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The Preparation and Kinetics of Lactate Dehydrogenase Attached to Water-Insoluble Particles and Sheets

By R. J. H. WILSON, G. KAY and M. D. LILLY
Biochemical Engineering Section, Department of Chemical Engineering, University College London, Torrington Place, London, W.C. 1

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1. The preparation of lactate dehydrogenase covalently attached to anion-exchange cellulose particles and sheets by use of a dichloro-*sym*-triazinyl dyestuff, Procion brilliant orange MGS, is described. 2. The stability and kinetic properties of these preparations were investigated. 3. An equation is derived to describe the change in concentration of a substrate when passed through a uniform bed of a substrate-inhibited enzyme. A number of theoretical curves are shown to illustrate the system. 4. A titrimetric assay for lactate dehydrogenase is described, and shown to be stoicheiometric over the range pH 5·0–9·2. 5. The results are discussed in relation to previous work, and the effects of charged groups on the support, and of the diffusion film surrounding any particle in suspension, are treated qualitatively.

Water-insoluble derivatives of enzymes have been made by a variety of physical and chemical methods. These techniques have been reviewed by Silman & Katchalski (1966). Previous publications have mostly concentrated on the methods of preparation of these derivatives and have tended to neglect the kinetic properties of the products, which can be markedly different from those of the free enzyme. Factors known to affect the apparent kinetic parameters of the attached enzyme include: (1) steric hindrance by the supporting matrix; (2) the presence of charged groups on the support; (3) the hydrophilic or hydrophobic nature of the support; (4) diffusion limitation due to the presence of diffusion films surrounding particles in fluids. Though the costs of isolation of the enzymes and manufacture of insoluble derivatives are high, the insoluble enzyme may show increased stability and can also be recovered at the end of the reaction process. Therefore it becomes possible to consider the use of these materials to perform biochemical conversions on laboratory and industrial scales. Attention is now being turned to the practical means of using fixed enzymes most efficiently, and to this end their kinetic properties have been studied in both stirred tanks (Lilly & Sharp, 1968) and packed beds (Bar-Eli & Katchalski, 1963; Lilly, Hornby & Crook, 1966). Certain enzymes have also been attached to porous sheets (Kay, Lilly, Sharp & Wilson, 1968), a method of possible practical advantage over packed beds.

Nearly all the information on kinetics and stability of these preparations has been obtained with proteolytic enzymes with single polypeptide chains and relatively low molecular weights. Thus before being able to generalize about the properties and advantages of insoluble derivatives of enzymes we considered it necessary to study the kinetics and stabilities of a variety of more complex enzymes. In the present paper the preparation and properties of two insoluble derivatives of lactate dehydrogenase are described.

THEORETICAL

The theoretical behaviour of a column or bed of insoluble enzyme particles that obey simple one-substrate Michaelis–Menten kinetics has been dealt with in detail by Lilly *et al.* (1966). They showed that when a substrate, concentration s_0, flows through a homogeneous bed of constant diameter at a rate Q (vol./unit time) the emergent substrate concentration, s_e, is given by the relationship:

$$s_0 - s_e = K'_m \ln(s_e/s_0) + C/Q \qquad (1)$$

where C is the reaction capacity of the column as mass/unit time and K'_m is the apparent Michaelis constant. If $s_0 - s_e$ is plotted against $\ln(s_e/s_0)$ for a constant flow rate the slope of the line will equal K'_m. Extrapolation to $\ln(s_e/s_0)=0$ gives C/Q.

For a single-substrate enzyme that is substrate-inhibited the simple kinetic equation is modified to the form:

$$v = \frac{k_3[E]}{1 + K_m/s + s/K_s}$$

where k_3 and [E] are respectively the rate constant for the

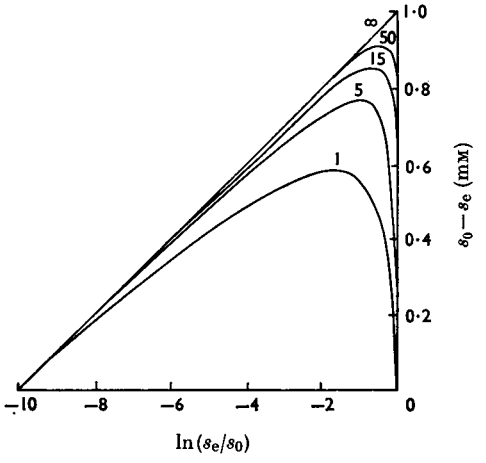

Fig. 1. Computed values of $s_0 - s_e$) plotted against $\ln(s_e/s_0)$ for $C/Q = 1{\cdot}0$ mM and $K'_m = 0{\cdot}1$ mM; Curves for the following values of K'_s (mM) are shown: 1, 5, 15, 50, ∞.

breakdown of the enzyme–substrate complex and the concentration of the enzyme. In a similar fashion to the derivation of eqn. (1) it can be shown that the corresponding relation for a column of insoluble substrate-inhibited enzyme is:

$$s_0 - s_e = K'_m \ln(s_e/s_0) + C/Q - (1/2K'_s)(s_0^2 - s_e^2) \qquad (2)$$

where K'_s is the apparent substrate-inhibition constant. Theoretical curves for various values of K'_s, K'_m and C/Q have been computed. A set of curves is shown in Fig. 1.

