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The Preparation and Properties of Pyruvate Kinase Attached to Porous Sheets, and the Operation of a Two-Enzyme Continuous-Feed Reactor

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1. The covalent attachment of pyruvate kinase to filter-paper disks and some kinetic properties of the resultant enzymically active porous sheets are described. 2. A two-enzyme reactor was constructed by using water-insoluble sheets of lactate dehydrogenase in conjunction with the above-mentioned sheets possessing pyruvate kinase activity.

A number of workers have described the properties of enzymes attached to water-insoluble supports by a variety of methods (see review by Silman & Katchalski, 1966). These solid-supported catalysts are of interest for both theoretical and practical reasons. First, they may form a model for enzymic reactions normally performed in restricted systems such as on an intracellular membrane, where electrostatic and diffusional effects are important. Secondly, they may be used as recoverable catalysts for performing certain biochemical conversions on laboratory or commercial scales. Continuous conversion of substrate may be achieved by using the fixed enzyme in the form of particles packed into a bed, a more efficient process than the use of continuous-feed stirred tanks (Lilly & Sharp, 1968). Lilly, Hornby & Crook (1966) have shown that for such a bed of enzymically active particles where the enzyme obeys simple Michaelis–Menten kinetics the overall system can be represented by the equation:

$$s_0 - s_e = K_m \ln(s_e/s_0) + C/Q \qquad (1)$$

where s_0 and s_e are the initial and effluent substrate concentrations respectively, K_m is the Michaelis constant, C is the reaction capacity of the column and Q is the flow rate. A plot of $s_0 - s_e$ against $\ln(s_e/s_0)$ gives a line of slope K_m and intercept on the $s_0 - s_e$ axis C/Q if the equation is obeyed. Certain enzymes have been attached to porous sheets, in the form of modified cellulose papers or cloths (Kay, Lilly, Sharp & Wilson, 1968), and these catalytic materials may be considered as special cases of packed beds when substrate-containing solutions are made to flow through the sheet. The present paper describes the preparation and properties of pyruvate kinase (EC 2.7.1.40) attached to filter-paper disks, and the operation of a two-enzyme reactor formed by coupling the pyruvate kinase reaction to similar disks with lactate dehydrogenase (EC 1.1.1.27) activity.

MATERIALS AND METHODS

Substrates. ADP (monosodium salt) and phosphoenolpyruvate (tricyclohexylammonium salt) were from Calbiochem Ltd., London, W. 1. NADH (disodium salt) was obtained from Boehringer Corp. (London) Ltd., London, W. 5.

Enzymes. PK* (308 units/mg.) as a suspension (5·5 mg./ml.) in $(NH_4)_2SO_4$, and LDH (band 1 isoenzyme; 5 mg./ml.; 426 units/mg.) were purchased from Calbiochem Ltd. The latter preparation was essentially free of PK activity.

Chemicals. *N*-(3-Aminopropyl)diethanolamine was from Aldrich Chemical Co. Ltd., Milwaukee, Wis., U.S.A. All other chemicals were A.R. grade where available.

*Preparation of DE*81*-cellulose–LDH.* LDH was attached to Whatman DE81-cellulose anion-exchange paper (H. Reeve Angel and Co. Ltd., London, E.C. 4) as described by Wilson, Kay & Lilly (1968).

*Preparation of dichloro-*sym*-triazinyl-cellulose paper.* Twelve disks, each of 2·5 cm. diam., were cut from Whatman no. 1 filter paper and soaked in 1 M-NaOH for 5 min. Excess of NaOH was removed and the papers were stirred gently in about 25 ml. of dioxan for 5 min. Cyanuric chloride (5 g.) was dissolved separately with magnetic stirring in 20 ml. of dioxan. The papers were added to this solution, followed by 25 ml. of water and after about 5 sec. by approx. 25 ml. of acetic acid. The disks were removed and then resuspended in 20 ml. of dioxan. After 2 min. a further 20 ml. of water and a little acetic acid were added. Gentle stirring was continued for 5 min. and then the papers were washed thoroughly with water and acetone before being dried and stored at 2° in a vacuum desiccator. The paper was much hardened by this process, probably as a result of a certain amount of cross-linking between cellulose chains. The disks were made slightly cationic by treatment for 7½ min. at

