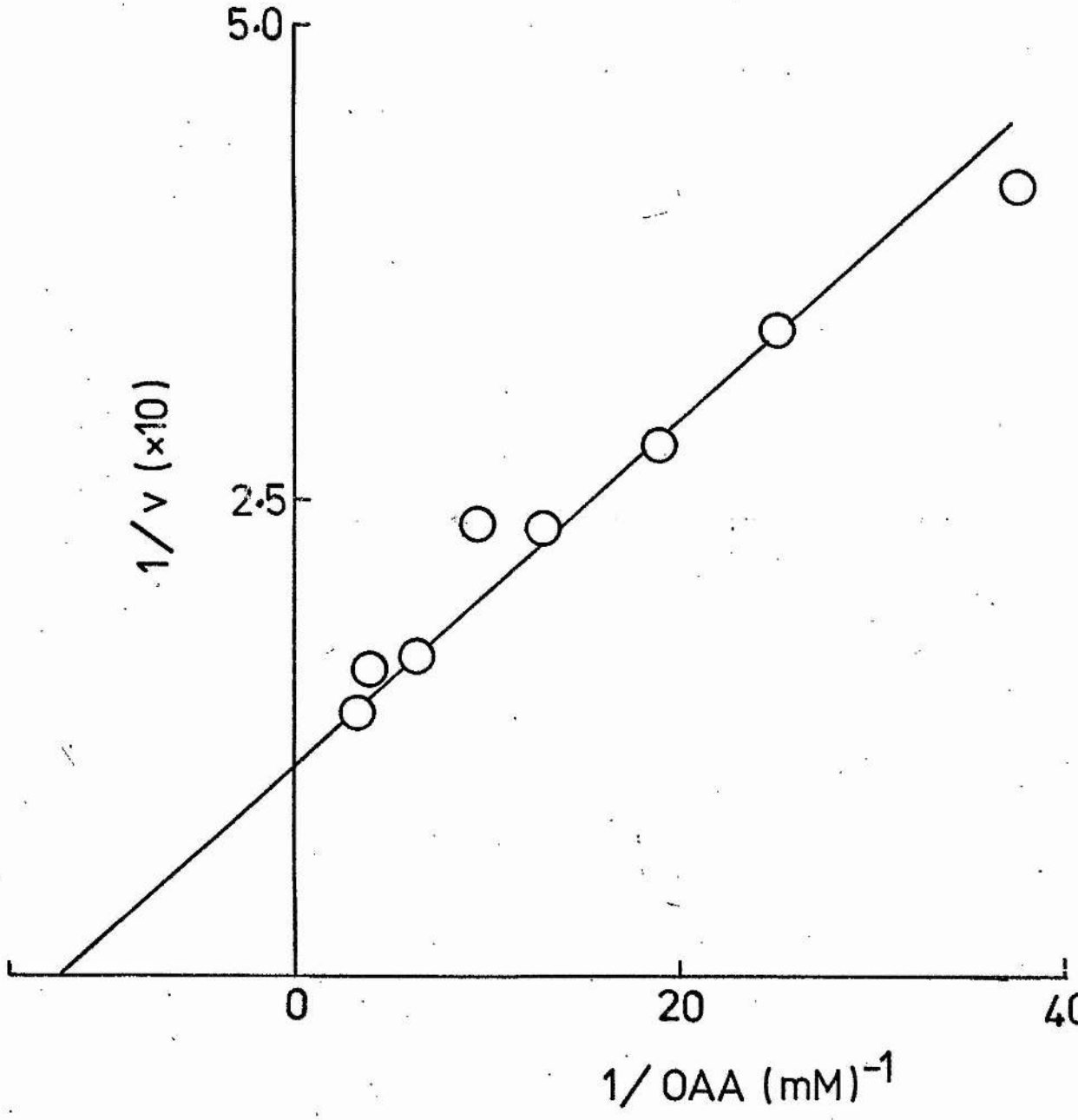


Fig. 63.

Primary reciprocal plot of initial velocity data for soluble MDH at pH8.4. NADH concentration was held constant at 150μM. Temperatures of assays were 25°C. Units of 'v' are micromoles per ml per min per mg enzyme.

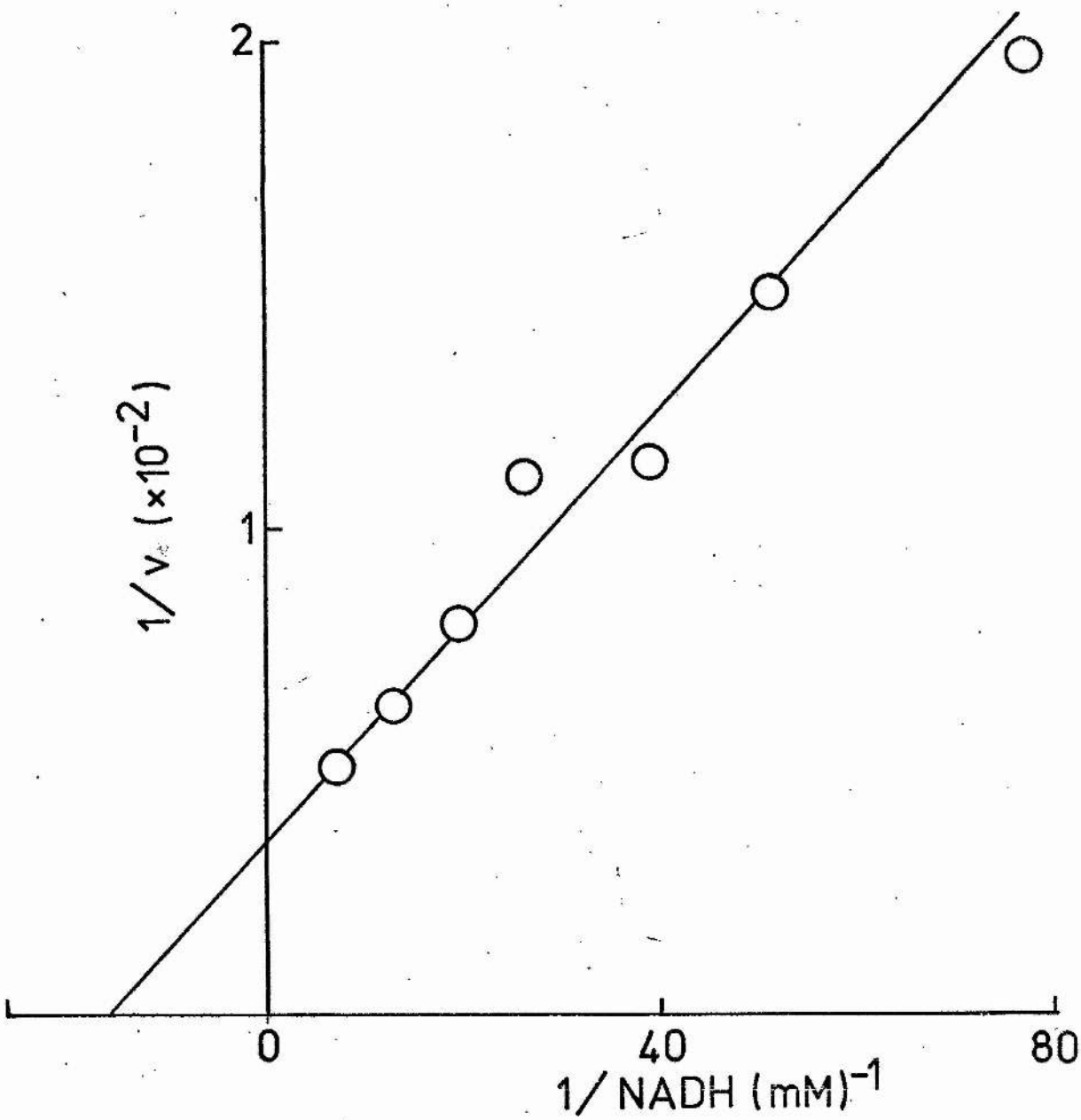


Fig. 64.

Primary reciprocal plot of initial velocity data for the "reduced" preparation at pH8.4. Oxalacetate concentration was held constant at 250μM. Temperatures of assays were 25°C. Units of 'v' are micromoles per ml per min per mg enzyme-support.

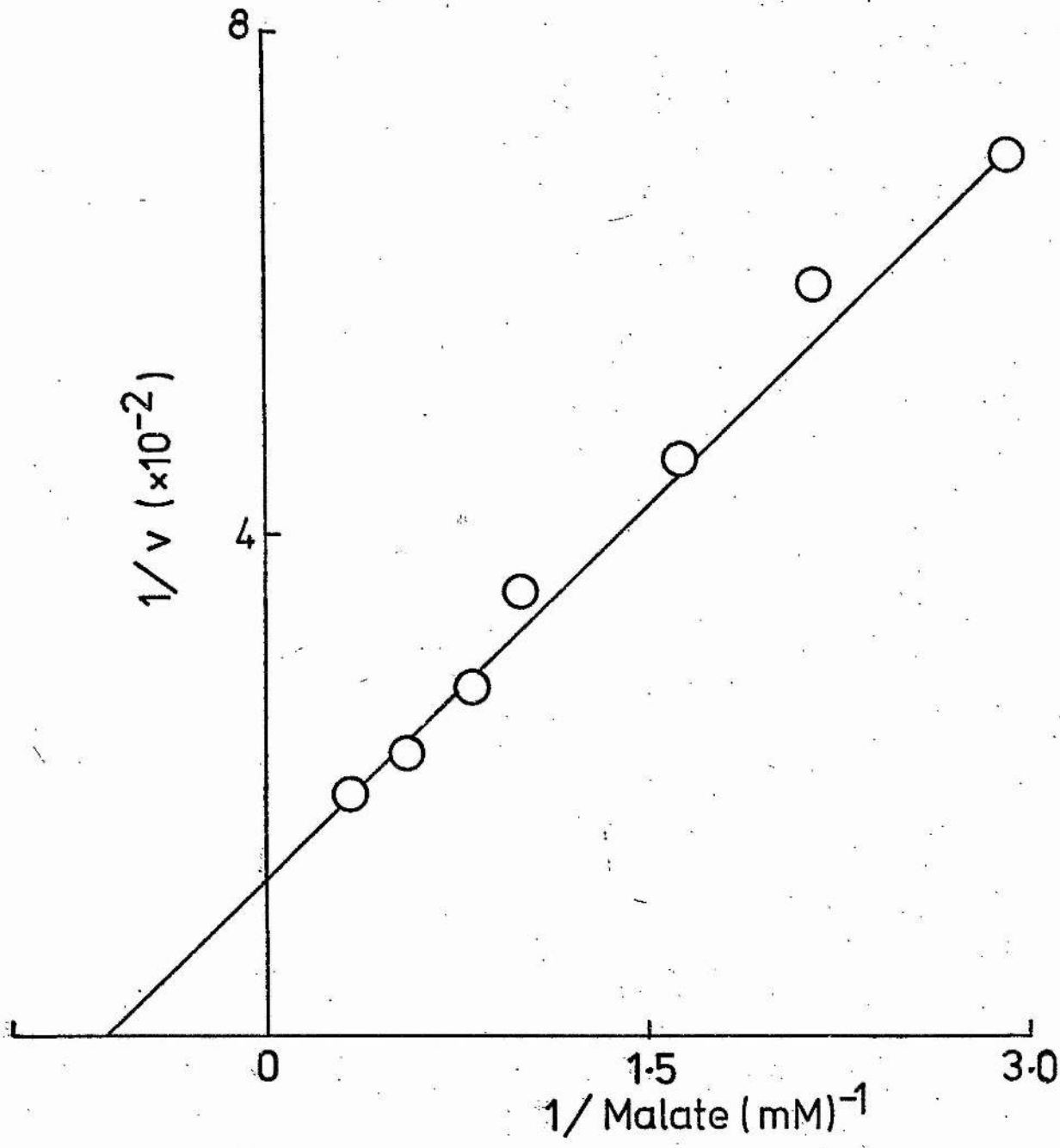


Fig. 65.

Primary reciprocal plot of initial velocity data for the "unreduced" preparation at pH8.4. NAD^+ concentration was held constant at 600µM. Temperatures of assays were 25°C. Units of 'v' are micromoles per ml per min per mg enzyme-support.