MATERIALS AND METHODS

Substrates and other chemicals. NADH was obtained from Calbiochem Ltd. (London, W. 1) or Boehringer Corp. Ltd. (London, W. 5). Solutions of NADH were made up in deionized water and stored on ice before use in titrator experiments, or in deionized water made slightly alkaline with KOH for other purposes; they were used within 1 day. Sodium pyruvate was A grade from Calbiochem Ltd.; bovine serum albumin fraction V was from Armour Pharmaceutical Co. Ltd. (Eastbourne, Sussex); dichloro-*sym*-triazinyl dyestuff, Procion brilliant orange MGS, was from Mayborn Products Ltd. (London, S.E. 26); Whatman microgranular anion-exchanger DE52-cellulose and anion-exchanger chromatography paper DE81-cellulose were obtained from H. Reeve Angel and Co. Ltd. (London, E.C. 4). All other chemicals were A.R. grade where available. Water was glass-distilled and, where appropriate, further deionized by passage through a Permutit Deminerolit mark 7 cartridge deionizer (The Permutit Co. Ltd., London, W. 4).

Measurement of protein. Bovine serum albumin was measured in solution by the method of Itzhaki & Gill (1964), a standard curve being constructed from known concentrations of the protein.

Preparation of monochloro-sym-triazinyl-cellulose derivatives. (*a*) Dyed DE52-cellulose. Procion brilliant orange (0·25g.) was dissolved in 750ml. of water, and 50g. (wet wt.) of DE52-cellulose was stirred in. After 15min. stirring at room temperature, 2g. of anhydrous Na_2CO_3 was added, and stirring was continued for 1hr. The product was filtered and washed with four 250ml. lots of M-NaCl, followed by four 250ml. lots of water. Washings were pooled and the volume was noted. The E_{480} value of the combined washings was read after 2 days were allowed for hydrolysis. The product was meanwhile freeze-dried, weighed (11·6g.) and stored at 2°. The $E^{1\%}_{1\,cm.}$ at 480mμ of the alkali-hydrolysed dye was found to be 256, indicating that the product contained 2·1% (w/w) of the dye.

(*b*) Dyed DE81-cellulose paper. A strip of DE81-cellulose paper (6cm. × 46cm.) was immersed as a coil in 250ml. of a fresh aqueous solution of Procion brilliant orange (0·2mg./ml.) contained in a 400ml. beaker. After 10min. with gentle agitation, 0·655g. of anhydrous Na_2CO_3 was added and agitation at room temperature continued for 45min. The liquid was then decanted off and the paper rinsed with six 250ml. lots of M-NaCl over a period of 1½hr., until most of the uncombined dye was removed. The paper was then rinsed with six 250ml. lots of water, drained and freeze-dried. Uncombined dye was measured in the washings as described above. The product, containing 1·4% (w/w) of dye, weighed 2·38g. Inspection of the paper after drying showed that dyeing was largely confined to the outer surface layers of the paper.

*Preparation of LDH.** A 1ml. sample of LDH suspension [10mg./ml. in $(NH_4)_2SO_4$ soln., 150units/mg. (Koch–Light Laboratories Ltd., Colnbrook, Bucks.)] was dialysed with stirring overnight at 2° against 2l. of 3mM-potassium phosphate buffer, pH7·4, containing 0·03mM-EDTA. The final volume of the dialysis residue was 1·32ml.

Preparation of insoluble LDH derivatives. (*a*) DE52-cellulose–LDH. A 2g. sample of active dyed DE52-cellulose was stirred into 20ml. of 0·1M-sodium borate buffer, pH8·75, and 0·9ml. of dialysed LDH solution was added. Stirring was continued at about 25° for 25hr. The cellulose was filtered, and rinsed with a little 0·1M-sodium borate buffer, pH8·75, with two 30ml. lots of M-NaCl and with 30ml. of 0·1M-NaOH–M-NH_4Cl buffer, pH8·6. The solid was then suspended in 25ml. of the NaOH–NH_4Cl buffer and stirred for 30min. before being left at 2° overnight to convert any remaining uncombined chloro compounds into the corresponding amine derivatives. The cellulose derivative was next filtered and rinsed with two 30ml. lots of M-NaCl and two 30ml. lots of water. It was then suspended in 200ml. of water in a 250ml. cylinder, shaken and allowed to stand for 30min. The fines were then decanted off and the process was repeated once. The final product was then stored at 2° in M-NaCl–0·03mM-EDTA.

