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A COUPLED ENZYME NYLON TUBE REACTOR PYRUVATE KINASE–LACTATE DEHYDROGENASE SYSTEM

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A coupled enzyme nylon tube reactor has been made by simultaneously immobilizing rabbit muscle pyruvate kinase (EC 2.7.1.40) and lactate dehydrogenase (EC 1.1.1.27) onto the inside surface of a nylon tube initially derivatized with poly (L)-lysine to serve as a spacer molecule. The enzymes were covalently linked to the amino groups of the poly lysine spacer molecule by cross-linking with glutaraldehyde. The coupled enzyme system may be used in routine analysis to determine the concentrations of PEP, ADP, pyruvate, and NADH in a given solution and also to modify radioactively labeled nucleotides. The kinetic properties of the enzymes appear to be partially diffusion controlled as shown by an inverse relationship of K_m(app) values and activities to the flow rate of substrates through the tube reactor. This coupled enzyme system may be used as an indicator system when used in conjunction with other enzymes to complete a sequence of catalytic steps. An example of this is demonstrated in this paper by linking a nylon tube supported acetate kinase (EC 2.7.2.1) to this coupled enzyme system so that the three enzymes function in a series that facilitates the estimation of acetate.

INTRODUCTION

The technique of immobilizing enzymes onto the surface of insoluble polymers, studies of the fundamental properties of such immobilized molecules and their application studied earlier by Sundaram, Tweedale, and Laidler (1), Laidler and Sundaram (2), and Sundaram (3), has given a new spur to enzymology. Technically difficult problems in basic research and novel applications in research and industry have become approachable by this method. One development that holds bright prospects for application in routine analysis was the demonstration by Sundaram and Hornby (4) and Sundaram, Igloi, Wasserman, Hinsch, and Knoke (5) that urease bound to the inside surface of nylon tubes may be used as a catalytic reactor coil for analytical purposes by using the flow-through principle. Since then, this principle has been extended to many enzyme systems by Allison, Davidson, Gutierrez-Hartman, and Kitto (6), Campbell, Hornby, and Morris (7), Sundaram and Apps (8), and Sundaram (9,10). Recently the use of

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immobilized enzyme nylon tube reactors in routine clinical analysis of blood serum components such as urea, citrulline, uric acid, lactate, and pyruvate was demonstrated (5,11,12). However, coupled enzyme systems involving two or more enzymes catalyzing a sequence of reactions (13,14) offer a challenging prospect for exploitation in the immobilized form in a nylon tube. This paper deals with one such system where pyruvate kinase (PK) and lactate dehydrogenase (LDH) have been simultaneously immobilized on the inside surface of a nylon tube. Other enzymes, such as a variety of kinases, may be linked to this coupled enzyme system for the analysis of specific substrates such as triglycerides, creatine, and creatinine.

The same enzyme system, that is, PK–LDH, has been coupled to porous glass beads by Newirth, Diegelman, Pye, and Kallen (15), and filter paper disks by Wilson, Kay, and Lilly (16). However, this investigation was undertaken since a tubular polymer is more attractive for use in routine analysis because of its obvious advantages in handling. We use the following abbreviations: PEP—phosphoenolpyruvate, ADP—adenosine diphosphate, DTT—α-Dithiothreitol.

EXPERIMENTAL

Materials and Methods

$Nylon_6$ tube of 1.0-mm inner diameter was purchased from Portex Ltd., Hythe, Kent, U.K. Pyruvate kinase (PK) and lactate dehydrogenase (LDH), both rabbit-muscle enzymes, were obtained separately from Boehringer GmbH, Tutzing, the former at 200 U/mg and the latter at 550 U/mg. A mixture which is specified as having a protein ratio of 3:1 and activity ratio of 1:1 was also obtained. The activity of this mixture was specified at about 200 U/mg with phospoenolpyruvate (PEP) as substrate. Acetate kinase (AK) with specific activity of 200 U/mg and NADH and PEP were also supplied by Boehringer GmbH. ADP and ATP were supplied by Pharma Waldhof GmbH, Germany, and poly (L)-lysine ($n = 8000$) was a Miles-Yeda Ltd., Israel product.