* Abbreviations: PK, pyruvate kinase; LDH, lactate dehydrogenase.

room temperature in 20ml. of a solution (pH7·0) containing 82·5mM-*N*-(3-aminopropyl)diethanolamine and 2·5M-NaCl. The reaction was stopped by the addition of 10ml. of 1M-HCl. The papers were washed next with 5M-NaCl, followed by water and acetone, and then replaced in the vacuum desiccator.

Preparation of PK. A 0·25ml. sample of PK was dialysed overnight at 4° against 2l. of 3mM-potassium phosphate buffer, pH7·4, containing 0·03mM-EDTA and 0·375mM-$MgSO_4$. The final volume of dialysis residue was approx. 0·4ml. This solution was diluted with 1ml. of 0·1M-potassium phosphate buffer, pH7·4.

Preparation of no. 1-cellulose–PK. Six of the dichloro-*sym*-triazinyl-cellulose paper disks prepared as described above were laid in a Petri dish, and 0·1ml. of the PK solution was pipetted on to each disk. After 50min. at approx. 25° the disks were suspended in about 15ml. of a solution containing 30mM - *N* - (3 - aminopropyl)diethanolamine, 2·1mM-EDTA, 11mM-$MgSO_4$ and 43mM-potassium phosphate buffer, pH7·8. After being left at room temperature for 24hr. to displace chlorine atoms remaining on the cellulose with amino groups, the disks were left at 2° for a week. After this treatment the disks of no. 1-cellulose–PK were rinsed and subsequently stored in a buffer solution, pH7·4 (at 25°), containing 0·1M-tris, 1M-KCl and 1mM-EDTA.

Assay of no. 1-cellulose–PK sheets. The enzymically active disks were assayed at 25° in a Teflon and stainless-steel filter holder (Gelman Co. Ltd., Ann Arbor, Mich., U.S.A.) by a method similar to that used for the assay of DE81-cellulose–LDH disks (Wilson *et al.* 1968). Degassed reaction mixture solutions, pH7·4, normally containing 91mM-tris, 91mM-KCl, 0·91mM-EDTA, 5·45mM-$MgSO_4$, 0·545mM-phosphoenolpyruvate, 0·215mM-NADH and ADP at various concentrations, were pumped through the disks at a constant flow rate. The total conversion of phosphoenolpyruvate into pyruvate was tested usually by adding an excess of LDH, in a negligible volume, to 3ml. of the effluent contained in a silica cuvette and noting the equilibrium change in E_{340} in a Hilger–Gilford reaction-kinetics spectrophotometer (Hilger and Watts Ltd., London, N.W.1). Alternatively, the LDH was added continuously to the effluent and the equilibrium change in E_{340} noted. Corrections were made for volume changes incurred by this method. Concentrations of ADP, the limiting substrate, were measured accurately by the addition of an excess of both free PK and LDH to a cuvette containing the perfusate. The equilibrium change in NADH concentration deduced from the decrease in E_{340} was assumed to be a measure of the initial ADP concentration.

Previously unused no. 1-cellulose–PK sheets were perfused at 25° with a buffer (pH7·4) containing 0·1M-tris, 1M-KCl and 1mM-EDTA until no PK activity was detected in the effluent. The sheets were rinsed through with this buffer after experiments and stored in it at 2°.

Assay of the two-enzyme system. Two-enzyme reactors were constructed by taking one or more sheets of both no. 1-cellulose–PK paper and DE81-cellulose–LDH paper and placing them in the holder. Reactors so formed were perfused with similar solutions to those used for the assay of no. 1-cellulose–PK disks. Production of lactate was measured directly from the decrease in E_{340} of the effluent solution, and pyruvate production by adding an excess of PK-free LDH as described above. All assays were performed at 25°.