(*b*) DE81-cellulose–LDH. Eight disks of dyed DE81-cellulose paper (2·5cm. diam.) were laid in a Petri dish. Then 0·42ml. of dialysed LDH solution was added to 5ml. of 0·1M-sodium borate buffer, pH8·75, and this mixture was poured over the disks. The dish was gently agitated at intervals for 28hr. at about 25°. After this time the liquid was removed and the disks were rinsed for 30min. with three 5ml. lots of M-NaCl followed by two 5ml. lots of 0·1M-NaOH–1M-NH_4Cl buffer, pH8·6, over a period of 1hr. They were then left in 10ml. of this buffer at 2° overnight. The liquid was then drained off and the disks were rinsed for

* Abbreviation: LDH, lactate dehydrogenase.

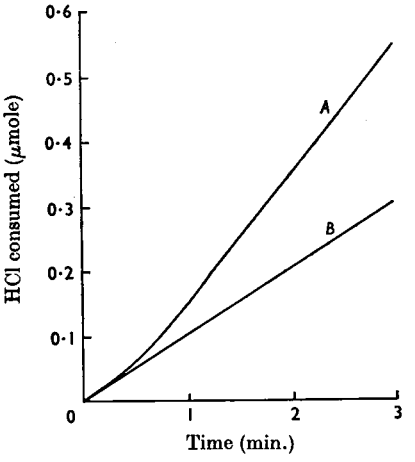

Fig. 2. Course of the titrimetric assay for arbitrary quantities of DE 52-cellulose–LDH (curve *A*) and free LDH (curve *B*). The reaction was started by the addition of NADH in 0·1ml. The reaction mixture contained: NaCl, 0·98M; EDTA, 0·03mM; sodium pyruvate, 1·15mM; NADH, approx. 0·1mM. The final volume was 46ml. The pH value was 7·4 and the temperature 25°.

2½ hr. with several lots of M-NaCl and stored at 2° in M-NaCl–0·03mM-EDTA.

Measurement of enzymic activity. (*a*) DE 52-cellulose–LDH. DE 52-cellulose–LDH was assayed titrimetrically in the 50ml. water-jacketed glass vessel of a Radiometer TTT1c titrator (Radiometer, Copenhagen, Denmark). The temperature was maintained at 25°. The titrant used was usually 5mM-HCl, and the vessel contents were stirred at a constant high speed with a modified glass stirrer to improve mixing. All solutions were made with deionized water and equilibrated with N_2 where practicable; N_2 was also blown over the surface of the liquid. Corrections were made for the slow losses of acid, probably as CO_2, that were normally found. Reaction was generally started by the addition of NADH in a small volume, e.g. 0·1ml. Typical traces from the titrator for both DE 52-cellulose–LDH and the free enzyme assayed at low pyruvate concentration are shown in Fig. 2. Initial rate of reaction was taken from the linear portion of the curve for each assay. After reaction the insoluble enzyme was removed by suction on to a 1cm.-diam. filter stick (no. 3 sinter) and normally used again for the next assay. Free enzyme was assayed in the same system by substituting a suitable quantity of the enzyme, in a small volume, for the insoluble derivative.

The stoicheiometry of the reaction was tested in the 25ml. vessel of the titrator over the range pH5·0–9·2. The solution in the vessel was equilibrated as described above and contained, in a final volume of 4·05ml., the following substances: NaCl, 3m-moles; EDTA, 0·09μmole; NADH, 4·2μmoles; sodium pyruvate, 4·0μmoles. The reaction was started by addition of LDH (0·1ml.) that had been passed through a short column of Sephadex G-15 (Pharmacia, Uppsala, Sweden), to remove $(NH_4)_2SO_4$, and then adjusted to the appropriate pH value. The reaction was followed to completion by titration with 20mM-HCl and the total amount of acid consumed was calculated. The results showed that the process was essentially stoicheiometric over the pH range measured, 1 mole of OH^- ions being produced for each mole of pyruvate present.

(*b*) DE 81-cellulose–LDH. One or more DE 81-cellulose–LDH disks were placed in a Teflon and stainless-steel filter holder of the same diameter (Gelman Instrument Co., Ann Arbor, Mich., U.S.A.). These disks were protected from damage and dirt by sandwiching them between two disks of Whatman no. 1 filter paper. They were perfused with a suitable reaction medium at a constant rate by using a Technicon peristaltic pump (Technicon Instruments Co. Ltd., Chertsey, Surrey). Care was taken to remove all air bubbles from inside the holder by application of sudden suction with a syringe; all solutions were also degassed before use by application of a mild vacuum. New DE 81-cellulose–LDH disks were perfused with M-NaCl until no LDH activity could be detected in the effluent. The amount of reaction was measured by comparing the E_{340} value of the effluent at dynamic equilibrium with that of the original inlet mixture, by using a Hilger–Gilford reaction-kinetics spectrophotometer (Hilger and Watts Ltd., London, N.W. 1).