Preparation of the Nylon–Poly Lysine Copolymer

The poly (L)-lysine derivative of $nylon_6$ tubing was made by initially activating the tubing by methylation (17). Coiled up tubing was filled with $(CH_3)_2SO_4$ and immersed in a boiling water bath for exactly 4 min and the reaction stopped by dipping the tube in a melting ice bath. The tube was carefully emptied into ice cold methanol and washed with cold methanol

several times, followed by deionized water. Then it was filled with poly (L)-lysine solution at a 2 mg/ml concentration made up in pH 7.1 phosphate buffer ($I = 0.1$) and left for 4 h at room temperature. After the reaction was complete the tube was washed with deionized water and filled with a freshly made 1.25% (v/v) solution of glutaraldehyde, in pH 9.4 bicarbonate buffer and left standing for 5 h at room temperature. The tube was then washed well with deionized water. At this stage the tube is ready for the immobilization of enzymes.

Coupling of PK–LDH to Nylon–Poly Lysine Copolymer

Of the enzyme mixture, supplied as a suspension in 3.2 M $(NH_4)_2SO_4$ at a concentration of 4 mg/ml, 0.5 ml was spun off and the pellet dissolved in 1 ml of pH 7.2 phosphate buffer (0.02 M). The tube was filled with this enzyme solution and left at 4°C for about 15 h. This solution and the first washing of the tube with about 10 ml of the coupling buffer were collected in order to estimate the amount of bound protein. The nylon tube was then washed with a liter of 1 M NaCl to remove any ionically adsorbed protein followed by a perfusion with deionized water. The tube was stored at 4°C and filled with pH 7 phosphate buffer containing 0.85 mM DTT.

The same procedure was used in coupling PK-LDH of different ratios mixed together from individual enzymes just before coupling.

Acetate Kinase Immobilization

Of acetate kinase (AK), supplied as a 1 mg/ml suspension in 3.2 M $(NH_4)_2SO_4$, 0.5 ml was centrifuged and the pellet dissolved in 1 ml of pH 7 phosphate buffer ($I = 0.1$) containing 1 mM EDTA and 0.85 mM DTT. This solution was filled into a 1-meter long nylon tube which had previously been converted to a nylon polyethyleneimine (nylon-PEI) derivative by coupling a 1% v/v solution of PEI in pH 9.2 bicarbonate buffer ($I = 0.1$) to a methylated tube and then activated with glutaraldehyde; a procedure similar to the coupling of PK-LDH to nylon–poly lysine copolymer. The tube was washed after 15 h at 4°C with the coupling buffer, 1 M NaCl, and water, and stored with the coupling buffer.

Routine Method for the Production of Radioactively Labeled ATP with the PK–LDH Reactor

Twenty-five milliliters of an assay mixture consisting of 1.36 mM [^{14}C]ADP, 2 mM PEP, 3.65 mM $MgCl_2$, and 3.8 mM KCl were kept recycling through the nylon tube reactor at a flow rate of 1.5 ml/min for 3 h at

22°C. This solution was then run through a DEAE-Sephadex A-25 column (15.0 × 1.5 cm) with a 0.1 to 0.6 M linear gradient (2 × 250 ml) of triethylammonium bicarbonate buffer in the cold room. The effluent peak of ATP emerged at a buffer concentration of approximately 0.42 M buffer.

RESULTS AND DISCUSSION

The Coupled Enzyme System

The two enzymes, pyruvate kinase and lactate dehydrogenase, functioning together in that sequence serve as an ideal indicator system for assaying metabolites such a triglycerides, creatinine, and creatine when used in conjunction with enzymes that are specific for these substrates. Such an approach in routine analysis involving several enzymes reacting in a sequence would involve systems with complicated kinetics. In the immobilized form additional physical factors such as diffusion and electrostatic effects will perturb the system considerably. There is initially, therefore, a need to optimize the performance of the PK–LDH indicator system immobilized in nylon tubes before multienzyme systems are set up. This coupled enzyme system which terminates the sequence of a reaction is monitored by the drop in concentration of the cofactor NADH (Eq. 2) and must therefore show a suitably high turnover rate. The results discussed here are of experiments designed toward such an optimization with a demonstration of the use of this indicator system in conjunction with acetate kinase to assay acetate.