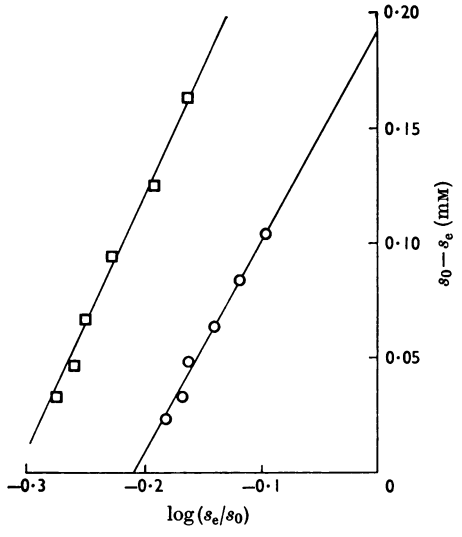

Fig. 1. Variation of activity of one no. 1-cellulose–PK sheet with ADP concentration. The amount of ADP converted, s_0-s_e, is plotted against $\log(s_e/s_0)$ for various ADP concentrations at two KCl concentrations: ○, 0·91M; □, 91mM. The flow rate was 0·346ml./min./cm.^{2}. Values of reaction capacity, C, and apparent K_m calculated from these curves are given in Table 1. Further details are given in the text.

RESULTS

No. 1-cellulose–PK

Variation of activity with ADP concentration and determination of reaction capacity and of apparent K_m for ADP. The conversion of ADP into ATP by one no. 1-cellulose–PK sheet was investigated, at a constant flow rate, for various perfusate concentrations of ADP and at two concentrations of potassium chloride. ADP concentration varied between 0·068mM and 0·516mM. The results are shown in Fig. 1 where total conversion of ADP, s_0-s_e, is plotted against $\log(s_e/s_0)$. Values for reaction capacity, C, and apparent K_m were obtained as described in the introduction. These values are compared in Table 1 with values for V and K_m obtained for the free enzyme assayed in the spectrophotometer under similar conditions by using excess of LDH as indicator and following the reaction at 340mμ.

Two-enzyme reactor

Effect of interchanging enzymically active sheets. Two of each of the DE81-cellulose–LDH sheets and the no. 1-cellulose–PK sheets were placed in the holder with the normal backing papers of Whatman

no. 1 paper to protect them from damage and dirt. These disks were equilibrated for 30min. by perfusion with a solution of low ionic strength, consisting of 91mM-potassium chloride and 0·91mM-EDTA in 91mM-tris buffer, pH 7·4. The papers were then perfused at constant flow rate with the reaction mixture containing 0·516mM-ADP. After assay of the effluent, the order in which the sheets were arranged in the filter holder was altered and the assay then repeated. The order of the sheets was changed several times, and the results are shown in Table 2 in chronological order, the last experiment being a check of the first. Great care was taken to remove air bubbles from between the sheets by applying sudden suction with a syringe before assay, but this was difficult with several sheets in place and small errors may be present in the results for this reason.

Effect of varying flow rate. Two no. 1-cellulose–PK sheets followed by one DE81-cellulose–LDH sheet were placed in the holder and perfused for 30min. at 25° with the 91mM-tris buffer, pH 7·4, containing 91mM-potassium chloride and 0·91mM-EDTA. They were then perfused as described above at various flow rates with the reaction mixture containing either 0·258mM- or 0·516mM-ADP. Results, evaluated as above, are shown in Figs. 2, 3 and 4. Values for C and apparent K_m (ADP) for no. 1-cellulose–PK sheets were calculated from pairs of values obtained at constant flow rate (shown in Fig. 3) assuming the validity of eqn. (1). These results are given in Table 3.

DISCUSSION

The method of attachment described above for the coupling of PK to filter-paper disks is based on that of Kay & Crook (1967) for the attachment of chymotrypsin to cellulose particles. Kay *et*

Table 1. *Kinetic constants for PK and no. 1-cellulose–PK paper*

The flow rate for no. 1-cellulose–PK experiments was 0·346ml./min./cm.^{2}. C is the reaction capacity, V the maximal velocity.