RESULTS

Test of attachment method with bovine serum albumin. Adsorbed protein is often difficult to remove entirely from cellulose particles and it was thought desirable to demonstrate that covalent linkage was achieved by the method described above for the attachment of LDH to DE 52-cellulose, and that physically bound protein had been removed by the washing procedure used. The coupling of bovine serum albumin to DE 52-cellulose was investigated since LDH is expensive and available only in small amounts. A sample (1·84g.) of dyed DE 52-cellulose, and a similar amount of DE 52-cellulose previously converted into the chloride form by reaction overnight with concentrated sodium chloride solution containing excess of hydrochloric acid to neutralize released OH^- ions, were each stirred with 42·4mg.

Table 1. *Attachment of bovine serum albumin to DE 52-cellulose*

	Protein (mg.) released	
Washing solution	From control	From DE 52-cellulose–bovine serum albumin
(1) Filtrate and buffer	4·12	1·10
(2) 1M-NaCl	30·4	21·5
(3) 1M-NaCl	4·11	3·94
(4) 1M-NaCl	0·88	1·05
(5) 5M-NaCl	0·86	0·89
Total	40·37	28·48
Protein remaining	2·03	13·92

of bovine serum albumin in 20ml. of 0·1M-sodium borate buffer, pH 8·75. After 24hr. at about 25° both preparations were filtered and washed thoroughly with approx. 30ml. of various solutions. The protein released was measured and the results are shown in Table 1.

Thermal stability of DE 52-cellulose–LDH. Approx. 150mg. (dry wt.) of DE 52-cellulose–LDH was suspended in M-sodium chloride–0·03mM-EDTA, and kept on ice. A 10ml. quantity of the same solution was placed in a boiling-tube held in a water bath and brought to the required temperature. Then 10–25mg. (dry wt.) of DE 52-cellulose–LDH was added via a filter stick, and the suspension was magnetically stirred for 20min. The insoluble enzyme was then removed by using the filter stick and a water pump and immediately transferred to a tube containing 4ml. of ice-cold sodium chloride–EDTA solution. This experiment was performed at various temperatures. Corresponding experiments were performed with the free enzyme in the same sodium chloride–EDTA solution: 3ml. quantities in boiling-tubes were plunged into the bath and stirred for the same time as above, before being plunged into an ice bath. The DE 52-cellulose–LDH and the free enzyme were both assayed in the titrator, the samples of the former subsequently being washed with water and dried at 90° before being weighed to determine the quantity used. The results are shown in Fig. 3 as percentage inactivation with temperature at constant time.

Apparent V and K_m values of DE 52-cellulose–LDH for pyruvate. An apparent value for the

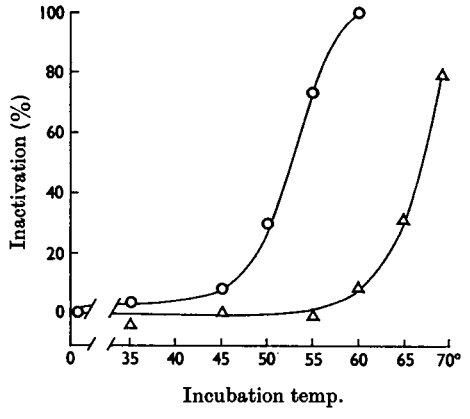

Fig. 3. Thermal inactivation of DE 52-cellulose–LDH (△) and free enzyme (○). In both cases the enzyme was held at the given temperature for 20min. Further details are given in the text.

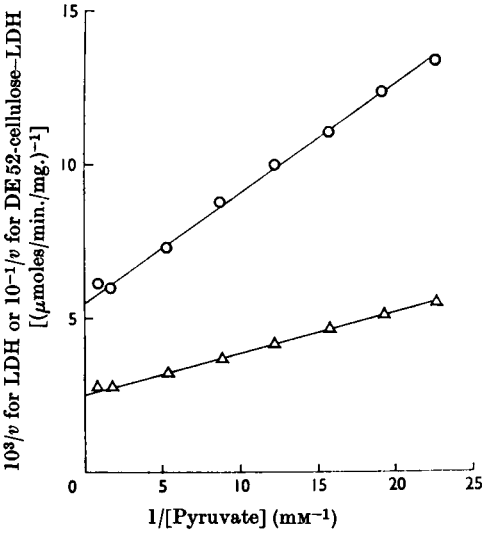

Fig. 4. Determination of V' and K'_m values. Plots of reciprocal velocity against reciprocal sodium pyruvate concentration are shown for DE 52-cellulose–LDH (△) and free LDH (○) at 0·24M-NaCl concentration. The reaction mixture also contained: EDTA, 0·03mM; NADH, approx. 0·1mM. The final volume was 46ml. The pH value was 7·4 and the temperature 25°.

Table 2. *Kinetic constants for LDH and DE 52-cellulose–LDH*

All assays were performed at pH 7·4 and 25°. Experimental details are given in the text.