Chemistry of the Coupling Reaction

The process of the activation of nylon by *O*-alkylation and further coupling of a nucleophilic group has been described by Sundaram (17) and Morris, Campbell, and Hornby (14). Although enzymes may be coupled directly to nylon activated by this procedure a copolymer of nylon–poly (L)-lysine was made so that the polyaminoacid would act as a spacer molecule separating the enzymes from the polymer surface. The enzymes are subsequently cross-linked to the spacer molecule with glutaraldehyde. This should minimize any steric hindrance of the substrates that may arise from the enzymes being very close to the polymer surface and should prevent a possible denaturation of the enzymes due to a close contact with the rather hydrophobic surface of nylon.

In the second step, where methylated nylon is treated with polylysine, the ideal pH for the reaction is closer to 9.5, for it is the ionized form of the amino group that acts as an efficient nucleophile in reacting with the

alkylated nylon. However, when an enzyme is coupled directly to the alkylated nylon, the choice of a pH for coupling depends upon its isoelectric point and the effect of pH on its stability. Thus with urease, which has an isoelectric point of about 5.1, Sundaram, Igloi, Wasserman, Hinsch, and Knoke (5) found that above pH 5 the amount of protein coupled steadily increased up to pH 9. Below pH 5 and above pH 9 there was a noticeable drop in the amount of protein coupled. On the other hand, in most cases when an enzyme is coupled to a polymer to which one arm of glutaraldehyde has already been linked, the optimal pH for enzyme coupling is found to be around pH 7 to 8.

Principles of Operation of the PK–LDH Reactor

The coupled enzyme system catalyzes the sequential reaction as follows:

$$\text{PEP} + \text{ADP} \rightleftharpoons \text{pyruvate} + \text{ATP} \quad (1)$$

$$\text{pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{LDH}} \text{L-lactate} + \text{NAD}^+ \quad (2)$$

LDH acts as the indicator enzyme and the course of the reaction may be followed at 340 nm by the disappearance of NADH absorbance. Measured against time, this would give an estimate of the pyruvate kinase activity. The specific activity of the coupled-enzyme reactor tube at a given flow rate was estimated by an assay mixture consisting of an enzyme saturating concentration of all the substrates as follows: 1.36 mM ADP, 2 mM PEP, 2.4 mM NADH, 3.65 mM $mgCl_2$, 0.85 mM DTT, 1 mM EDTA, and 3.8 mM KCl.

Assay and Application

Using the principle outlined above, the coupled enzyme system nylon tube reactor may be used for routine estimations of the concentration of PEP, ADP, and NADH. The tube reactor may also be used to estimate pyruvate, lactate, NADH, and NAD concentrations using the second enzyme, LDH, alone.

The assays were carried out by perfusing the substrate through the nylon tube reactor connected to a peristaltic pump which controlled the rate of flow. Aliquots were collected from time to time and the optical density measured at 340 nm. All assays were carried out at $22 \pm 1°C$.

pH Optima of Enzymes in the PK–LDH Reactor

The immobilized PK in the reactor tube showed optimal activity at pH 7, the activity at pH 8 being only 10% less than at pH 7. LDH showed a

broader optimum such that, if needed, pyruvate could be estimated with equal efficiency between pH 7 and pH 9. However, the combined system of the two enzymes had a pH optimum at pH 8, activities at pH 7 and 9 being 10% and 15% less than at the optimum. Thus all the kinetics for the PK–LDH system were studied at pH 8. Gestrelius, Mattiasson, and Mosbach (18) found that the pH optimum of an immobilized coupled enzyme system varied when the ratio of activities of the two enzymes in the original coupling solution was varied. This was perhaps because the two individual enzymes in their study showed pH optima well separated by 1.6 units whereas in the present investigation it is found that the second enzyme in the sequence shows a rather broad pH optimum between pH 7 and 9 that overlaps the first. Perhaps because of this no noticeable shift in the optimum pH of the coupled enzyme was seen even when the activity ratio was altered in the immobilization.