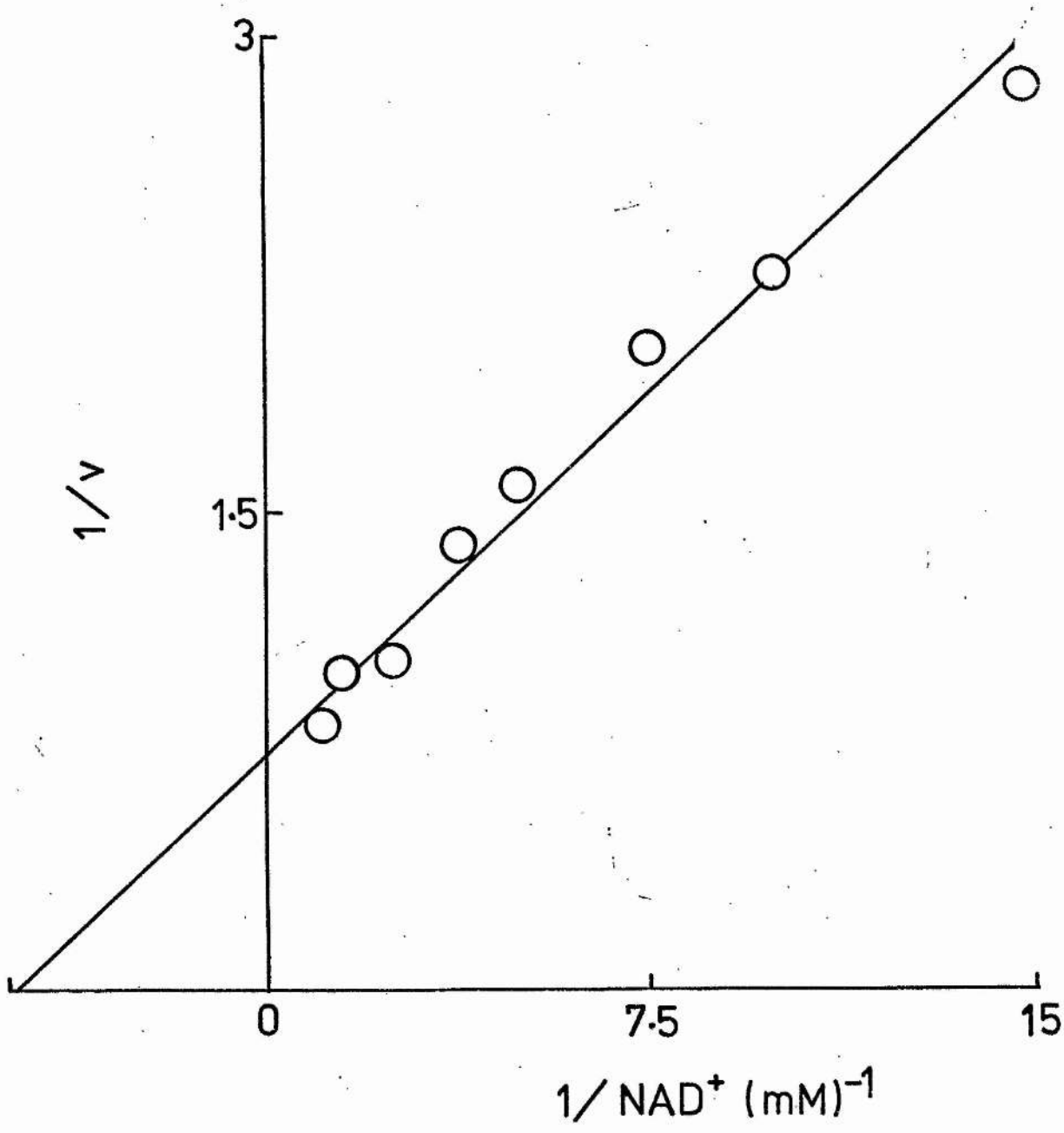


Fig. 66,

Primary reciprocal plot of initial velocity data for the "3mg" preparation at pH8.4. Malate concentration was held constant at 3mM. Temperatures of assays were 25°C. Units of 'v' are micromoles per ml per min per mg enzyme-support.

Table 18.

Derivative	Substrate	K_m' [1]	V_{max}' [2]
Soluble	OAA	79	8.85
	NADH	83	12.06
	Malate	1850	1.810
	NAD^+	200	1.320
"3mg"	OAA	152	0.026
	NADH	125	0.034
	Malate	1100	0.0053
	NAD^+	330	0.0050
"Reduced"	OAA	82	0.027
	NADH	63	0.028
	Malate	2100	0.0040
	NAD^+	180	0.0020
"Unreduced"	OAA	114	0.034
	NADH	133	0.055
	Malate	1430	0.0074
	NAD^+	170	0.0035

[1] K_m' : units of micromolar for all substrates.

[2] V_{max}' : units of micromoles NADH formed or oxidised per ml per min per mg (enzyme or enzyme-support).

Table 19(b).

Enzyme	Derivative	Relative Forward Rate	Relative Reverse Rate
MDH	Soluble	1	6.6
	"Reduced"	1	7.0
	"Unreduced"	1	7.0
	"3mg"	1	6.5

Fig. 67.

Effect of incubation at 40°C on the stability of soluble MDH (○), "unreduced" derivative (△), "reduced" derivative (□) and "3mg" derivative (▭).

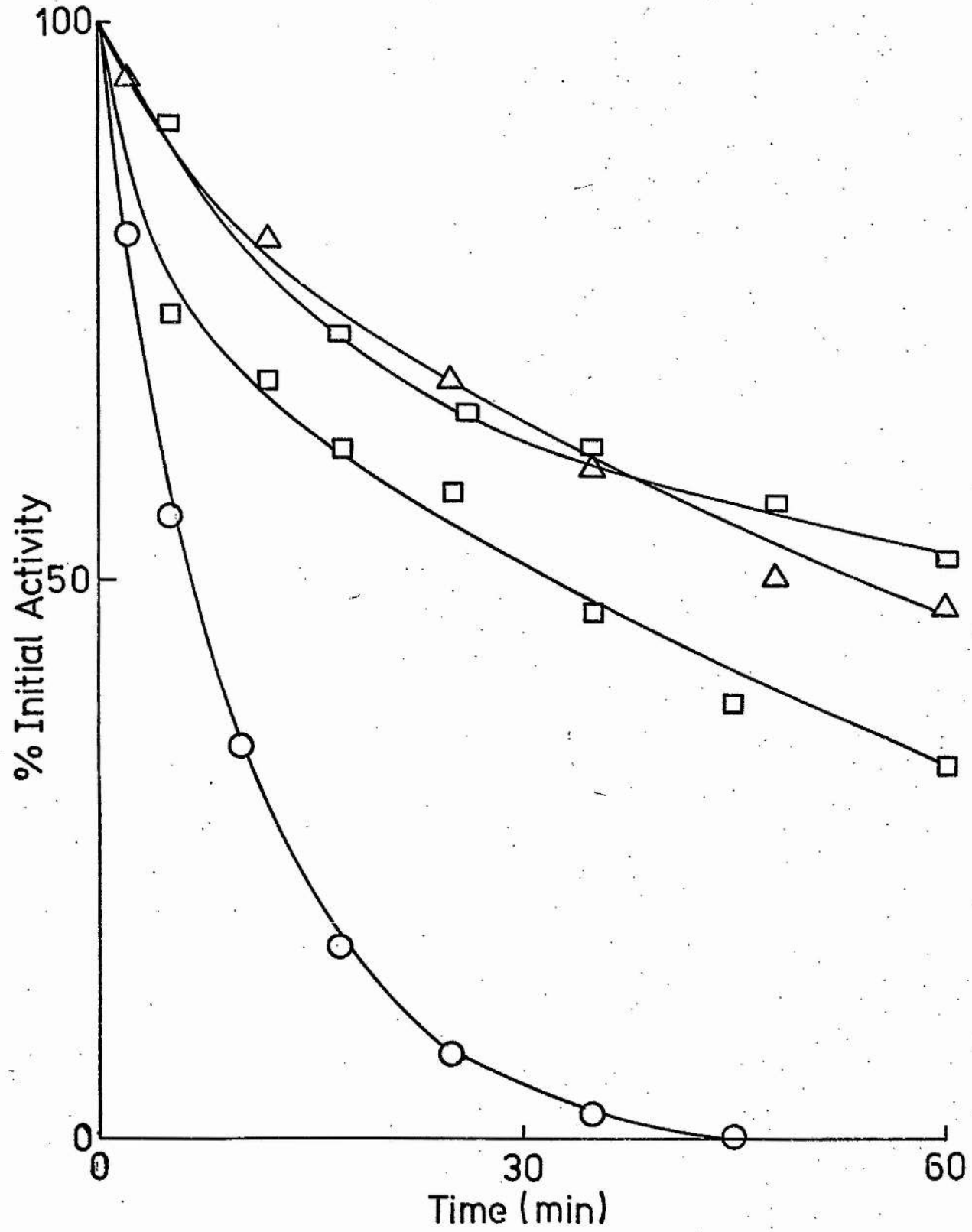
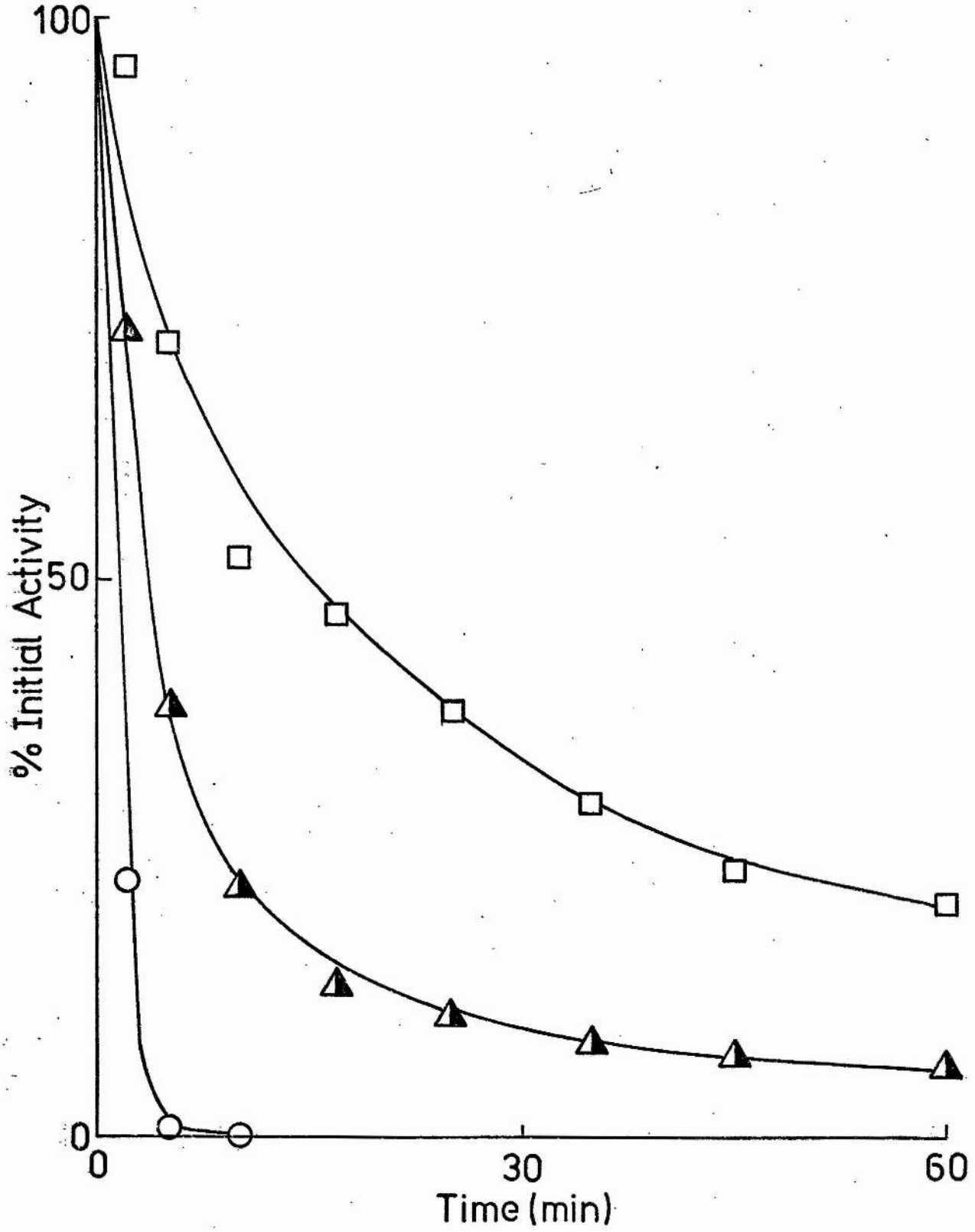

100
50
0
% Initial Activity
0
30
60
Time (min)

Fig. 68.