	LDH		DE 52-cellulose–LDH	
	0·24M-NaCl	0·98M-NaCl	0·24M-NaCl	0·98M-NaCl
K_m for pyruvate (mM)	0·071	0·115	0·055	0·057
K'_s for pyruvate (mM)	7±1	10±1	20±2	50±2
V' (μmoles/min./mg.)	189	149	0·041*	0·029*
V' at 0·24M-NaCl / V' at 0·98M-NaCl	1·27		1·38	

* Calculated per mg. dry wt. of material.

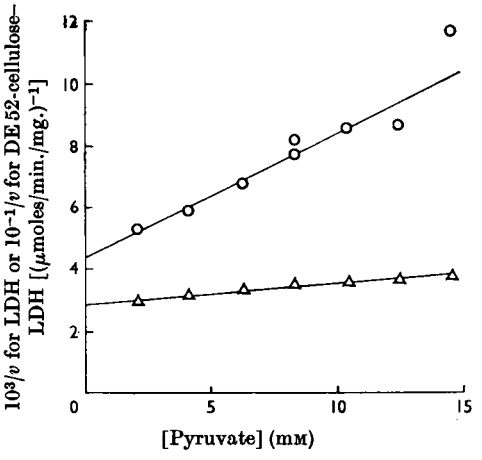

Fig. 5. Determination of K'_s value. Plots of reciprocal velocity against sodium pyruvate concentration are shown for DE 52-cellulose–LDH (△) and free LDH (○) at 0·98M-NaCl concentration. Other details were as for Fig. 4.

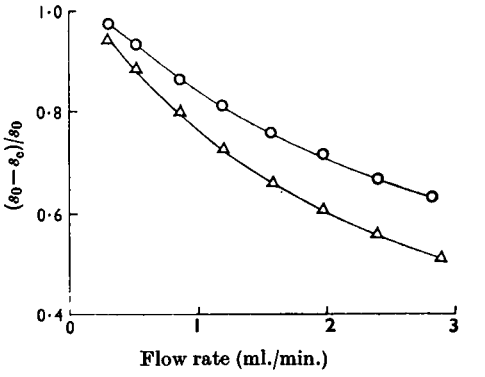

Fig. 7. Variation of the proportion of pyruvate converted into lactate by a single DE 81-cellulose–LDH paper disk perfused at various flow rates is shown for two initial values of sodium pyruvate concentration. The perfusate contained: potassium phosphate buffer, 8·6mM; EDTA, 0·83mM; NADH, approx. 0·21mM; NaCl, 0·83M; sodium pyruvate, 0·0833mM (○) or 0·165mM (△). The pH value was 7·4 and the temperature 25°. Values of K'_m and C calculated from the points on this curve are shown in Table 3.

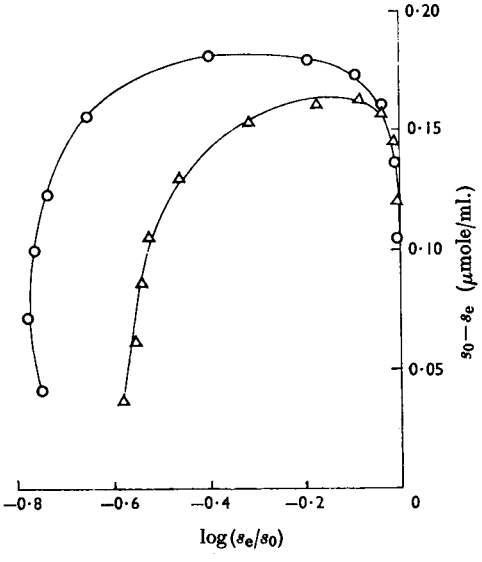

Fig. 6. Variation of activity with sodium pyruvate concentration at constant flow rate for a single DE 81-cellulose–LDH paper disk perfused at two ionic strengths. The perfusate also contained: potassium phosphate buffer, 8·6mM; EDTA, 0·83mM; NADH, approx. 0·25mM; NaCl, 0·083M (○) or 0·83M (△). The pH value was adjusted to 7·4 with KOH. The temperature was 25° and the flow rate was 2·0ml./min./cm.^{2}.

Michaelis constant, K'_m, with respect to pyruvate was obtained at pH 7·4 and 25° for the insoluble enzyme at two ionic strengths and compared with corresponding values for the free enzyme. Assays were performed with various pyruvate concentrations, and initial rates were obtained from the titration curves. The results, presented as Lineweaver–Burk plots, are shown in Fig. 4. Values of V' and K'_m were obtained from these graphs and are given in Table 2. Assays at lower ionic strengths were found to be impracticable owing to an increasing tendency for DE 52-cellulose–LDH to stick to the surface of the vessel and electrodes as the ionic strength was lowered.

Apparent substrate inhibition constant of DE 52-cellulose–LDH for pyruvate. Assays were performed for the insoluble and free enzyme, as for the determination of K'_m outlined above but with much higher pyruvate concentrations. K'_s values, determined from plots of the reciprocal of the velocity against pyruvate concentration (Fig. 5), are given in Table 2.