Calibration of the Enzyme Ratio and the Optimization of Activity

Several coupled enzyme reactor tubes of the PK–LDH system were made starting with solutions of the two enzymes mixed in different ratios. The activities of these reactor tubes are shown in Table 1. The activities of PK in these reactors were measured with PEP as the substrate assaying for pyruvate produced by the dinitrophenylhydrazine method of Kachmar and Boyer (19). LDH activity was measured with pyruvate and NADH and the activities of the coupled enzyme system were assayed with NADH as the cofactor. All these activities were measured at enzyme saturating levels of 1.6 mM PEP and pyruvate concentrations.

As long as the total amount of protein in the coupling mixture is not a limiting factor, the kinetics of the immobilized coupled enzyme system will

TABLE 1. **Properties of Different PK–LDH Reactors**[a]

Reactor (number)	Protein (ratio)	Activity (ratio)	% PEP conversion		% Pyruvate conversion	Total protein coupled(mg)
			PK	PK–LDH		
1	3:1	1:1	—	6.86	22	0.51
2	1:2	1:5.5	25.8	39.2	54	0.52
3	2:1	2:1.1	33.0	15.3	17	0.50
4	1.6:5	1:8.6	30.4	68.0	62.3	0.58

[a] Protein estimations were made by the Lowry method (21) and OD measurement at 280 nm of the enzyme solution. In conjunction with this the activity disappearing from the enzyme solution remaining after coupling was used as an additional cross-check on protein estimations. All activities discussed in text and tables are for reactors 1 meter in length unless otherwise specified. Assays were done at 22°C.

depend chiefly on the protein and activity ratios of the two enzymes as well as their specific activities. LDH is an indicator enzyme and unless it is in excess the observed reaction rate of the combined system will not be governed by the activity of PK alone but also by the activity of LDH. Thus in Reactor 1 even though the activity ratio of PK:LDH is 1:1, there is no guarantee that they will be immobilized in a 1:1 ratio since various other factors influence the actual amount of the two enzymes coupled. In fact it is obvious that the system is rate limited by LDH in Reactor 1 since, upon increasing the LDH concentration in the starting mixture as seen in Reactor 2, the rate increases considerably. However, the rate attains a maximum of 68% in Reactor 4 where PK:LDH is at 1:8.6. On the other hand, keeping