PK Concn. of KCl (M)	V (μmoles/min./mg.)	$\frac{V(1)}{V(2)}$	K_m (ADP) (mM)
(1) 0·091	685		0·52
(2) 0·91	178	3·84	0·46
No. 1-cellulose–PK			
Concn. of KCl (M)	C (μmoles/min./cm.^{2})	$\frac{C(1)}{C(2)}$	K_m (ADP) (mM)
(1) 0·091	0·12		0·48
(2) 0·91	0·066	1·80	0·385

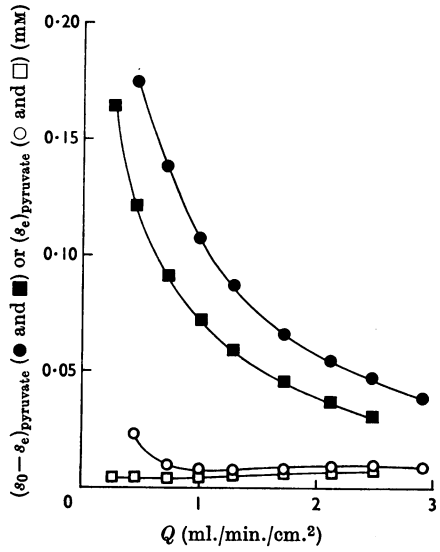

Fig. 2. Kinetics of the two-enzyme reactor $A_1A_2B_1$ (see Table 2): variation of product formation with flow rate Q. The amounts of both lactate (● and ■) and pyruvate (○ and □) in the effluent are shown for two concentrations of ADP in the perfusate: ● and ○, 0·516mM; ■ and □, 0·258mM. Further details are given in the text.

Table 2. *Two-enzyme reactor: effect of varying the order of the enzymically active sheets*

The flow rate was 1·13ml./min./cm.^{2}. A, no. 1-cellulose–PK; B, DE81-cellulose–LDH.

Expt. no.	Order of sheets (Flow→)	ADP converted (mM)	Lactate formed (mM)	% conversion of ADP into ATP	% conversion of pyruvate into lactate
1	$A_1B_1A_2B_2$	0·173	0·163	34	94
2	$B_1A_1A_2B_2$	0·167	0·152	32	91
3	$A_1A_2B_1B_2$	0·167	0·161	32	97
4	$A_1B_1B_2A_2$	0·162	0·100	31	62
5	$B_1A_1B_2A_2$	0·158	0·093	31	59
6	$B_1B_2A_1A_2$	0·153	0·004	30	3
7	$A_1B_1A_2B_2$	0·166	0·159	32	96

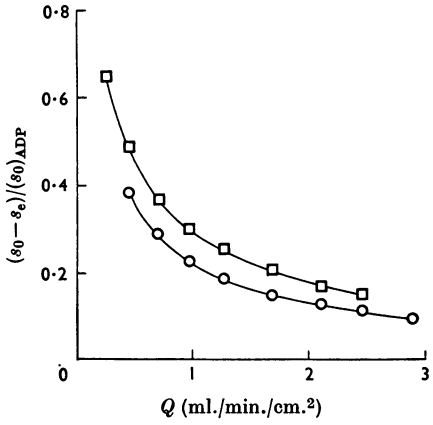

Fig. 3. Kinetics of no. 1-cellulose–PK in the two-enzyme reactor $A_1A_2B_1$ (see Table 2): variation of conversion of substrate with flow rate Q. The proportion of ADP converted into ATP is shown for the two disks perfused at two ADP concentrations: ○, 0·516mM; □, 0·258mM. Further details are given in the text.