Variation of activity of DE 81-cellulose–LDH with substrate concentration. A single DE 81-cellulose–LDH paper disk was perfused at a constant rate with reaction mixtures of two ionic strengths and various pyruvate concentrations. Equilibrium values of the change in NADH concentration were measured as described in the Materials and Methods section. The initial concentration of pyruvate in each perfusate was measured accurately by converting the pyruvate completely into lactate with an excess of LDH. The results are plotted in Fig. 6 in a similar way to the theoretical curves of Fig. 1.

Table 3. *Apparent kinetic parameters of DE 81-cellulose–LDH: variation with flow rate*

Experimental details are given in the text.

Flow rate, Q (ml./min.)	K'_m for pyruvate (mM)	C (μmole/min.)
0·29	0·09	0·12
0·51	0·14	0·23
0·86	0·15	0·33
1·20	0·14	0·36
1·58	0·13	0·39
1·98	0·12	0·42
2·40	0·13	0·47
2·82	0·12	0·49

Variation of flow rate with DE 81-cellulose–LDH. A single DE 81-cellulose–LDH paper disk was perfused at various flow rates with two solutions containing different concentrations of pyruvate. The results, expressed as the proportion of pyruvate converted against flow rate for two pyruvate concentrations, are shown in Fig. 7. If it is assumed that the system obeys eqn. (2) and that K'_s is sufficiently high to be an unimportant factor, then it is possible to calculate values of K'_m and C for the various flow rates employed from the points obtained at two different substrate concentrations. Values of K'_m and C calculated from the points shown in Fig. 7 are given in Table 3.

DISCUSSION

Though a wide variety of chemical methods have been published for the production of water-insoluble derivatives of enzymes, most of the enzymes that have been attached so far are stable proteases or amylases. The kinetics of these insoluble derivatives have been treated in detail only for trypsin (Levin, Pecht, Goldstein & Katchalski, 1964), ficin (Hornby, Lilly & Crook, 1966) and chymotrypsin (Lilly & Sharp, 1968), all of which are proteolytic enzymes. Active insoluble preparations of LDH, a more complex and labile enzyme with a larger molecular weight (approx. 135000), have now been made. As with CM-cellulose–ficin (Hornby *et al.* 1966), the DE 52-cellulose derivative of LDH was more stable than the free enzyme when exposed to high temperatures (Fig. 3). This may not be a general feature, since Goldman, Kedem, Silman, Caplan & Katchalski (1968) found that papain cross-linked into collodion membranes was more heat-labile than the native enzyme. There was no significant loss in the activity of DE 52-cellulose–LDH or of DE 81-cellulose–LDH sheet stored at 2° for 3 months and used at intervals for various experiments at 25°.

The method of coupling enzyme to support described in this paper for LDH is a logical variation of the technique outlined by Kay & Crook (1967) for the attachment of chymotrypsin to a carboxymethylamino-*sym*-triazinyl-cellulose formed by reaction of 2-carboxymethylamino-4,6-dichloro-*sym*-triazine with cellulose powder. The reaction of M-type Procion dyes with cellulose under suitable conditions produces a monochloro-*sym*-triazinyl-cellulose that should be reactive towards nucleophilic groups on a protein under mild alkaline conditions.

The use of the M-type dyes for enzyme fixation on cellulose has been demonstrated here for LDH. The results of the fixation experiment with bovine serum albumin (see Table 1) show that covalent coupling was achieved between protein and support. The choice of the positively charged anion-exchange cellulose supports used here was dictated by two considerations: first, the need to adsorb the protein on the support so that it is available to the reactive groups on the insoluble matrix; secondly, to provide an electrostatic environment around the enzyme that would attract the two negatively charged substrates, NADH and pyruvate. The strongly negatively charged dye is also attracted by the positive support, and reaction between them would be expected to be faster than with ordinary uncharged cellulose, owing both to the increased concentration of dye at the surface of the cellulose particles and to an elevated pH value in the same region relative to the bulk of the surrounding liquid. Recent work also suggests that the reaction of the chlorine atoms of the dichloro-*sym*-triazine ring may be catalysed by the diethylaminoethyl groups constituting the positive centres of the DE 52- and DE 81-cellulose products (G. Kay, M. D. Lilly, R. J. H. Wilson & A. K. Sharp, unpublished work).