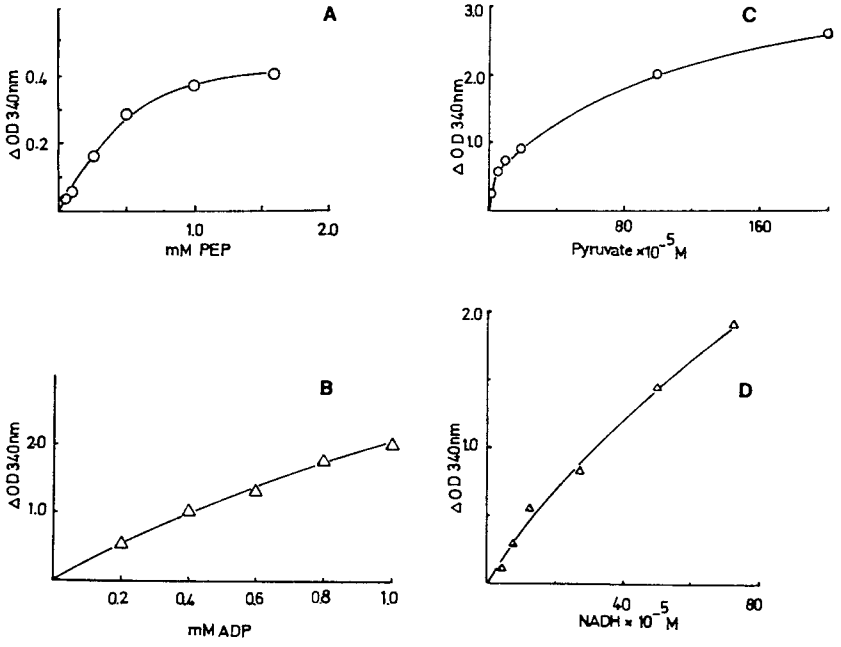

FIG. 1. (A). Estimation of PEP at a concentration range of 0.02 to 2 mM. Concentrations of ADP, NADH, $MgCl_2$, DTT, KCl, and EDTA were kept constant at levels mentioned in the text in 38 mM Tris-HCl buffer, pH 8.0, at 22°C. (B). Estimation of ADP at a concentration range of 0.2 to 1.0 mM. The rest of the assay mixture containing of PEP, NADH, $MgCl_2$, DTT, KCl, and EDTA was of the same composition as described in the text in 38 mM Tris-HCl buffer, pH 8.0, at 22°C. (C). Estimation of pyruvate at a concentration range of 0.04 to 2 mM. Assay mixture consisted of 2.4 mM NADH and 0.85 mM DTT in 38 mM Tris-HCl buffer, pH 8.0, at 22°C. This procedure uses LDH alone. (D). Estimation of NADH at a concentration range of 0.05 to 0.8 mM. Assay mixture consisted of 2 mM pyruvate and 0.85 mM DTT in 38 mM Tris-HCl buffer, pH 8.0, at 22°C. This procedure uses the second enzyme, LDH, alone.

the concentration of LDH constant as in Reactor 1 but doubling that of PK nearly doubles the rate of conversion of PEP as seen for Reactor 3.

For Reactors 2 to 4 the K_m(app) values for PEP measured with NADH are reduced to from 0.25 to 0.4 mM resulting in a steepening of the $[v]$ versus $[S]$ plot.

Figure 1 shows the calibration curves obtained when PEP, ADP, pyruvate, and NADH of different concentrations were perfused through the coupled-enzyme nylon tube reactor keeping the concentrations of the other substrates in the assay mixtures constant. A flow rate of 1.5 ml/min was used for the perfusion of substrates in these experiments. At this flow rate there was a turnover of about 23% and 13% of the two substrates, pyruvate and PEP, respectively, which increased to about 53% and 86% when the flow-rate was reduced to 0.15 ml/min (Fig. 2). After the reactor tube had lost some of its activity after being subjected to some unfavorable storage conditions over a period of 5 months it showed a flow-rate dependence in activity with a turnover of 10% and 8% of pyruvate and PEP, respectively, at 1.5 ml/min. This increased to 18% and 35% at a 0.15 ml/min flow-rate (Fig. 2). All activities were measured with substrates at saturation levels.

Kinetics of the PK–LDH Reactor

Table 2 compares some results of the kinetic studies carried out with the PK–LDH system with those obtained with LDH alone. The former catalyzes the conversion of PEP and the latter acts upon pyruvate. Measurements of kinetic constants, that is, the K_m (app) values, were made at three different

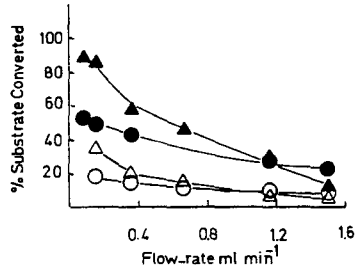

FIG. 2. The effect of flow rate on the activities of the coupled-enzyme system and lactate dehydrogenase. Triangles represent coupled-enzyme system activity; circles, lactate dehydrogenase activity. Filled symbols show the substrate composition at full saturation levels and NADH at 2.4 mM in pH 8 Tris buffer (I = 38 mM); open symbols, the results of experiments done with the tube reactor after it had lost some activity during storage. With reduced activity, substrate composition was at saturation levels and NADH at 0.66 mM for the coupled-enzyme system. Pyruvate was at 1.8 mM and NADH at 0.66 mM in pH 8.0 Tris buffer (38 mM) for lactate dehydrogenase.