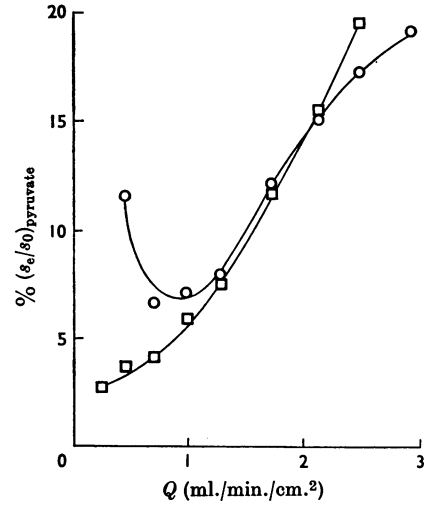

Fig. 4. Kinetics of the two-enzyme reactor $A_1A_2B_1$ (see Table 2): variation of the proportion of pyruvate not converted into lactate by DE81-cellulose–LDH with flow rate Q. The percentage $(s_e/s_0)_{pyruvate}$ is plotted against flow rate Q for the two ADP concentrations: ○, 0·516mM; □, 0·258mM. Further details are given in the text.

al. (1968) noted that it is advantageous to perform the coupling reaction under conditions such that the support matrix is oppositely (net) charged to the enzyme, so that the protein is more easily adsorbed. It was for this reason that the dichloro-*sym*-triazinyl-cellulose papers, formed as shown above,

Table 3. *Variation of C and apparent K_m values for no. 1-cellulose–PK with flow rate*

Reaction capacity, C, was calculated for one disk.

Flow rate (ml./min./cm.²)	K_m (ADP) (mM)	C (μmoles/min./cm.²)
0·45	0·39	0·88
0·71	0·43	1·0
0·99	0·40	1·1
1·28	0·37	1·1
1·71	0·33	1·1
2·11	0·44	1·3
2·47	0·48	1·4

were allowed to undergo partial reaction with *N*-(3-aminopropyl)diethanolamine before reaction with PK. This treatment should lead to the production of a positively charged support, attractive towards PK at the pH used for the coupling since this enzyme has an isoelectric point of 6·6 (Warner, 1958). For parallel reasons it was thought desirable to have the final enzymically active PK disks positively charged, so that the negatively charged substrates, ADP and phosphoenolpyruvate, should be attracted into the cellulose structure. Any remaining chlorine atoms on the substituted cellulose were therefore replaced by further reaction with the amine compound. Attempts had been made earlier to couple PK to Whatman DE81-cellulose anion-exchange paper by the simpler method of Wilson *et al.* (1968) that had proved successful for the attachment of LDH. It was found, however, that the enzyme lost virtually all of its activity when adsorbed on to the highly positively charged support, an observation that confirms the finding of Reynard, Hass, Jacobson & Boyer (1961) that PK is totally inactivated by adsorption on to DEAE-cellulose.

The no. 1-cellulose–PK sheets appear to obey eqn. (1) at any particular flow rate (Fig. 1). The slightly lower values for K_m that were found for the attached PK compared with the free enzyme (Table 1) may be explained by the presumed electrostatic attraction of the support for both substrates (Hornby, Lilly & Crook, 1968; Goldstein, Levin & Katchalski, 1964). It also appears from the results shown in Table 3 that the reaction capacity of the disks increases with the flow rate, though the apparent K_m remains more or less constant. These results are similar to those obtained with DE81-cellulose–LDH (Wilson *et al.* 1968), but in that case the system was complicated since eqn. (1) was not obeyed. Lilly *et al.* (1966) investigated the behaviour of CM-cellulose–ficin particles packed into columns and found that the reaction capacity remained almost constant but that the apparent K_m decreased with increased flow rate. These

observations were explained by changes in the thickness of the diffusional film surrounding each particle (Hornby *et al.* 1968). In the same way, increased flow rates through the papers would be expected to decrease the thickness of the film and hence decrease the apparent K_m. Lilly & Sharp (1968) have reported that the maximum reaction velocity of CM-cellulose–chymotrypsin assayed in a stirred tank increases with turbulence, though they also found a corresponding decrease in apparent K_m values and a dependence on substrate concentration that are not present with the no. 1-cellulose–PK sheets. It is possible that at low flow rates liquid passes preferentially through a limited number of pores in the paper. At high flow rates the liquid may flow more evenly through the sheets, leading to an apparently increased value for the reaction capacity.