Catalysis of the reaction of the monochloro Procion H-type dyes with cellulose by tertiary amine compounds was clearly shown by Dawson (1964), who also found catalysis of alkaline hydrolysis of the dye in aqueous solution. Zerlotti (1967) subsequently showed that cross-linking of a protein matrix by reaction with Procion M-type dyes is enhanced by the presence of a tertiary amine. That the tertiary amine groups of the DE 52- and DE 81-cellulose products should act in this way is therefore not unlikely. This possibility may explain why the quantity of protein fixed here is small, being less than 1% of the product weight for bovine serum albumin (Table 1) after 24hr. at 25°. It is possible that many of the chlorine atoms on the *sym*-triazine rings were displaced by alkaline hydrolysis or cross-linking reactions during the preparation of the dyed celluloses described above. Adsorption of the protein before reaction, mentioned above, is likely to be an important factor governing the success of an attachment procedure. The

isoelectric points of both bovine serum albumin (Mahler & Cordes, 1966) and LDH (Nielands, 1952) are known to be far below the pH value used during the coupling technique, and therefore the proteins can be assumed to be negatively charged during attachment. The fixation of the negatively charged dye will decrease the net positive charge on the DEAE-cellulose; computation of the magnitude of this effect is not possible as information on the nature of the dye moiety of the Procion dye used in the experiments is not available. However, empirical observations of the adsorbing capacity of DE 52- and DE 81-cellulose for this dye lead to the conclusion that the ratio of positive charges to negative charges at pH values near neutral is about 5:1 in the preparations used here.

Binary-substrate enzymes attached to solid supports have not been investigated before this paper, except for a limited study of alcohol dehydrogenase by Manecke (1962), and of LDH and glucose oxidase entrapped in polyacrylamide gels by Hicks & Updike (1966). In the theoretical and practical investigations of the kinetics of LDH in this paper it has been assumed that the second substrate, NADH, if kept at a concentration well above its K_m value with respect to the free enzyme, can be disregarded in analysis of the kinetic parameters involving pyruvate. Hakala, Glaid & Schwert (1956) showed that at low pyruvate concentrations, where no substrate inhibition occurs, the reaction fits the formulation:

$$\frac{V}{v} = 1 + \frac{K_A}{[\text{Pyruvate}]} + \frac{K_B}{[\text{NADH}]} + \frac{K_{AB}}{[\text{Pyruvate}][\text{NADH}]} \quad (7)$$

Values for K_m determined from Lineweaver–Burk plots at high [NADH] will be slightly larger than the true kinetic constant K_A; similarly for V, the determined value is necessarily less than the true constant. This will not affect the comparisons between K'_m and V' values reported here unless the apparent values of constants K_B and K_{AB} are drastically increased when the enzyme is attached to the support. K'_s values should not be affected in this fashion.

The effect of charged groups on the support matrix has been shown to have a marked bearing on measured kinetic parameters for a number of insoluble enzymes. This has been ascribed to a difference in the equilibrium concentrations of both charged substrate and H^+ ions at the surface of the matrix, as compared with the bulk of the solution. Goldstein, Levin & Katchalski (1964) developed a comprehensive theory to correlate their data on an apparent shift in the pH–activity profile of trypsin on a solid matrix. They showed in this paper and a previous one (Levin *et al.* 1964) that the shift was markedly dependent on ionic strength and could be virtually eliminated by increasing the salt concentration sufficiently. They explained this on the basis of an electrostatic gradient, ψ, existing between the support and the mass of the solution. According to the signs of the charges involved, both H^+ ions and substrate molecules (if ionized) would be either repelled or attracted to the surface. Increasing ionic strength would be expected to decrease the gradient, and hence diminish the apparent effect on pH–activity curves and on K'_m values. Goldstein *et al.* (1964) found good agreement between values of ψ as calculated from observations at the same ionic strength on the apparent change of K_m of trypsin for benzoyl-L-arginine amide and from the pH–activity shift.

Evidence has also accumulated to point to a second factor, namely diffusion, that may become important in certain circumstances. It is well known that particles in suspension are surrounded by films of essentially stagnant liquid through which molecules can pass only by diffusion. The apparent lag noticeable at low pyruvate concentrations in the reaction curve for DE 52-cellulose–LDH assayed titrimetrically possibly may be an indication of diffusional limitation though ion-exchange reactions on the cellulose also could have an effect (see Fig. 2). In this case both the substrate added to start the reaction and the H^+ and OH^- ions involved must pass through the diffusional film, and the attainment of dynamic equilibrium will be time-dependent. In addition, the pH value near the cellulose structure will rise at the start of the reaction by an amount related to the rate of reaction on the matrix as well as to the thickness of the diffusional film and other factors (Goldman *et al.* 1968). An enzymically active particle may thus become limited in its apparent catalytic activity simply because it can convert incoming substrate faster than the substrate can diffuse in. This effect might be expected to cause an anomalously low value for V'. Such a system has been demonstrated to operate for CM-cellulose–chymotrypsin acting on an uncharged substrate in a stirred tank (Lilly & Sharp, 1968). It was found that an apparent increase in V could be obtained merely by increasing the stirring rate. This is explained by the fact that the diffusion film around a particle is known to contract as turbulence in the suspending medium is increased. Lower values for V' were also found by Lilly *et al.* (1966) for CM-cellulose–ficin in columns compared with the same material in stirred suspension. This may be explained partly by occlusion of a part of the surface area of the particles owing to the close proximity in a packed bed. A more significant finding was their discovery that the apparent K_m of the enzyme changed in the columns

as a function of the flow rate, being highest at the lowest flow rate.