TABLE 2. **Kinetic Constants of the Immobilized Coupled-Enzyme System and Lactate Dehydrogenase Alone**[a]

Substrate	K_m (M)	K_m (app) (M) / Flow rate (ml/min) 0.15	1.15	1.5
ADP	3.0×10^{-4}	5.8×10^{-4}	5.26×10^{-4}	4.87×10^{-4}
PEP	7.0×10^{-4}	2.38×10^{-3}	1.07×10^{-3}	6.0×10^{-4}
Pyruvate	1.64×10^{-4}	3.85×10^{-4}	3.12×10^{-4}	1.96×10^{-4}
NADH (PK/LDH)	—	1.5×10^{-3}	9.5×10^{-4}	8.69×10^{-4}
NADH (LDH)	1.07×10^{-5}	1.47×10^{-3}	6.6×10^{-4}	6.25×10^{-4}
Lactate (LDH)	6.7×10^{-3}		4.55×10^{-5}	
NAD (LDH)	2.5×10^{-4}		1.33×10^{-3}	

[a] Kinetic data for pyruvate kinase are from McQuate and Utter (20). The K_m (app) for PK with PEP as a substrate, estimated by the DNPH method, was around 1.8×10^{-3} M for all the reactors. All K_m (app) values were determined over a 20- to 40-fold range of substrate concentration depending upon the substrate. Reactor type 1 (see Table 1) was used in these kinetic measurements.

flow rates of 0.15, 1.15, and 1.5 ml/min. The k_m (app) values of the negatively charged substrates PEP, ADP, and pyruvate are not significantly different from the corresponding K_m values for these substrates in free solution, whereas NADH shows markedly increased K_m (app) values. This increase is a consequence of the electrostatic interactions between the positively charged polymer and NADH. The other noticeable feature in these experiments is the increase in K_m (app) values with decreasing flow rates for all the substrates. This is consistent with the fact that activity also increases with decreasing flow rates as already seen in Fig. 2. This phenomenon, observed previously in other studies (20), is caused by the reactions being partially diffusion controlled.

These observations are in contrast with our earlier findings, Sundaram and Hornby (4), and more recently with nylon tube supported NAD kinase by Sundaram and Apps (8), where both K_m (app) and K_{cat} values were found to be unaffected by flow rates of substrate. In both cases the enzymes were coupled directly onto nylon without a spacer and this suggests that the enzymes had been coupled as a layer close to the polymer surface. An added consideration is that the substrate for urease is uncharged. In the present study the polylysine chain acting as a spacer may well enhance the diffusion effects. Often diffusion combined with electrostatic interactions perturbs the kinetics more than does diffusion alone (2).

The shape of the calibration curves remain essentially the same when the flow rates are varied, and for a routine estimation of the substrates a control may be run each day before starting with unknown samples. Also,

the cost of operation in routine analysis may be considerably reduced by lowering the NADH concentration in the assay mixture (Fig. 2 and Fig. 3A,B, and C). Since the determinations are based on a kinetic assay the total turnover of substrates will depend upon the specific activity of the reactor coil and the flow rate.

Storage and Operational Stability

The storage and operational stabilities of the PK–LDH reactors were quite reasonable. When the reactors were being used for several hours almost daily at room temperature there was no appreciable loss in activity in the first 2 months. Subsequently the reactors were stored at 4°C without any buffer in them for another 3 months. At the end of this period only 25% of the former activity was left which increased to 65% upon washing well with Tris ($I = 38$ mM) buffer containing EDTA and DTT. This slow recovery of