Effect of incubation at 50°C on the stability of soluble MDH (○), "unreduced" derivative (△), "reduced" derivative (▲) and "3mg" derivative (□).


100
50
0
% Initial Activity
0
30
60
Time (min)

General Discussion

Chapter 7

7.1. General

7.1.1. Introduction

Enzymes immobilised on macromolecular supports have been comprehensively reviewed (Silman and Katchalski, 1966; Melrose, 1971). Procedures for immobilising enzymes include encapsulation (Chang, 1972), gel-inclusion (Hicks and Updike, 1966) and covalent attachment to an insoluble support such as nylon, polystyrene or cellulose (Hornby et al., 1970). It is with the last mentioned of these methods that most of the work described in this thesis has been carried out. Changes in Michaelis parameters, and in enzyme stability, arising from immobilisation are discussed below.

7.1.2. Effect of Immobilisation

Gabel et al. (1971) found that changes in conformation occurred when chymotrypsin was immobilised on Sepharose. Since storage of a soluble enzyme also may result in small conformational changes uncovering different reactive groups, it is probably very difficult to obtain completely reproducible immobilised enzyme preparations - reproducible in this context referring to activity, Michaelis parameters and stability.

The activity and Michaelis parameters of an immobilised enzyme will be influenced by the amount of enzyme attached. This can be determined by a number of methods. It can be found by measuring the supernatant protein spectrophotometrically (Kay and Lilly, 1970), by acid hydrolysis of the enzyme-support (Crook et al., 1970) or a modification of the former method, by determining protein concentration and activity before coupling, and the protein activity after coupling.

The nature of bonding between enzyme-protein and support depends largely on the bifunctional coupling reagent used. When glutaraldehyde is used as the bifunctional reagent, the main amino acid residue involved is lysine (Habeeb and Hiramoto, 1968; Ogata et al., 1968). The former authors also showed that glutaraldehyde reacts with sulphydryl groups, phenolic groups and imidazole rings.

Enzymes, when immobilised, are subjected to microenvironmental effects created by the support (Gestrelius et al., 1973). These effects can be due to steric hindrance (Hornby et al., 1966), partitioning of the substrate (Laidler and Bunting, 1973) and diffusional effects (Rovito and Kittrell, 1973; Weibel and Bright, 1971). The activity of an immobilised

enzyme is a measure of the combined activity of all the individual immobilised enzyme molecules differentially influenced by the three environmental effects mentioned above. If, for example, a hundred enzyme molecules are immobilised, there is a high probability that they will have attached in a range of modes, with a corresponding range of Michaelis parameters and activities. A similar situation would arise if adsorption and covalent bonding of enzyme were occurring simultaneously on the same support molecule.

Another way of looking at the problem of activity is to consider that an enzyme molecule attached at only one amino acid residue is more likely to retain most of its activity than if it were attached at two or more amino acid residues.

7.1.3. Economic Considerations

The cost of materials in any experiment is an important consideration. Enzymes are expensive reagents to use and this is why normally only one or two mg of enzyme were used in the couplings. The properties of the immobilised enzymes may well change when a large amount of enzyme is attached to a support. Unfortunately, although the stirring cuvette is an ideal way of assaying

enzymes immobilised on microgranular supports, a high coenzyme concentration is necessary for saturating assay conditions for the dehydrogenases. This makes the assay expensive compared to a nylon tube assay (Hornby et al., 1972).

7.1.4. Assay Methods

Advantages of the stirring cuvette for assaying immobilised dehydrogenases on microgranular supports are that the rate obtained is a direct measure of the enzyme-support activity and that the volume of substrates and buffer required for the assay is small (3.5ml). The other main methods of measuring dehydrogenase activity on microgranular supports are either by packing in a column, or using a stirred slurry (Laidler and Bunting, 1973). In both cases, the eluants are measured spectrophotometrically by using a silica flow-cell, for which about 10ml of reagent solutions are required. The former method requires a fast flow rate, since if a slow flow rate is used, a large change in optical density, difficult to measure without dilution, occurs as a result of NADH formation. Unfortunately a fast flow rate causes "packing down" of the microgranular supports resulting in slower flow rates. For the latter method, the change in optical density can be altered by

varying the flow rate, thereby changing the time that the substrate solution is in the presence of the immobilised enzyme. One other distinct advantage of the stirring cuvette over these methods is that the former method involves only an overhead stirrer and a cuvette; the latter methods involve pumps, flow-cells, columns and lengths of tubing.

7.2. Chemistry of the Immobilisation Process

7.2.1. Support Materials

Three support materials were used to immobilise the dehydrogenases studied in the present work. Two, cellulose and nylon, were insoluble in water, whereas the third, PEI, was soluble in water. A common feature is that all are hydrophilic structures, PEI being most, and nylon least, hydrophilic.

Three cellulose structures were used for immobilising enzymes. The first of these, "AE-Cellulose", described in Section 2.14.1.1, was not used after initial experiments with YADH since CDI is an expensive reagent. Instead of the "AE-Cellulose", a commercially available aminoethyl cellulose, Cellex-AE, was used. This cellulose was used to prepare immobilised derivatives of YADH, LDH and MDH. The third cellulose used for immobilising YADH was DEAE-cellulose, the coupling reagent in this case being modified cyanuric chloride. It was hoped,

using this coupling reagent to attach YADH to DEAE- and to CM-cellulose and cellulose; these are positively charged, negatively charged, and neutral supports respectively. YADH could be attached to the latter two supports but the product activity was too low to permit detailed kinetic studies. It was found that no adsorption of enzyme occurred on any cellulose used as a support material.

In direct contrast to this, when NP/3 Nylon Powder was used as a support material, much adsorption occurred. This property has been employed in the brewing industry where nylon powder is added to clarify beer. NP/3 Nylon Powder is a very porous material but has only a quarter of the number of reactive groups of Cellex-AE and consequently less enzyme is attached. As can be seen from Fig. 2, the carboxyl groups of the NP/3 Nylon Powder are esterified with methanol. This esterification removes the negative charge on the carboxyl group and allows coupling of the protein to the activated support. If the negative charge was not removed, this would cause electrostatic repulsion between the protein anion and support at all pH values above the isoelectric point. This would prevent coupling. Adsorption of protein, however, would still occur by hydrogen, hydrophobic (owing to the

nylon methylene groups) and electrostatic bonding.

PEI is a multibranched polymer which has a molecular weight of 20,000 - 30,000. It is crosslinked very quickly by glutaraldehyde to form macromolecular compounds with molecular weights greater than a million.

Sephadex, being hydrophilic, was also considered as a support for the dehydrogenases studied. It was found, however, to be too fragile for use in the stirring cuvette. Polystyrene was not considered as a support material as it is a very hydrophobic material.

7.2.2. Coupling Reagents

Two bifunctional coupling reagents were used for immobilising enzymes on each of the supports mentioned above. The lesser used of these two was the ethanolamine derivative of cyanuric chloride (see Section 3.3 for its preparation) with which YADH was coupled to DEAE-cellulose. Disadvantages of this reagent are that it is not commercially available and that the cyanuric chloride parent compound is rather obnoxious to work with. Another disadvantage is that coupling to DEAE-cellulose has to be carried out in acetone-water (50% v/v) in which the cyanuric chloride derivative is soluble,

whereas with glutaraldehyde all couplings are carried out in water. An acetone-water mixture causes celluloses to contract reducing the availability of reactive groups. An advantage that this coupling reagent has over glutaraldehyde is that its structure is defined. This means that one hydroxyl group on the support will couple with one reactive group on the modified cyanuric chloride molecule, the remaining reactive group of which couples to an amino acid residue on the protein. When glutaraldehyde is coupled to Cellex-AE, two or three amino groups may react with one oligomeric glutaraldehyde molecule.

In recent years, many immobilised enzyme derivatives have been prepared using the bifunctional reagent, glutaraldehyde, for coupling an enzyme to a support. In some preparations, the enzyme has been suspended with the support and then glutaraldehyde added. This results in the formation of both enzyme-enzyme bonds and support-enzyme bonds (Habeeb, 1967). In other preparations, the support has been treated with glutaraldehyde, thoroughly washed, and then resuspended in a buffer containing the enzyme (Inman and Hornby, 1972; Filippusson et al., 1972; Robinson et al., 1971). This latter method ensures that no enzyme-enzyme crosslinking occurs.

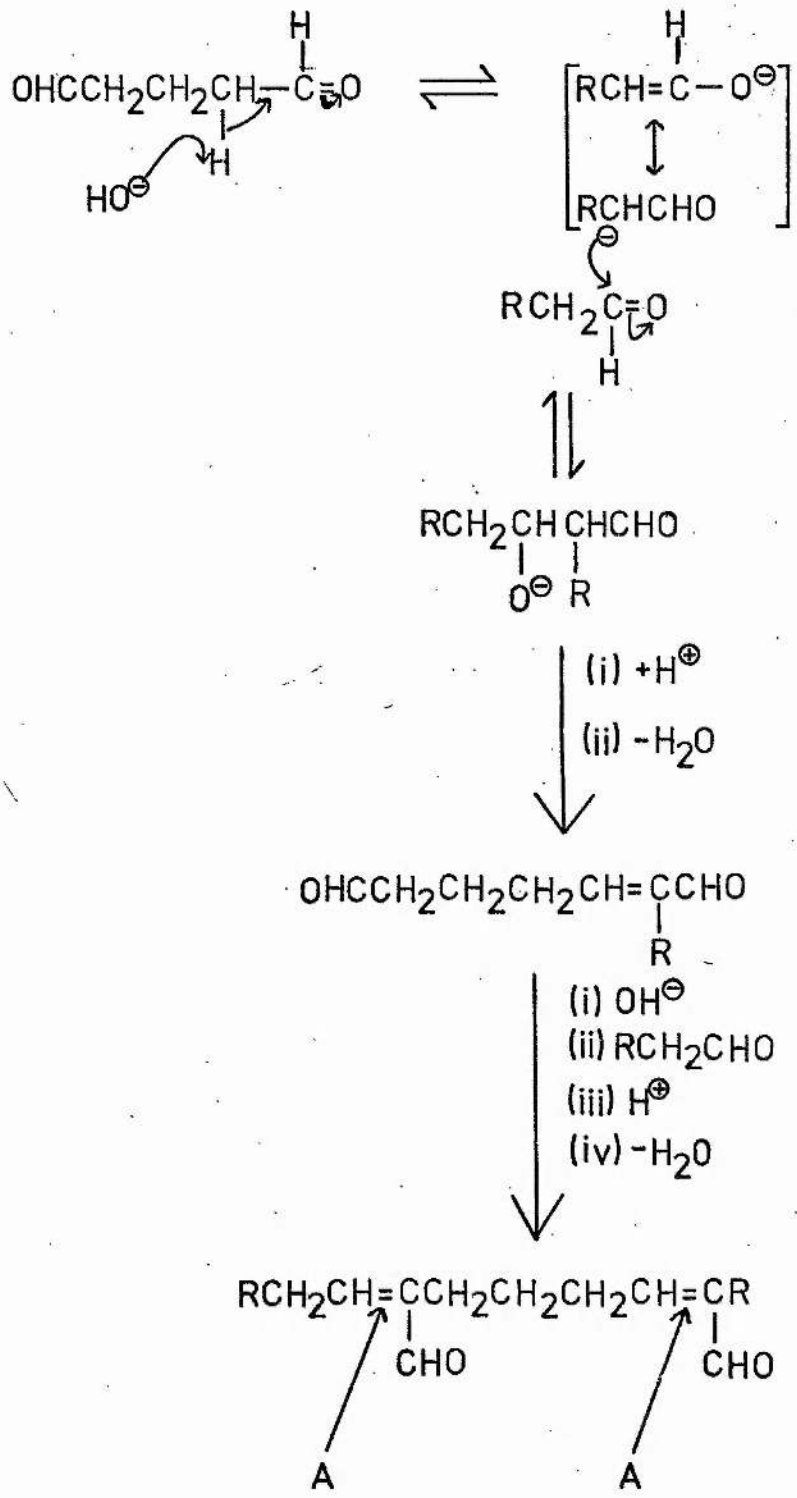
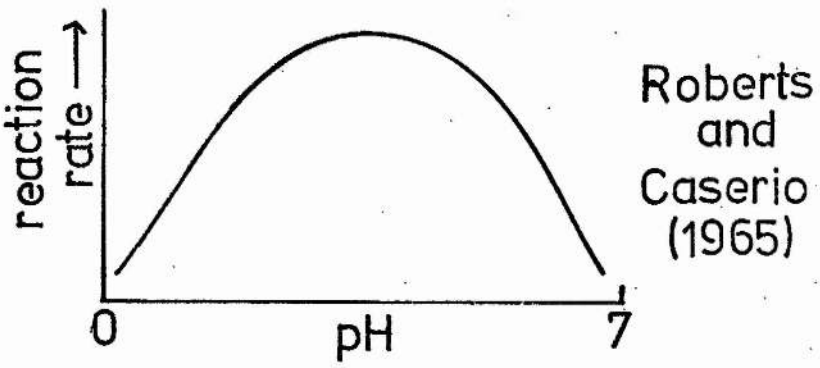


Fig. 69.