So far in discussions of electrostatic and diffusional effects it has been assumed that the true kinetic parameters of a bound enzyme are similar to those of the native enzyme. This may not necessarily be so. Most covalently bound enzymes have not shown anything approaching 100% retention of activity after coupling. Though this may be due partly to diffusion effects and steric hindrance of some active sites it is likely that some individual enzyme molecules may be attached in a manner such as to alter the tertiary structure slightly without destroying enzymic activity. This could result in true changes in K_m and k_3 for the molecule with respect to its substrate, and though a particular value for a kinetic parameter may be measured it will only be a net value for a large number of enzyme molecules bound to their support in a great number of different ways. An argument of this type may be advanced to explain the apparent anomaly found in the relative changes in K'_m and K'_s for LDH between the soluble and insoluble states, as reported in this paper. Although K'_m became smaller when the enzyme was attached, K'_s conversely increased appreciably. It can be postulated that binding of the enzyme molecules as a whole tended to occlude or destroy the second (and inhibitory) binding sites for pyruvate, believed to be present in this enzyme, in preference to the active sites. This would cause an apparent increase in K_s overall, provided that loss of the second binding site did not destroy enzymic activity. LDH is now known to be composed of four sub-units each containing one active site, and therefore the insoluble derivatives may owe a proportion of their activity to sub-units that are not attached directly to the support; the possibility also exists that much of the attached enzyme is in the dimeric form. Dissociation of the heart enzyme occurs at high ionic strength (Millar, 1962) and at both high and low pyruvate concentrations (Hathaway & Criddle, 1966). The apparent rise in K_s may also be explained by the results obtained by Chilson, Kitto, Pudles & Kaplan (1966). These authors found that an active intermediate, possibly the dimer, was formed when urea-inactivated heart LDH was reactivated slowly by dilution and addition of mercaptoethanol, and that this intermediate was considerably less inhibited by high concentrations of pyruvate than was the normal enzyme. A shift of pH value in the vicinity of the cellulose matrix could not account for both an increased K'_s and a decreased K'_m. Winer & Schwert (1958) found that both K_m and K_s values of LDH increased considerably, and by similar proportions, as the pH was increased. Evidence for a pH shift was difficult to obtain from pH–activity curves, owing to the lack of a true optimum pH for the reverse reaction (Winer & Schwert, 1958). Additionally, at pH 5·0 and below, the DE 52-cellulose–LDH and the free enzyme rapidly and permanently lost their activity.

The combined effects of both diffusion and electrostatic gradients on K'_m values have been treated theoretically by Hornby, Lilly & Crook (1968). Diffusion limitation was shown to increase K'_m values, whereas an electrostatic gradient caused an apparent increase or decrease according to the nature of the charges involved. The fall in the apparent K_m value for DE 52-cellulose–LDH may indicate that the electrostatic effect was the dominating factor here. K'_s values should be affected to a similar degree by the electrostatic gradient, but the diffusion effect is likely to be less important owing to the higher range of substrate concentrations involved.

Diffusional and charge effects should have no effect on V' values. The results (Table 3) for values of K'_m and C, calculated from eqn. (1) for DE 81-cellulose–LDH, do not support this thesis. There appears to be an increase in C with flow rate but no change in K'_m, in contravention of the results of Lilly *et al.* (1966) with CM-cellulose–ficin columns. This conclusion must be treated with caution, however, since the system does not appear to be obeying eqn. (2), at least at low s_0 values. This is apparent from a comparison of the results of varying s_0 at constant flow rate Q shown in Fig. 6 with the theoretical curves shown in Fig. 1. Lilly & Sharp (1968) obtained Lineweaver–Burk plots for CM-cellulose–chymotrypsin that, although straight at values of s_0 below the apparent K_m, curved towards the origin at high s_0 values. They explained these observations by suggesting that in the interior of the particles there was a significant amount of enzyme that took an increasing part in the reaction as s_0 increased.

Gutfreund, Cantwell, McMurray, Criddle & Hathaway (1968) showed that at high pyruvate concentrations heart LDH forms an abortive ternary complex, LDH–NAD^+–pyruvate. This complex forms over a period of about 1 min. during the reaction of pyruvate and NADH, and causes a decrease in reaction rate. A similar time-dependent decrease in reaction rate at high pyruvate concentrations was found during the titrimetric assay for LDH but was not noticeable when DE 52-cellulose–LDH was assayed. It is not clear whether this form of inhibition accounts for the results shown in Fig. 5, where initial rates were measured. However, with the DE 81-cellulose–LDH, the NAD^+ concentration leaving the sheet was sufficiently high that this type of inhibition probably occurred and may account partly for the results shown in Fig. 6.

A fuller understanding of the kinetics of insoluble

derivatives of enzymes is dependent upon experiments performed on systems that may be satisfactorily interpreted mathematically, though the work described in this paper does serve to illustrate some of the complexities involved and especially the importance of charged support materials in determining the apparent kinetic parameters of the attached enzyme.

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