During the present experiments no. 1-cellulose–PK sheets showed an unexpected instability. The papers appeared to lose some activity after use, though during an experiment the fixed enzyme seemed to be stable. The long-term stability of unused disks was markedly better than that of used disks. Recent work on the effect of various cations and anions on the perturbation of tryptophyl residues in PK, and also on the stability of the enzyme, may be relevant (Wilson, Evans & Becker, 1967). Tris, in particular, seems to cause the enzyme to assume an inactive and less stable conformation, though K^+ ions stabilize PK in the active form. It may be significant that runs with no. 1-cellulose–PK were often performed at low K^+ ion concentrations, but in 0·1 M-tris, whereas the papers were stored at the same concentration of tris, but in strong potassium chloride solution. The presence of Mg^{2+} ions in the storage solution might have improved stability, since this cofactor also forms a complex with the enzyme, causing a conformational change in the tertiary structure (Suelter & Melander, 1963; Mildvan & Cohn, 1965). The marked difference in the effect of potassium chloride concentration on the maximal velocity of the enzyme between the free and attached states (Table 1) may indicate a relative stabilizing of the protein in the active conformation when covalently linked to the cellulose.

The extension of work on single enzymes to the simple two-enzyme reactor described in the present paper gives an indication of the complexity of such systems. It is possible to make some general observations from the data in Table 2. Expt. 6 shows that back mixing, whether due to diffusion, fibre overlap or pump fluctuations, is almost negligible. Thus sheet B_1 plays no part in Expt. 2 though the amount of pyruvate unconverted into lactate is still small. In Expts. 1 and 3, where both LDH sheets participate in the reaction, a higher conversion efficiency was obtained in Expt. 3. The kinetics of the complete system are rather complex, however, since the substrate concentration seen by the second enzyme is dependent on the reaction caused by the first. Fig. 4 shows the way in which the proportion of pyruvate, formed by the no. 1-cellulose–PK sheets, that was not converted into lactate by DE81-cellulose–LDH varied with flow rate. At an ADP concentration of 0·258mM a greater proportion of pyruvate was converted into lactate as the flow rate decreased, though more pyruvate was actually produced by the no. 1-cellulose–PK sheets (Fig. 2). When a higher concentration of ADP was used there was an optimum flow rate (approx. 1ml./min./cm.^{2}) for the most efficient conversion of pyruvate.

It is possible that some cellular systems may operate in a similar sequential fashion, where the different enzymic steps are carried out separately. More complex models of possible metabolic pathways may be constructed soon with the aid of 'insoluble' enzymes, and the introduction of gross diffusional and electrostatic effects should prove interesting. The use of fixed enzyme systems for continuous analysis of substrates could also prove rewarding.

REFERENCES

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

Hornby, W. E., Lilly, M. D. & Crook, E. M. (1968). *Biochem. J.* **107**, 669.

Kay, G. & Crook, E. M. (1967). *Nature, Lond.*, **216**, 514.

Kay, G., Lilly, M. D., Sharp, A. K. & Wilson, R. J. H. (1968). *Nature, Lond.*, **217**, 641.

Lilly, M. D., Hornby, W. E. & Crook, E. M. (1966). *Biochem. J.* **100**, 718.

Lilly, M. D. & Sharp, A. K. (1968). *Chem. Engr*, **215**, CE12.

Mildvan, A. S. & Cohn, M. (1965). *J. biol. Chem.* **240**, 238.

Reynard, A. M., Hass, L. F., Jacobson, D. D. & Boyer, P. D. (1961). *J. biol. Chem.* **236**, 2277.

Silman, I. H. & Katchalski, E. (1966). *Annu. Rev. Biochem.* **35**, 873.

Suelter, C. H. & Melander, W. (1963). *J. biol. Chem.* **238**, PC4108.

Warner, R. C. (1958). *Arch. Biochem. Biophys.* **78**, 494.

Wilson, R. H., Evans, H. J. & Becker, R. R. (1967). *J. biol. Chem.* **242**, 3825.

Wilson, R. J. H., Kay, G. & Lilly, M. D. (1968). *Biochem. J.* **108**, 845.