Aldol condensation of glutaraldehyde. Michael addition can occur at A for both the enzyme and the support.

R is $OHC.CH_2CH_2-$

Glutaraldehyde has several advantages as a bifunctional coupling reagent. It is a relatively non-toxic liquid which is easy to handle; it is also cheap and readily available. One disadvantage is that its precise structure in solution is not known. Korn (1972) has proposed that three molecular species are involved in binding of protein to a support. These three molecular species, which are in equilibrium with each other, are free glutaraldehyde (I), the cyclic hemiacetal of its hydrate (II), and oligomers of this (III). (III) is believed to be converted to (I) and (II) on dilution. Richards and Knowles (1968) have proposed that glutaraldehyde polymerises in aldol condensations (see Fig. 69) to give α,β-unsaturated aldehydes. The expected interaction of glutaraldehyde with amine to give a Schiff's base is not favoured under the coupling conditions used. At pH7.4 an imine dissociates very rapidly - in fact, the maximum velocity of the condensation reaction of an amine with a carbonyl compound occurs at about pH3.5 (Roberts and Caserio, 1965).



Richards and Knowles (1968) have also proposed that the reaction of protein with these aldol condensation products proceeds by Michael-type additions across the double bond in the aldol (see Fig. 69).

In one report (Dixon et al., 1973), the reaction of support and protein with glutaraldehyde has been postulated as Schiff's base formation. It would not seem to be reasonable to consider this the only reaction to be occurring, as active glutaraldehyde dimers and trimers, whether formed by aldol condensations or by cyclisation, are known to be present (Korn, 1972). The Schiff's base reaction between an aldehyde and an amine results in a very labile bond. But no protein, either active or inactive, has been found in the supernatant of the immobilised enzyme preparations. Some immobilised enzyme preparations were reduced with sodium borohydride with the aim of stabilising the Schiff's base by reduction. The yellow colour which appears on treatment of the support with glutaraldehyde disappears during this reduction procedure. This suggests that either Schiff's bases or the double bonds formed during an aldol condensation are being reduced. Habeeb and Hiramoto (1968) have found that, not only lysine, but also cysteine, tyrosine

and histidine form bonds with glutaraldehyde. It is difficult to visualise how Schiff's bases could be formed by these amino acid residues and glutaraldehyde. Richards and Knowles (1968) also found that the bonds formed between lysine and glutaraldehyde are resistant to acid hydrolysis.

It is proposed, therefore, that most of the bonds between enzyme proteins and macromolecular supports, involving glutaraldehyde as the bifunctional reagent, are formed by Michael additions. This involves attack by the amino group (Fig. 69) on the support at the carbon-carbon double bonds formed during the aldol condensation of glutaraldehyde, followed by attack by a nucleophilic group of the protein at the same or a different double bond.

One problem that could arise when the support is activated with glutaraldehyde, is that the reaction of the amino groups with the double bonds of the condensation products might produce a double bond-single bond shift resulting in the reaction of other monomers of glutaraldehyde condensation products already attached to the support. This would form a partial "coat" on the surface of the support. This small polymer of glutaraldehyde on the surface of the support could react with a further two or three

amino groups of the support. This would reduce the amount of protein which could be attached. In fact, using , instead of glutaraldehyde, the bifunctional coupling reagent, diethyladipimidate (Pinner, 1892), where it is known that a single bifunctional molecule reacts with one amino group on the support and with one amino group on the protein, then, under identical coupling conditions, five times more enzyme can be attached (D. L. Morris, unpublished results).

Another problem with glutaraldehyde is its change in structure on storage. New batches of glutaraldehyde were stored in 100ml quantities in the frozen state at -20°C to avoid spontaneous polymerisation of the glutaraldehyde. A 100ml quantity was thawed and stored at 4°C when required for laboratory use. Since, for most coupling experiments, only one or two ml were required, the glutaraldehyde used at the beginning of a 100ml batch might not have the same properties as that at the end of a batch.

7.2.3. Enzyme Immobilisation and Activity

Three dehydrogenases, YADH, LDH and MDH, were used in all immobilisation experiments. These enzymes were kept as lyophilised preparations

Table 19.

Enzyme	Derivative	% Soluble Enzyme Activity Retained
YADH	"200µg"	18
	"Reduced"	8
	"Unreduced"	13
	"5mg"	4
LDH	"2.5mg"	6
	"1.5mg"	4.5
	PEI-LDH (I)	3.6
	PEI-LDH (II)	3.6
	"Reduced"	6
	"Unreduced"	6
	Cellex-AE-PEI-LDH	5
MDH	"Reduced"	1
	"Unreduced"	1
	"3mg"	2

or as ammonium sulphate suspensions until required for coupling. The purification procedure on Sephadex G-25 (see Section 3.1.1.1) used for the ammonium sulphate suspensions of LDH was not used for purifying a similar suspension of MDH since it was subsequently found that the presence of ammonium sulphate in the coupling stage made virtually no difference to the activity of the immobilised enzyme obtained.

When an enzyme is immobilised on a support, generally speaking it will lose activity for the reasons mentioned in the General Discussion. It is necessary, therefore, to prepare immobilised derivatives which retain as much of the soluble enzyme activity as possible. Table 19 lists most of the preparations described in Chapters 4, 5 and 6 and shows the percentage retention of soluble enzyme activity by the immobilised preparations. The support specific activities of the MDH derivatives are much lower than those for LDH and for YADH. Summarising the results for the "unreduced" preparation of each of the enzymes, it is seen that the ratio of their activities is 13 : 6 : 1 for YADH, LDH and MDH respectively. If it is remembered that MDH is a dimer whereas LDH and YADH are tetramers, then, assuming each enzyme molecule

to be attached through a single subunit to the support and that this attached subunit becomes inactivated, there remains a three-fold excess of potentially reactive subunits for the latter two enzymes. However, the soluble enzyme activities are in the ratio 1 : 1.5 : 2.5 for YADH, LDH and MDH respectively. If the enzymes were immobilised through a single subunit as stated above, the immobilised derivatives of YADH, LDH and MDH (under identical experimental conditions for all three enzymes) would have activities in the ratio 0.75 : 1.12 : 1.25 respectively i.e. the immobilised MDH derivative would be the most reactive. In fact the ratio obtained was 13 : 6 : 1. This suggests that the MDH and LDH are being covalently bound to the support at amino acid residues which are near the active sites or are important to the structural integrity of the enzymes. Alternatively, LDH and MDH may bind through a higher proportion of their subunits than YADH. In the case of MDH, this could result in inactivity of a proportion of the attached molecules. Only towards the end of the coupling reaction, when few glutaraldehyde molecules remain, would MDH attach through only one subunit. If, however, as suggested also, the glutaraldehyde forms a "coat" on the support, this

Table 20.

Enzyme	Derivative	% Initial Protein Coupled	Activity (U/g)
YADH	"Reduced"	42	59
	"Unreduced"	43	106
LDH	"Reduced"	40	65
	"Unreduced"	40	64
MDH	"Reduced"	70	21
	"Unreduced"	73	27

would increase the probability of multiple subunit attachment.

In the early stages of the present work, when it was thought that glutaraldehyde formed Schiff's bases with both support and enzyme, reduction with sodium borohydride was used in an attempt to enhance the stability of the immobilised enzyme preparations; it is a primary objective that an enzyme should be more stable than the original soluble enzyme. Table 20 shows the activity of "unreduced" and "reduced" immobilised preparations of YADH, LDH and MDH. All the derivatives were prepared from coupling suspension containing 1mg enzyme. Whereas both YADH and LDH preparations coupled about 40% of the initial protein present, the MDH preparations coupled 70% although in the last case, the support specific activity was very much lower. This suggests that much of the MDH, on immobilisation, is inactive or has a very low activity. The effect of sodium borohydride reduction on the immobilised enzyme activities is most marked with YADH and least with LDH with MDH showing a slight effect. The drastic decrease with YADH has been explained by Wallenfels and Sund (unpublished result) (see Section 4.1.1). Sodium borohydride, however, must be modifying

groups in YADH which are important in catalytic activity since Wallenfels and Sund (1957) found that the activity of YADH preparations depended on the number of free sulphydryl groups but they also found that, on reduction of YADH with sodium borohydride, the number of sulphydryl groups increased and the enzyme turnover number decreased. The reducing agent may be reacting with disulphide bonds which are structurally or catalytically important. Sodium borohydride is a strong reducing agent. It might be worth testing some weaker reducing agents e.g. the ethoxy-substituted lithium aluminium hydrides, for a selective effect on the disulphide bonds. LDH, on the other hand, contains no disulphide bridges (Levi and Kaplan, 1971) and as would be expected, does not show any change in activity on reduction. This should also apply to MDH (Thorne and Kaplan, (1963) found no disulphide bridges). But the reduced derivative, in this case, is 30% less active than the unreduced derivative. This suggests that sodium borohydride may be modifying the protein in some other way.

One would expect that, if the number of reactive groups on a support were increased, an increase in the support specific activity would occur. Fig. 10 shows that a maximum support specific activity of 68U/g after 75min

coupling time was attained for immobilising YADH on "AE-cellulose" whereas YADH immobilised on Cellex-AE had a support specific activity of 100U/g after 75min coupling time. The latter did not reach a maximum activity until 180min at which time the support had a specific activity of 140U/g. This result was unexpected since, as can be seen from Table 1, "AE-cellulose" has three times the amino group content of Cellex-AE. The increased amount of glutaraldehyde covalently linked to "AE-cellulose" may well form multiple bonds with the protein, causing distortion and inactivation of the subunits. Datta et al. (1973) proposed that one of the reasons why lysozyme loses activity on coupling to diazotised polyacrylamide is that the presence of too many reactive groups on the polyacrylamide surface resulted on a denaturation of the attached lysozyme. More links also mean less flexibility of the enzyme on the support. The longer coupling time required for attachment of YADH to Cellex-AE could be due to the presence of fewer glutaraldehyde attachment points than on "AE-cellulose". Alternatively, with "AE-cellulose", the protein may attach quickly, but free glutaraldehyde molecules may then slowly react causing distortion and lowering the support specific

activity as suggested above. With fewer of these free glutaraldehyde molecules available in Cellex-AE, there will be less chance of this distortion occurring.

When the protein concentration in a coupling is increased then this results in an increase in support specific activity, as seen in Table 5 for YADH. 5mg YADH was the maximum amount of enzyme used in a coupling but a limit of activity would probably be reached owing to the nature of the bifunctional coupling reagent. As stated above, (Section 7.2.2), an increase in support specific activity occurred when YADH was coupled to DEAE-cellulose using modified cyanuric chloride as the bifunctional coupling reagent. In contrast to glutaraldehyde, this reagemt reacts with only one hydroxyl group on the support and one nucleophilic group on the enzyme; thus more enzyme can be attached, resulting in higher activities. The coupling of the modified cyanuric chloride to DEAE-cellulose has to be carried out in acetone-water at 50°C. Table 6 shows the variation of activity with coupling time. The lower activities obtained for the longer times are probably due to the hydrolysis of the remaining chloro groups on the triazine ring (see Fig. 1(b)), preventing attack of a nucleophile. A similar reason could hold for the decrease in support specific

activity when the coupling time of YADH to activated DEAE-cellulose was varied (Table 7).

When an enzyme is immobilised by covalent attachment to cellulose derivatives or nylon powder, it changes phase, and part of its loss in activity is probably due to its being in an unnatural environment. A derivative with a higher activity would be expected if the enzyme were immobilised on a support which kept it in the aqueous phase. For this purpose, PEI was chosen. This support has a large amino group content (see Table 1). As expected this support immobilises a large amount of protein and the resulting "soluble immobilised" enzyme is more active than the corresponding insolubilised derivatives (Table 12). Although high protein contents and activities were obtained, several disadvantages became apparent. One of these is in the Sephadex G-200 column procedure (Section 3.1.2) used to separate "bound" from "unbound" LDH. This step is carried out at room temperature and, whereas the activity loss for LDH is very small, that for YADH is greater. For this reason, immobilisation of YADH to PEI was not carried out. A factor determining the amount of protein immobilised was the ease with which the activated PEI precipitated, ... enzyme molecules probably acting as crosslinks. A protein concentration greater than

0.2mg LDH per ml coupling buffer resulted in precipitation of PEI-LDH complex which could not be redissolved on dilution with buffer.

The higher activities obtained by this "soluble immobilisation" could also be due to less distortion of the enzyme occuring since the LDH, on immobilisation, does not change phase. This would appear to be reasonable because, when PEI-LDH was immobilised on activated Cellex-AE, a low activity preparation was obtained. The loss in activity of the support, could however, be due to bonds being formed between this Cellex-AE and LDH as well as between Cellex-AE and PEI. This would hinder approach of the substrates towards the active sites. This view is supported by the finding that when LDH is attached to PEI-activated Cellex-AE, the activity of the derivative, now in a hydrophilic environment, is much higher (Table 12).

The amounts of enzyme which can be immobilised depends on a number of factors. One is the pH of the coupling solution. A pH must be chosen that will allow coupling of the enzyme to a support to proceed fairly rapidly but without undue denaturation of the enzyme during the coupling. Figs. 12, 37, and 58 show the effect of varying the pH of the coupling buffer upon the support specific activities obtained for the immobilisation of

YADH, LDH and MDH respectively on Cellex-AE. Maximum support specific activities were obtained at pH7.4, pH7.6 and pH8.4 for YADH, LDH and MDH respectively. The isoelectric points for the three enzymes are pH5.4 for YADH (Hayes and Velick, 1953), pH4.5 - 4.8 for LDH (Nielands, 1952) and pH6.1 - 6.4 for MDH (Wolfe and Nielands, 1956). It is seen that the coupling pH at which maximum activities for immobilised LDH and MDH is about two to three pH units on the alkaline side of their respective isoelectric points. The effect is less clear cut for YADH which shows a broad maximum.

Fig.11 shows the effect of varying the pH of the buffer used in activating Cellex-AE with glutaraldehyde; this shows a very broad maximum. Fig. 11 was derived from results using YADH as a test enzyme. Although it cannot be assumed that identical results would be obtained using LDH and MDH as test enzymes, nevertheless, activation of Cellex-AE was carried out at pH7.4 in subsequent couplings of all three enzymes.

Fig. 14 shows the effect of varying the time of the glutaraldehyde activation step. This was carried out only for YADH. The coupling time for optimum activity was so determined for the immobilisation of LDH and MDH. Fig. 14 shows a plateau after about 3min for YADH. The results presented in Table 17 show that this

assumption may not be justified for MDH. When the amount of enzyme in the coupling suspension was trebled, the support specific activity doubled, although the amount of protein coupled scarcely increased. This suggests that the duration of glutaraldehyde activation for MDH may be critical.

Similar arguments may hold for Figs. 10, 13 and 15 and Table 4 which show the variation of support specific activities with coupling time, glutaraldehyde concentration, enzyme concentration and ionic strength respectively, using YADH as the test enzyme. Although the conditions derived for YADH were used for both LDH and MDH it cannot be assumed that these enzymes would give identical results.

LDH was immobilised on NP/3 Nylon Powder and derivatives of this support. Low activities were obtained for these preparations for the reasons stated in Section 5.3. This, however, was not important. All that was required was that in each of the couplings the same initial protein concentration was the same, and all coupling conditions were the same. As the object of the experiment was to study the effect of modification of the activated NP/3 Nylon Powder on the immobilised enzyme, a high activity was not important. The most active preparation (Table 13) was that which, after protein had been coupled, was not subsequently treated

with N,N-dimethylaminopropylamine, γ-animobutyric acid or n-butylamine. The three derivatives which have these different compounds attached have all similar support specific activities. In all cases, some protein is covalently attached as the activities have all increased with respect to the blank coupling. As seen from Table 13, only 3% of the free enzyme activity was retained when LDH was immobilised in NP/3 Nylon Powder. This is a similar value to that obtained when LDH is immobilised on PEI but is half the activity retained when LDH is immobilised on Cellex-AE (see Table 19). The support activity that it being measured, however, is not due entirely to covalently bound protein but is also due to adsorbed protein. This causes two completely different reaction rates on the same nylon particle. As the total activity measured is the sum of two types of immobilised enzyme activity, kinetics become more complicated.

7.3. Stability

7.3.1. Coupling Conditions and Stability

When an enzyme is coupled to a macromolecular support, the pH of the solution and the time of coupling are critical in that the enzyme being coupled should be stable at the coupling pH during the time course of the coupling. Since most of the couplings

involving YADH, LDH and MDH were carried out for 90min or 120min, the stability at 4°C of the free enzymes was studied in various buffers over a pH range from 5 to 10.2 during a period of 2h. Generally, if the bonding involves nucleophilic groups, the more enzyme immobilised. Coupling can be carried out at pH9.0 for LDH (Fig. 38) and at pH10.2 for MDH (Fig. 60) without any loss of soluble enzyme activity. YADH, however, is a less stable enzyme and a compromise has to be found between its pH stability and the most suitable pH for immobilising it. Another consideration is the buffer used. For example, Tris buffer could not be used for coupling enzyme to glutaraldehyde-activated Cellex-AE, since the amino group on the Tris would compete with the enzyme for attachment to the glutaraldehyde.

7.3.2. Storage Conditions and Stability

Immobilised enzymes are generally stored as a wet suspension at 4°C. A buffer is chosen so that the immobilised derivative retains its original activity for as long as possible. Immobilised derivatives of YADH, LDH and MDH on Cellex-AE were studied in the pH range 5.4 - 9.4 over a period of five weeks. For both YADH and LDH, when the soluble enzyme is unstable,

then the immobilised enzyme is equally unstable; but where the soluble enzyme is stable, then the immobilised enzyme is slightly more stable. Anomalous results are obtained for LDH at pH5.4 and pH9.4 where the immobilised preparations are very much more stable.

This latter observation can be applied to autoanalytical circuits, in which a continuous supply of NADH is required. For instance, in the glutamic oxalacetic transaminase reaction, oxalacetate can be measured using NADH and MDH (J. Campbell, 1974). The equilibrium for the LDH reaction lies in the direction of NADH formation at pH9.4, and as immobilised LDH is quite stable at this pH, a column of Cellex-AE-LDH can be used as an NADH generator. The NADH produced is used to measure continuously the formation of oxalacetate. This technique is cost-saving since NADH costs twice as much as NAD^+. The NADH formed will also be purer as it has been prepared in situ.

MDH shows results similar to those obtained for YADH and LDH at pH values where the soluble enzyme is unstable. But at pH values pH7.4, pH8.4 and pH9.4, where the soluble enzyme is stable, the immobilised enzyme is less stable than the soluble enzyme. There would, therefore, appear to be no benefit to be derived from immobilising MDH on Cellex-AE with regard to its long

term stability at 4°C. A reason for this apparent difference between MDH and the other two enzymes could be, that in addition to the pH effect, there may be slow chemical reactions between unsubstituted glutaraldehyde residues and active protein subunits. It is assumed (Section 7.2.3) that the protein attaches to the support through one subunit. It is proposed here that the second subunit may also bind to the support when the conformation of the whole protein molecule is affected by pH. At pH5.4, there can be some Schiff's base formation (Roberts and Caserio, 1965). This effect would also mean that both subunits of MDH were covalently bound and inactivated whereas, with LDH and YADH, two of the subunits still remained unbound.

Summarising Figs. 17, 18, 40, 61 and 62: YADH immobilised on Cellex-AE would be best stored in phosphate buffer (pH7.5); LDH in phosphate buffer (pH6.4); and MDH in pyrophosphate buffer (pH9.4).

7.3.3. Borohydride Reduction and Stability

The effect of sodium borohydride reduction on the long term stability of YADH, LDH and MDH immobilised on Cellex-AE was studied. Figs. 19, 39 and 59 show the storage stability of the "reduced" derivative is not enhanced compared to the stability of the "unreduced" derivative. Differences in the stability of "reduced" and "unreduced" preparations

do appear at higher temperatures and these will be discussed later.

As stated above (Section 7.2.3) the purpose of the sodium borohydride treatment was to reduce any aldol condensation double bonds, and also to reduce any Schiff's bases. This should prevent any slow reactions between the immobilised enzyme and support. Stabilisation of any Schiff's bases formed would prevent distortion of the protein molecules with a further lowering of the specific activity of the enzyme-support. LDH immobilised on Cellex-AE gives the most stable derivative, retaining 65% of its original activity after 13 weeks, whereas YADH, under the same experimental coupling conditions, retains only 40% after 6 weeks and MDH only 50% after 4 weeks.

When YADH is immobilised on DEAE-Cellulose using modified cyanuric chloride as the bifunctional reagent, a preparation is obtained with a much higher support specific activity than YADH on Cellex-AE. But, as can be seen in Fig. 19(b), it is far less stable, even at $4^{o}C$. This is probably due to the presence of the heterocyclic ring. Cyanuric chloride was modified with ethanolamine to make it slightly hydrophilic, creating a more favourable environment for attachment of YADH. If 2-amino-4,6-dichloro-s-triazine (Kay and

Lilly, 1970) had been used as a coupling reagent instead of 2-ethanolamino-4,6-dichloro-s-triazine, the YADH derivatives would probably have lost activity more quickly, since the former compound is less hydrophilic.

7.3.4. Temperature and Stability

All the stabilities discussed so far have been for storage at 4°C. When immobilised enzymes are required to provide coenzymes for autoanalytical techniques, as previously mentioned (Section 7.3.2), the temperatures used are generally between 25°C and 37°C. The effect of temperature on the stability of all three soluble enzymes, on YADH and MDH on Cellex, and on LDH on Cellex and PEI, was studied. There have been reports on the comparative effect of temperature on the stability of soluble and immobilised enzymes, (von Specht et al., 1973; Epton et al., 1973; Mori et al., 1973; Barker et al., 1968). All of the enzymes were hydrolases and in all cases, the soluble enzyme was less stable than the immobilised derivative. This is not always true; trypsin, when immobilised on Cellex-AE, was found to be less stable at 40°C than the free enzyme in solution (D. L. Morris, unpublished results). LDH immobilised on PEI was found to be less stable than the soluble enzyme at 45°C and 50°C (See below). Improved stability is important

in aiding the recovery and the re-usability of the immobilised preparations. Increased stability is also important in compensating for activity losses during coupling.

Figs. 35, 56 and 67 show the loss of activity of soluble and immobilised derivatives of YADH, LDH and MDH at 40°C and 45°C; the results at 50°C are presented in Figs. 36, 57 and 68. With two exceptions, soluble LDH is less stable than any of its immobilised derivatives at 45°C and 50°C. The "2.5mg" derivative, however, shows a heat inactivation pattern very similar to that for LDH. The PEI derivative is less stable than the soluble enzyme. As expected, both the soluble enzymes and their derivatives are inactivated far more quickly at the higher temperature.

The effect of immobilisation on stabilising the enzyme is most marked with MDH. This is the opposite effect from storage at 4°C (Section 7.3.2). There is evidence that, in the cell, MDH is bound in some way to the mitochondrial membrane which stabilises it against inactivation at 37°C. Callahan and Kosicki (1967) have found MDH to be intimately associated with a lipid which is complementary to the tertiary structure of the enzyme.

The effect of sodium borohydride reduction on the stability of the immobilised enzymes is most marked at 50°C for both the "1.5mg" derivative of LDH and the "reduced" derivative of YADH. For immobilised MDH, however, it does not have much effect. This difference could be due to the tetrameric structure of the first two enzymes whereas the MDH is a dimer. Reduction may place a constraint on the freedom of movement of the enzyme attached to the support. For YADH and LDH, there would probably still be two or three subunits unattached to the support, but with MDH, the enzyme might require freedom of movement of one subunit for activity. The increased inactivation for the "unreduced" derivatives may be due to promotion of increased bond formation by the higher temperatures. This would cause further distortion of the proteins and loss of activity.

The one immobilised preparation that is less stable than the soluble enzyme is the PEI derivative of LDH. This decreased stability could be due to attachment of the LDH to PEI though all four subunits. When such a derivative is heated, the PEI structure would change shape and the bound LDH would lose activity more quickly than the soluble enzyme. This change of shape is decreased when the PEI, with or without LDH attached, is immobilised on Cellex-AE (Figs. 56 and 57).

Saito (1972) proposed the model of the "active dimer" for LDH i.e. two protomers act in cooperation as an active dimer in a tetramer of LDH. Both protomers must be active for the dimer to be active. The lower activity of the PEI-LDH preparations might be explained. If LDH is bound to Cellex-AE through one or two of its subunits, it still has three or two subunits which can be dissociate. Two active protomers from separate LDH molecules could then recombine to form an active dimer which would reassociate with subunits immobilised on the support giving active immobilised LDH. If, however, LDH immobilised on PEI is largely crosslinked, there would be fewer protomers available for dissociation and reassociation as active dimers.

This might also explain the increased stability of LDH immobilised on Cellex-AE and the characteristic heat inactivation profiles. If the enzyme is immobilised by two subunits, both of which are active but have a much decreased activity, the other two remaining protomers would be capable of dissociation and reassociation as suggested above. Loss of activity would be at the same rate as with soluble enzyme protomers. The "active dimer" attached to the support will retain its activity; this would account for the plateau of the heat inactivation profile.

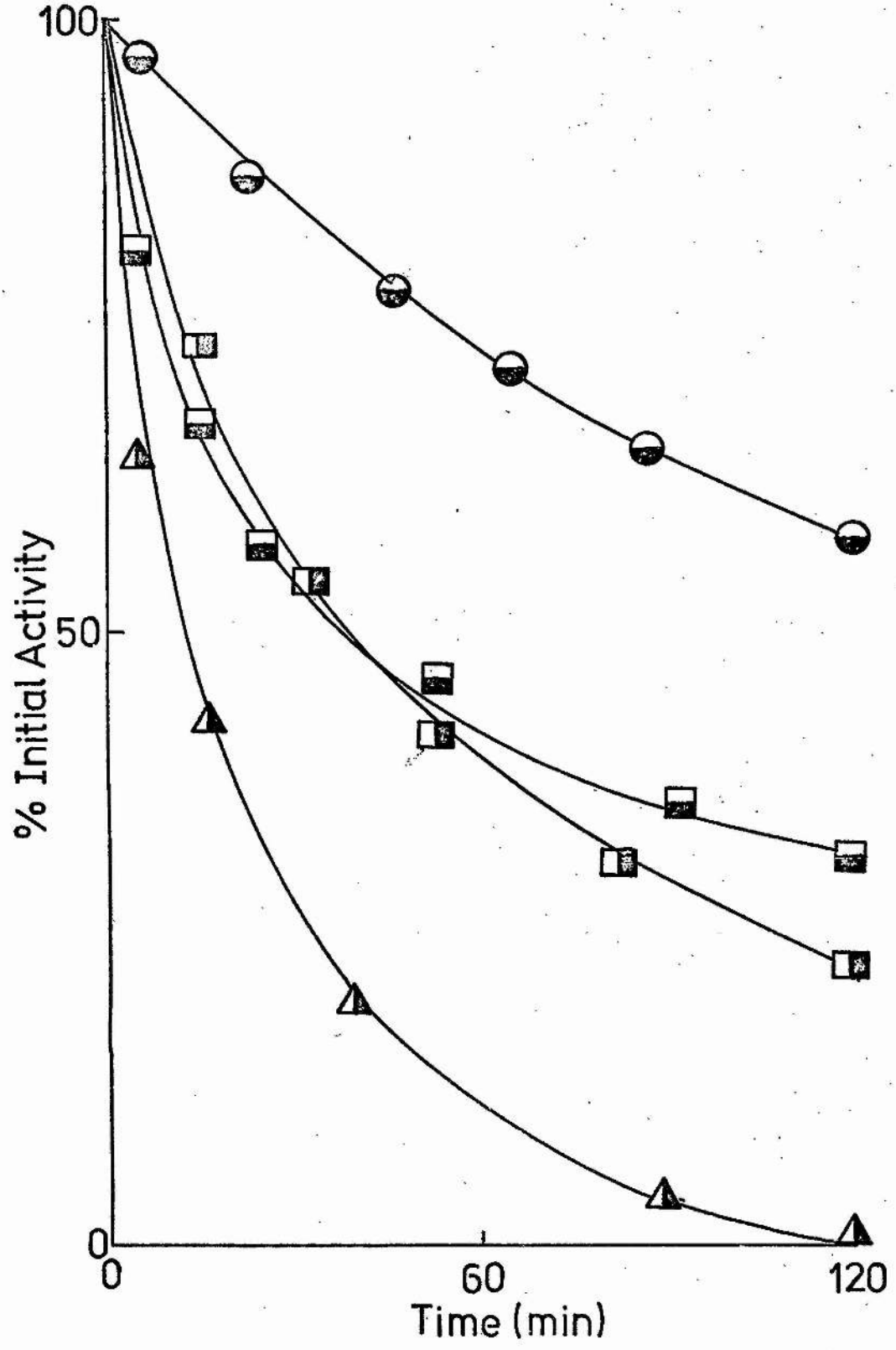
The amount of enzyme that is present in the initial coupling, or that has been attached, does not determine how quickly the immobilised preparation will lose activity. The heat inactivation profiles vary with the enzyme. For instance, the "3mg" MDH preparation is the most stable at 50°C (Fig. 68). The results for the "200µg" and "5mg" YADH preparations (Fig. 35) illustrate that the use of a larger amount of protein does not necessarily result in the attachment of the enzyme in a more active conformation. Obviously each enzyme has its own inactivation properties.

One of the problems in studying heat inactivations is that the value of K_m' and hence V_{max}' may change as the immobilised enzyme loses activity; the curve obtained may not be the true heat inactivation profile. Michaelis parameters would have to be worked out on samples from different time intervals on the heat inactivation profile.

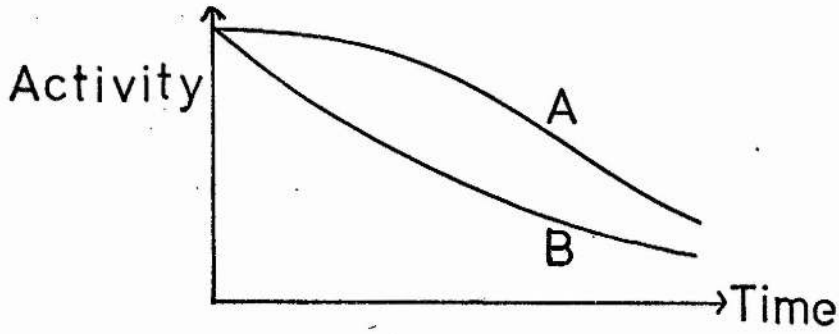
When these heat inactivation studies were carried out, an attempt was made to assay both the soluble and immobilised enzymes under substrate saturating conditions. With the majority of the immobilised preparations of LDH and MDH, as the concentration of NADH present was only about twice K_m', activity changes were not being measured under saturating conditions.

Fig. 70.

Effect of incubation at 45°C (▭) and at 50°C (△) on the stability of soluble LDH; also the effect of incubation at 45°C (⊖) and at 50°C (□) on the stability of Cellex-AE-LDH. Unfilled symbols represent the activities obtained by assaying in the reverse direction (see Section 2.12.2); filled symbols represent the activities obtained by assaying in the forward direction. The assay in the forward direction was as described in Section 2.12.2, but with lactate (3.5M) replacing the stock pyruvate solution and NAD^+ (84mM) replacing the stock NADH solution.


100
50
0
% Initial Activity
0
60
120
Time (min)

If, as is the case with soluble LDH, the reaction in the reverse direction is very fast and in the forward direction relatively slow, and a large amount of LDH is coupled to the support, the influence of diffusion on the reaction rate and on the heat inactivation profile should be detectable.



If the reverse reaction is faster than the rate of diffusion, then no loss in support specific activity will be seen until the rate of the enzyme reaction is less than the rate of diffusion (Curve A). Conversely, for the forward reaction, which is slower than the rate of diffusion, then the loss in activity will be seen immediately (Curve B).

There was no difference in the rate of loss of activity, both at 45°C and 50°C between the forward and reverse directions when LDH was immobilised on Cellex-AE using glutaraldehyde as the coupling agent (Fig. 70).

7.4. Kinetics

In the past few years a wealth of information has accumulated concerning the association of soluble enzymes with solid matrices. A major difficulty, that has hindered progress in the understanding of immobilised enzymes, has been the lack of precise information concerning the types and concentrations of catalytically active species in the immobilised molecule. The changes in physical and chemical properties of the enzyme after immobilisation are complicated by the heterogeneity of the system. Changes in the kinetic parameters of enzymes after immobilisation are due to conformational changes in the enzyme structure (Gabel et al., 1971), changes in reaction environment (Goldstein et al., 1964; Gestrelius et al., 1973), specific interactions between the substrate and the matrix and between protons and the matrix (Hornby et al., 1966), the effect of intrapore diffusion for porous solids (Rovito and Kittrell, 1973; Weibel and Bright, 1971) and finally, attachment of enzyme to a matrix pore inaccessible to macrosubstrates.

For kinetic studies carried out on the three dehydrogenases, the last factor mentioned above would not be important since all substrates and

coenzymes are small. The effect of an unstirred layer is probably also negligable since an increase in the stirring rate has no effect on the activity of an immobilised enzyme. Diffusional effects probably alter the Michaelis parameters of immobilised enzymes. Two types of diffusion are possible: the first is film diffusion of the substrate from the bulk solution to the support surface; the second is internal diffusion of the substrate through the catalyst and chemical reaction at the active sites. Viscosity may also be important in diffusional effects and, for the three enzymes studied, is most likely to have an effect on the Michaelis constants for NAD^+ for both immobilised YADH and LDH since the second substrates, ethanol and lactate respectively, are present in high concentrations. It is not very meaningful to characterise an immobilised enzyme by measuring its K_m' and V_{max}' under a single set of conditions; therefore two types of experiments were carried out. The first consisted of immobilising YADH on DEAE-cellulose and LDH on NP/3 Nylon Powder and studying the variation of K_m' and V_{max}' with pH. The results were compared with those obtained for the soluble enzyme. The second type of experiment involved measuring the Michaelis parameters at pH7.4 for different amounts of YADH, LDH and MDH immobilised on Cellex-AE. Comparisons were made with the soluble

enzymes. The effect of sodium borohydride reduction on immobilised derivatives of each of the three enzymes was also studied. The results obtained for YADH, LDH and MDH immobilised on Cellex-AE are summarised in Tables 9, 14 and 18 respectively. These results were obtained from double reciprocal plots which were linear in all cases except for NAD^+ and ethanol for the "200µg" preparation where they were concave towards the x-axis. In this case, the values of K_m' and V_{max}' are approximations. Table 9, which presents the data for YADH, shows that, for all the substrates except acetaldehyde, the K_m' values increase on immobilisation. Ethanol has a K_m' of 22mM for soluble YADH at pH7.4, i.e. an ethanol concentration of 2mM (one tenth of K_m') is still a saturating concentration of substrate relative to the values of K_m' for the other substrate and the coenzyme. It is not understood why the K_m' value for ethanol has increased on immobilisation of the enzyme. Lactate (see Table 14) also has a K_m' in units of millimolar for soluble LDH, but immobilisation of LDH on Cellex-AE or PEI causes no notable change in K_m'. For all YADH preparations the K_m' values for NAD^+ increase on immobilisation. This is probably due to a change in the local microenvironment of the enzyme when it is immobilised to the support. The increase in the values of K_m' for

NADH could be explained similarily, but the diffusion could also have an effect since the K_m' for NADH is low compared to that for NAD^+ and ethanol.

K_m' values for acetaldehyde for all preparations of immobilised YADH are decreased compared to the soluble enzyme except for the "unreduced" derivative which has a K_m' value similar to the soluble enzyme. Sodium borohydride treatment of the immobilised YADH preparations lowers the values of K_m' of all four substrates relative to the values obtained for the "unreduced" derivative. This suggests that the reduction procedure is chemically modifying part of a subunit of YADH which is involved in substrate or coenzyme binding. Sodium borohydride reduction has a more marked effect on the kinetic parameters of an immobilised enzyme preparation, than increasing the protein content of the support.

Table 14 presents the Michaelis parameters for LDH immobilised on Cellex-AE and PEI; the K_m' values differ only slightly. The K_m' values for PEI-LDH immobilised on Cellex-AE are, however, anomalous. The K_m' value for lactate does not change on immobilisation of LDH. The K_m' values for NAD^+ and NADH increase on immobilisation of LDH. This is to be expected, particularily for NADH, since it has a very low K_m'

for the soluble enzyme and will be affected by intrapore diffusion. The effect of solubility state of support is shown by the variation of K_m' for NADH. Whereas the K_m' value has increased ten-fold for the "1.5mg" preparation, it has only increased six-fold for LDH immobilised on PEI (Preparation I), and only two-fold for Preparation II. This is because the PEI is water-soluble and in the same phase as the coenzyme. The K_m' values for NAD^+ all increase on immobilisation except that for PEI-LDH (Preparation I), which has a value very close to that of the soluble enzyme. Although the K_m' values for pyruvate and for NAD^+ are similar for the soluble enzyme, the K_m' for pyruvate remains the same on immobilisation whereas the K_m' for NAD^+ increases on immobilisation. An explanation could be that the binding site for the coenzyme has been affected by a change in enzyme conformation on immobilisation whereas the binding site for pyruvate has been unaffected. Macpherson (1970) found that diffusion of NAD^+ to the enzyme caused a modification of the quaternary structure. If this modification was restricted as a result of immobilisation, this could also account for the increase in the K_m' value for NAD^+.

Sodium borohydride reduction appears to have little effect on the Michaelis parameters; the K_m' for NADH

for the "1.5mg" derivative (which is reduced) is half that for the unreduced "2.5mg" derivative. This could also be due to the different protein concentration on the support.

The K_m' values for MDH and its derivatives are presented in Table 18. No relation between K_m' values amd protein concentration on the support is obvious. There is, however, a difference between the K_m' values for the "reduced" and the "unreduced" derivatives. The values for the "reduced" derivative are quite similar to those for the soluble enzyme whereas the values for the "unreduced" and "3mg" derivatives are higher but are themselves similar. If modification of the protein conformation occurs during coupling, this modification may be lessened or removed by treatment with sodium borohydride. The overall picture derived from the results is that glutaraldehyde does not modify amino acid residues near the active centre.

Tables 9, 14 and 18 also present the value of V_{max}' for the derivatives studied. With one exception the values for the immobilised derivatives of all three enzymes are decreased by a factor of at least 10^3. When LDH is immobilised on PEI, however, V_{max}' is decreased by a factor of only 10 for both preparations.

Table 21.

Enzyme	Derivative	Relative Forward Rate	Relative Reverse Rate
YADH	Soluble	1	1.8
	"200µg"	1	10.7
	"Reduced"	1	19
	"Unreduced"	1	15
	"5mg"	1	9
LDH	Soluble	1	4.7
	"2.5mg"	1	4.5
	"1.5mg"	1	3.8
	PEI-LDH (I)	1	2.7
	PEI-LDH (II)	1	2.5
	AEC-PEI-LDH [1]	1	5.8
	AEC-(PEI-LDH) [1]	1	5.5
MDH	Soluble	1	6.6
	"Reduced"	1	7.0
	"Unreduced"	1	7.0
	"3mg"	1	6.5

[1] AEC : Cellex-AE.

In general, as the protein content of the support is increased, the V_{max}' values for immobilised YADH increase as do the values for the "2.5mg" and "1.5mg" derivatives of LDH. MDH, on the other hand, shows very little change in V_{max}' when the protein content of the support is increased. Both YADH and MDH "reduced" derivatives show decreased values of V_{max}' compared to the corresponding "unreduced" derivatives. Again this is evidence that sodium borohydride has affected the YADH and MDH conformations.

Table 21 compares the relative forward rates to the relative reverse rates of the three enzymes and their derivatives presented in Tables 9, 14 and 18. The forward rate is always taken as in the direction of NAD^+ reduction and the reverse rate as in the direction of NAD^+ formation. At pH7.4, the reverse rate is faster for all three enzymes. Immobilisation of LDH and MDH on Cellex-AE causes very little change in the ratio of the rates of the forward and the reverse directions. Immobilisation of LDH on PEI, however, halves the ratio; this again suggests a different method of attachment. The relative rates of the forward and reverse directions of YADH and its derivatives show large differences. The ratio of the forward to the reverse direction rates has increased for all the

derivatives, compared to the soluble enzyme. The results do not show any relation to the protein content of the support. The values of V_{max}' obtained for the forward direction of the "200µg" preparation are estimates because the double reciprocal plots were curved.

The conclusion derived from the data in Table 21 is that the coupling time of YADH to Cellex-AE enhances the oxidation of NADH and lessens the turnover of YADH in the direction of NADH formation whereas the coupling of MDH and LDH to Cellex-AE does not appear to affect the rates of the forward and reverse directions.

Consider the reversible enzyme-catalysed reaction

$$A + B \rightleftharpoons C + D$$

where the formation of C and D is much slower than the formation of A and B. Any diffusion in the system will have more effect on the formation of A and B, i.e. the faster reaction, than on the formation of C and D, i.e. the slower reaction.

Figs. 24, 25, 26, 27 and 28 show the variation of K_m' and V_{max}' with pH for soluble YADH and for YADH attached to DEAE-cellulose by a triazine link. It is believed that these are the first reported results for

the variation of Michaelis parameters with pH. It had been hoped that YADH could be attached to a variety of celluloses with different net charges, as was done by Kay and Lilly (1970) for chymotrypsin, using the triazine link, but the activities of YADH attached to cellulose and CM-cellulose were too low to proceed with this study. The K_m' and V_{max}' values for DEAE-cellulose-YADH can, therefore, be compared only with the soluble enzyme. Figs. 24 and 26 show that the values of K_m' for NAD^+ and NADH respectively both have minima between pH7.0 and pH8.0. The variation with pH in the shape of the curves could be due to dissociable groups on the enzyme surface. Bühner and Sund (1969), Harris (1964) and Jörnvall (1973) have all shown the presence of a cysteine residue at the active site. The sulphydryl group on cysteine has a pK of 8.3 when it exists free in solution. Although its pK will probably have changed when it is incorporated into the YADH molecule, it could be involved in the binding of coenzyme. The imidazole group of free histidine has a pK of 6.0. Histidine has been tentatively proposed as the amino acid adjacent to cysteine in the polypeptide chain of YADH (Jörnvall, 1973). Change in protonation of these two amino acid residues with pH could be responsible for the variation of K_m' for NAD^+ and NADH.

The K_m' for ethanol decreases with increasing pH and that for acetaldehyde is fairly constant until alkaline pH is reached where it increases rapidly. Both effects could also be explained by the properties of amino acid residues at the active site. If acetaldehyde reduction requires a protonated residue at the active site, at alkaline pH values, a higher concentration of acetaldehyde will be required to maintain the protonation. The opposite effect could explain the large increase in K_m' for ethanol at lower pH values.

The K_m' - pH relationship for these two substrates for YADH attached to DEAE-cellulose is very similar to that for the soluble enzyme. The following factors could be involved. Immobilisation of YADH on this support through the triazine coupling reagent may have no effect on the active site; and the presence of a positive charge on the support creates a microenvironment which minimised changes in K_m' values.

Table 8 shows the variation of V_{max}' with pH for both soluble and DEAE-immobilised YADH and also the ratio of the forward to the reverse directions. The rate of the reverse direction when YADH is immobilised on DEAE is higher than for the soluble enzyme. Fig. 28 shows

that V_{max}' for both the soluble enzyme and DEAE-cellulose-YADH decrease proportionately with pH changes. This implies that, although the V_{max}' for YADH has decreased by a factor of 10^3 on immobilisation, the active site has not been greatly modified. Any modification which has occurred has only enhanced the reverse rate relative to the forward rate.

As it had not been possible to attach YADH to celluloses with different charges, NP/3 Nylon Powder was used to couple LDH before reacting with a bifunctional molecule, one end of which was an amino group capable of reaction with the glutaraldehyde-activated nylon while the other end possessed a positive, negative, or neutral charge. These derivatives were examined over a pH range and the Michaelis parameters were compared with those for the soluble enzyme over the same pH range. At some pH values, however, the double reciprocal plots were curved. As NP/3 Nylon Powder was found to adsorb LDH physically as well as bind it covalently, there are, in general, two enzymes catalysing, with different reaction velocities, the same reaction. Both enzymes will influence the overall Michaelis parameters.

The initial reaction velocity (v) of an enzyme-catalysed

reaction depends on the substrate concentration (s) and is related to it by the Michaelis-Menten equation

$$v = \frac{Vs}{s + K_m}$$

where V is the maximum velocity under substrate saturating conditions and K_m is the substrate concentration required to give half the maximum reaction velocity.

Now, on a support, there are two enzymes, both catalysing the same reaction but which have been attached differently. Let these two enzymes (E_A and E_B) have Michaelis parameters K_A and K_B and V_A and V_B respectively. Therefore, for E_A

$$v_A = \frac{V_A s}{s + K_A}$$

and for E_B

$$v_B = \frac{V_B s}{s + K_B}$$

As both enzymes contribute towards the velocity measured, the total rate is

$$v_A + v_B = \frac{V_A s}{s + K_A} + \frac{V_B s}{s + K_B}$$

$$v_{A+B} = \frac{V_A s(s + K_B) + V_B s(s + K_A)}{(s + K_A)(s + K_B)}$$

$$\frac{1}{v_{A+B}} = \frac{s^2 + s(K_A + K_B) + K_A K_B}{s^2(V_A + V_B) + s(V_A K_B + V_B K_A)}$$

$$= \frac{1 + \frac{(K_A + K_B)}{s} + \frac{K_A K_B}{s^2}}{(V_A + V_B) + \frac{(V_A K_B + V_B K_A)}{s}}$$

This is of the form

$$y = \frac{1 + ax + bx^2}{d + cx}$$

where $y = \frac{1}{v_{A+B}}$, $x = \frac{1}{s}$, $a = (K_A + K_B)$, $b = K_A K_B$,

$c = (V_A K_B + V_B K_A)$ and $d = V_A + V_B$.

As K_A, K_B, V_A and V_B have positive values, a, b, c and d are positive. This equation describes a curve and the plots are concave downwards. It is relevant for a monosubstrate enzyme attached both physically and covalently to a support. This is a simpler solution than would be derived for a bisubstrate enzyme such as LDH, but it illustrates that, where an enzyme preparation has more than one K_m value, then there is a likelihood of curved double reciprocal plots.

Figs, 48 and 49 show the pH variation of the K_m' of lactate and NAD^+ for the soluble enzyme. Neither substrate shows very much variation of K_m' with pH except a slight increase towards alkaline pH values. Lactate would not be expected to show much change in K_m' as even at one-tenth K_m' the concentration of lactate present is 1.0mM. The binding of both substrates is relatively independent of pH over the pH range studied, a result similar to that obtained by Winer and Schwert (1963) for beef heart lactate dehydrogenase.

Figs. 46 and 47 show the pH variation of K_m' for pyruvate and NADH for the soluble enzyme. As proposed for YADH, there may be on the enzyme surface near the active site, dissociable groups which are required to be protonated on unprotonated depending on the substrate. It is known that there is a compulsory order for binding of substrate and coenzyme (Zewe and Fromm, 1962, 1965), the coenzymes binding before the substrate. Coenzyme binding causes a conformational shift and this may expose an amino acid residue, involved in binding the pyruvate, to the pH of the surrounding buffer. If this amino acid residue has a dissociable proton, an increase in pH would result in removal of this proton. If, however, the binding of

Table 22.

pH	Relative Forward Rate	Relative Reverse Rate
6.0	1	13.2
6.4	1	11.0
7.0	1	6.45
7.4	1	5.13
7.8	1	3.46
8.4	1	1.64
9.0	1	1.14

pyruvate requires a protonated amino acid, the increase in K_m' would be due to the higher concentration of pyruvate required to create the appropriate microenvironment on the surface of the LDH.

Fig. 50 shows the variation of V_{max}' with pH for soluble LDH. The curves have similar shapes to those of Winer and Schwert (1963). The rate of the forward direction is very much slower than that of the reverse direction (Table 22).

Figs. 52 and 53 illustrate the pH variation of K_m' for NADH and pyruvate for both the soluble enzyme and for LDH attached to NP/3 Nylon Powder. For pyruvate, the K_m' values show little difference between the soluble and immobilised enzymes. For NADH, however, immobilisation results in large changes. Except at alkaline pH, the K_m' value is increased at least ten-fold. For all the immobilised derivatives, the K_m' for NADH is in the region of 80μM. The very large increase is undoubtedly due to the diffusion effects.

Fig. 51 compares the values of V_{max}' for the NP/3 Nylon Powder derivatives and soluble LDH. All the curves show two peaks but only preparation I shows a similarity to soluble LDH in that it has a peak at pH7.4. The other three preparations have peaks at pH7.0.

Table 23.

pH	V_{max}' (sol) [1]	V_{max}' (Prep. I) [1]
6.0	362	0.0156
6.4	395	0.0198
7.0	535	0.0130
7.4	589	0.0166
7.8	523	0.0159
8.4	303	0.0124

[1] V_{max}' : units of micromoles NADH oxidised per ml per min per mg (enzyme or enzyme-support).

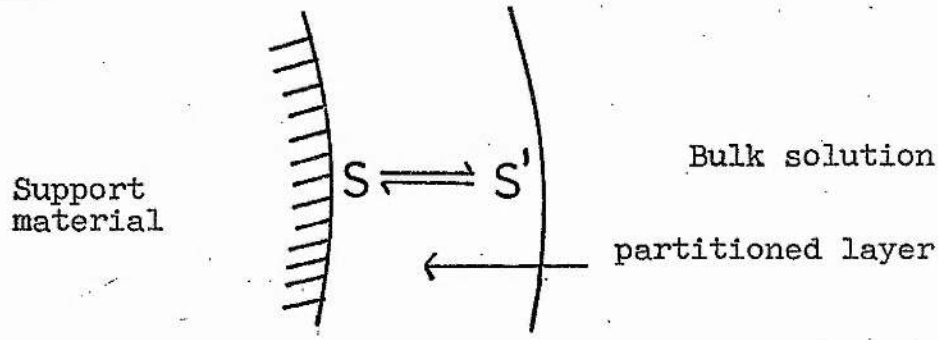
Table 23 presents the values of V_{max}' for soluble LDH and preparation I (as representative of the nylon-immobilised derivatives). It shows that V_{max}' has decreased by a factor of 10^4 compared to a factor of 10^3 for LDH immobilised on Cellex-AE.

The differences in the Michaelis parameters for the three hydrogenases are probably due to changes in comformation, diffusion, partitioning and,, in the case of YADH attached to DEAE-cellulose and of LDH attached to NP/3 Nylon Powder, also to local electrostatic effects arising from the positive charge on DEAE-cellulose and to the slightly hydrophobic nature of the nylon.

A better understanding of the alterations in the Michaelis parameters on immobilisation will require a detailed knowledge of enzyme structure and conformation.

As stated above, partitioning of the substrate can also occur. This means that the substrate concentration at the immobilised enzyme surface will be different from that in bulk solution. This is more likely to occur if the substrate and support are electrically charged (Goldstein et al., 1964). But the charge on the support, as well as attracting or repelling a

charged substrate, can also attract or repel protons changing the pH at the enzyme surface from that of the bulk solution. This would explain the pH optimum shifts noted for some immobilised enzymes (Gestrelius et al., 1973). Partitioning of the substrate may also occur if the support is hydrophobic and the substrate hydrophilic.



If S and S' are the substrate concentrations at the support and in the bulk solution respectively,

$$P \text{ (Partition Coefficient)} = \frac{S'}{S}$$

For an enzyme with one substrate (the bisubstrate enzyme is analogous)

$$v = \frac{Vs}{S + K_m}$$

where v is the rate of the reaction, S is the substrate concentration at the support, V is the maximum reaction velocity and K_m the true Michaelis constant.

Substituting for S

$$v = \frac{V.\frac{S'}{P}}{K_m + \frac{S'}{P}}$$

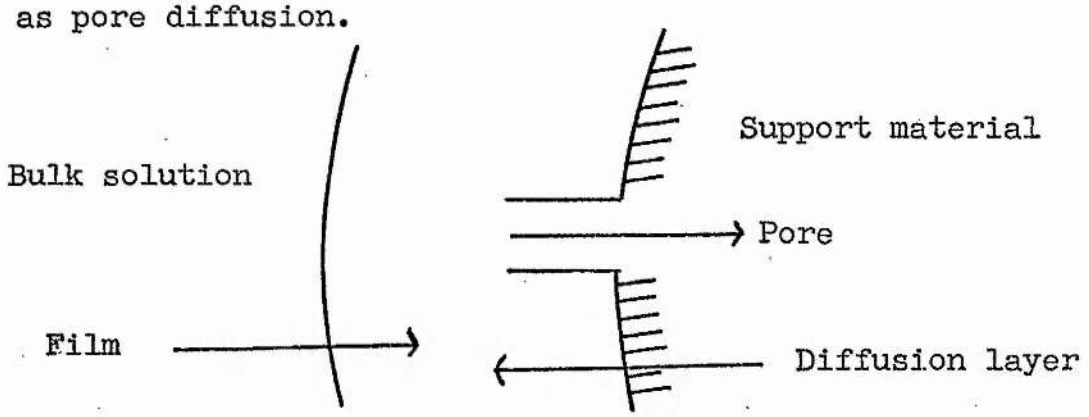
$$= \frac{VS'}{PK_m + S'}$$

This means that the measured Michaelis constant is PK_m and varies with the value of P.

In homogenous solution, there appears to be no diffusion limitation, even in the fastest enzyme reactions. But when the enzyme is immobilised and the substrate has to diffuse through the support, there are indications that diffusion could only be neglected for very slow enzyme reactions. Differences in the Michaelis parameters of LDH immobilised on cellulose have been attributed to diffusional effects (Wilson et al., 1968). Several quantitive treatments have appeared in the literature but these dealt only with monosubstrate enzymes (Laidler and Bunting, 1973).

Two different types of diffusion have been reported by Rovito and Kittrell (1973). The first of these is film diffusion and arises when the substrate molecules diffuse from the bulk solution to the support material. If, however, the support material contains pores, the substrate must diffuse through

these pores to the enzyme surface. This is known as pore diffusion.



The changes in K_m' were most marked for NADH and the increase is probably due to both partitioning and diffusional effects. Further understanding of this problem requires a fuller knowledge of enzyme structure and conformation and the effects on them of the immobilisation process.

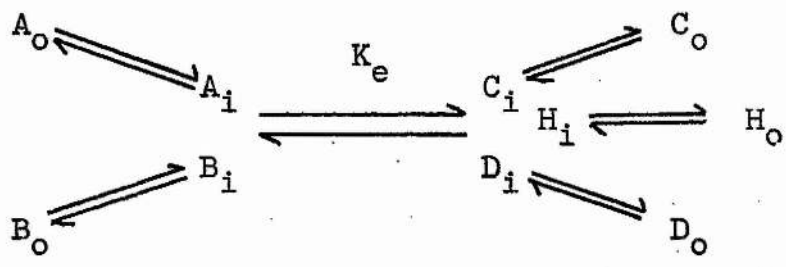
7.5. Equilibrium Studies

Equilibrium constants for soluble YADH and LDH and their immobilised derivatives were determined at 25°C by the method of Hakala et al.(1956). Tables 11 and 15 list the values obtained, and, without exception, the immobilised enzymes have lower equilibrium constants than those of the soluble enzymes. The effect of immobilisation of an enzyme on the equilibrium constant has not been previously reported.

In addition to the equilibrium constant, the K_m' values for the substrates and coenzymes of these two enzymes also change. A theory is developed, involving partition coefficients, relating the change in equilibrium constants on immobilisation to the change in K_m' values.

For a bisubstrate enzyme, such as YADH or LDH, immobilised on a macromolecular support, an equilibrium of all four substrates at any pH is eventually reached.

Schematically



where A., B., C. and D. are the four substrates; H is the hydrogen ion concentration; the subscripts 'o' and 'i' relate to the concentrations in the bulk solution and at the enzyme surface respectively; K_e is the equilibrium constant relating A_i, B_i, C_i, D_i and H_i i.e. the equilibrium constant of the soluble enzyme.

K_e' is defined as the measured equilibrium constant i.e. that determined from the equilibrium concentrations of A_o, B_o, C_o, D_o and H_o.

$$K_e' = \frac{C_o.D_o.H_o}{A_o.B_o} \qquad \text{--------(1)}$$

and

$$K_e = \frac{C_i.D_i.H_i}{A_i.B_i} \qquad \text{--------(2)}$$

The concentrations of substrates in the bulk solution and at the enzyme surface are related by partition coefficients.

$$P_A = \frac{A_i}{A_o} \qquad P_B = \frac{B_i}{B_o}$$

$$P_C = \frac{C_i}{C_o} \qquad P_D = \frac{D_i}{D_o} \qquad P_H = \frac{H_i}{H_o}$$

From equations (1) and (2)

$$\frac{K_e'}{K_e} = \frac{\frac{A_i}{A_o} \cdot \frac{B_i}{B_o}}{\frac{C_i}{C_o} \cdot \frac{D_i}{D_o} \cdot \frac{H_i}{H_o}}$$

$$= \frac{P_A \cdot P_B}{P_C \cdot P_D} \cdot \frac{1}{P_H} \qquad \text{----------(3)}$$

The partition coefficient (P) is related to the apparent Michaelis constant of the immobilised enzyme (K') and the soluble enzyme Michaelis constant (K) (Laidler and Bunting, 1973) by the equation

$$P = \frac{K}{K'}$$

Substituting in (3)

$$\frac{K_e'}{K_e} = \frac{K_A K_B K_C' K_D'}{K_A' K_B' K_C K_D} \cdot \frac{1}{P_H'} \qquad \text{----------(4)}$$

YADH and LDH were immobilised on Cellex-AE or PEI. The ionic charge on both supports is very small and the buffer used of sufficient ionic strength (I0.1)

Table 24.

Derivative	K_e [1]	$\frac{K_A}{K_A'}$	$\frac{K_B}{K_B'}$	$\frac{K_C'}{K_C}$	$\frac{K_D'}{K_D}$	K_e' [1]
"Reduced"	1.105	0.391	0.242	2.857	0.476	0.142
"Unreduced"	1.105	0.286	0.169	2.857	1.020	0.155
"5mg"	1.105	0.352	0.352	2.381	0.340	0.110

[1] Values of K_e and K_e' are multiplied by 10^{11}.

Table 25.

Derivative	K_e [2]	$\frac{K_A}{K_A'}$	$\frac{K_B}{K_B'}$	$\frac{K_C'}{K_C}$	$\frac{K_D'}{K_D}$	K_e' [2]
"2.5mg"	1.220	0.373	0.901	24.00	0.840	8.265
"1.5mg"	1.220	0.328	1.042	10.99	0.800	3.665
PEI-LDH (I)	1.220	0.901	1.242	6.667	1.205	10.960
PEI-LDH (II)	1.220	0.417	1.234	1.869	1.237	1.450
AEC-(PEI-LDH)[1]	1.220	0.427	0.794	5.848	0.340	0.822
AEC-PEI-LDH[1]	1.220	0.746	1.190	10.99	1.075	12.790

[1] AEC : Cellex-AE

[2] Values of K_e and K_e' are multiplied by 10^{12}.

Table 26.

Enzyme	Derivative	Theoretically determined Equilibrium Constant	Experimentally determined Equilibrium Constant
YADH [1]	Soluble	1.105	1.105
	"Reduced"	0.142	0.693
	"Unreduced"	0.155	0.780
	"5mg"	0.110	0.800
LDH [2]	Soluble	1.220	1.220
	"2.5"	8.265	0.956
	"1.5"	3.665	0.820
	PEI-LDH (I)	10.96	0.653
	PEI-LDH (II)	1.45	0.930
	AEC-PEI-LDH [3]	12.79	0.600
	AEC-(PEI-LDH) [3]	0.822	0.147

[1] Values multiplied by 10^{11}

[2] Values multiplies by 10^{12}

[3] AEC is Cellex-AE

to mask any charge effect of the support. As an approximation, the partitioning of the hydrogen ion can be ignored.

Using this approximation, for the soluble enzyme

$$K_e' = K_e \qquad \text{----------(5)}$$

For the immobilised enzyme

$$K_e' = K_e \cdot \frac{K_A}{K_A'} \cdot \frac{K_B}{K_B'} \cdot \frac{K_C'}{K_C} \cdot \frac{K_D'}{K_D} \text{------(6)}$$

All the variables on the right hand side of equation (6) can be determined experimentally and the value of K_e' calculated.

Tables 24 and 25 show the theoretical values for K_e' calculated for YADH immobilised on Cellex-AE and for LDH immobilised on Cellex-AE or PEI. Table 26 compares these theoretical K_e' values with the experimental values listed in Tables 11 and 15.

Equation (6) predicts the value of the equilibrium constant will vary with the change in K_m' values for the four substrates. For YADH, the values of the experimentally determined equilibrium constants are larger than those determined theoretically. The

converse applies for LDH for which, without exception, all the values of the theoretically determined equilibrium constants are larger.

This difference is due to the ratio of K_C' to K_C which are the immobilised and soluble enzyme Michaelis constants for NADH. The K_m' value for NADH increases six-fold or ten-fold when LDH is immobilised to an insoluble support but increases only two-fold or three-fold when YADH is immobilised on an insoluble support.

This may not be the only factor which causes a discepancy between the theoretical and experimental results. Although excluded in the approximations the hydrogen ion concentration may, in fact, be different at the support. Diffusion may also be an important consideration.

The theory which has been developed above, however, predicts the change in the equilibrium constant of a reversible enzyme-catalysed reaction which occurs if the Michaelis parameters for the substrates change when the enzyme is immobilised on a support.

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