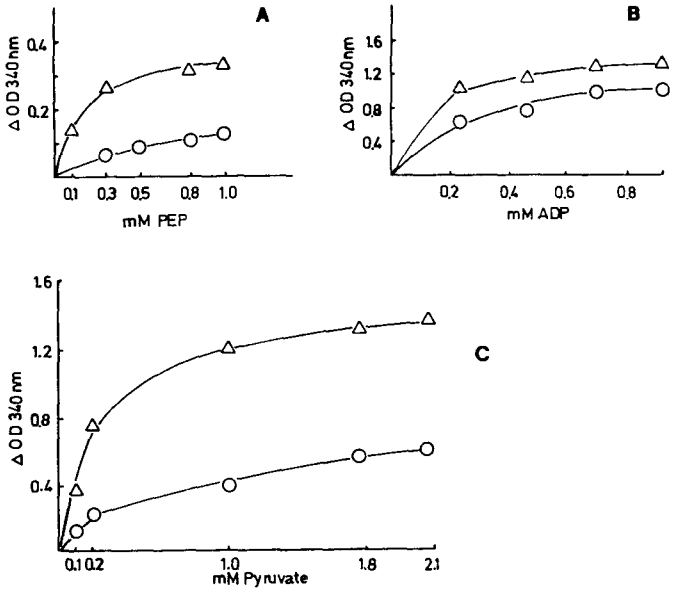

FIG. 3. (A). Calibration curves for estimation of PEP in the concentration range 0.1 to 1 mM. Concentrations of ADP, $MgCl_2$, DTT, KCl, and EDTA were kept at constant levels as mentioned in the text. The NADH concentration was at 0.66 mM in Tris buffer, pH 8.0 (38 mM) at 22°C: O–O, 1.15 ml/min; Δ–Δ, 0.15 ml/min flow rates, respectively. (B). Calibration curves for estimation of ADP in the concentration range of 0.2 to 1.0 mM. Concentrations of other substrates, $MgCl_2$, DTT, KCl, EDTA, and NADH as in (A). (C). Calibration curves for estimation of pyruvate in the concentration range of 0.1 to 2.1 mM. NADH concentration at 0.66 mM: O–O, 1.15 ml/min; Δ–Δ, 0.15 ml/min flow rates, respectively.

lost activity has been noticed with some other enzymes as well under similar circumstances and appears worthy of detailed study.

It was possible to find out approximately by how much the two enzymes deteriorated in activity during storage under the conditions mentioned above by measuring the activity of LDH alone with pyruvate. This experiment suggested that whereas pyruvate kinase possibly lost about 33% of its activity, LDH lost nearly 46% of its activity by this drastic treatment.

Application of the PK–LDH Reactor to the Synthesis of [^{14}C]ATP from [^{14}C]ADP

The experiment involving the production of [^{14}C]ATP showed a recovery of 81.1% by OD measurement and 82.1% by [^{14}C] counting. The yield was approximately 10 μmol of labeled ATP/h starting with [^{14}C]ADP. Thirty-four micromoles of [^{14}C]ATP carrying 7.98×10^6 counts were produced giving a specific activity of 2.35×10^5 counts/μmol/min.

This method is not only facile, but when used routinely cuts down the cost of production of the radioactively labeled nucleotide considerably. This is a particularly useful technique for a small laboratory which does not have a large selection of radioactively labeled compounds in stock.

Application of the PK–LDH Reactor as an Indicator System

There are various possibilities for using this coupled enzyme nylon tube reactor as an indicator system by combining with other enzymes such as kinases to make multienzyme systems for routine analysis of metabolites in clinical chemistry laboratories. This idea was tested by immobilizing acetate kinase (AK) in a separate 1-meter tube. This tube was linked to a PK–LDH tube and tested for acetate estimation by the following reaction sequence

$$\text{acetate} + \text{ATP} \overset{\text{AK}}{\rightleftharpoons} \text{acetyl-P} + \text{ADP} \tag{3}$$

$$\text{ADP} + \text{PEP} \overset{\text{PK}}{\rightleftharpoons} \text{pyruvate} + \text{ATP} \tag{4}$$

$$\text{pyruvate} + \text{NADH} + \text{H}^+ \overset{\text{LDH}}{\longrightarrow} (\text{L})\text{lactate} + \text{NAD}^+ \tag{5}$$

With reactor 1 of PK–LDH, acetate in the concentration range from 0.1 to 0.4 mM could be estimated with a turnover of only about 2% substrate at 0.4 mM acetate. On the other hand when the AK tube was linked with reactor 1 and the amount of pyruvate produced was determined by the DNPH method there was a turnover of 3.3% substrate. The three enzymes immobilized in the same tube were not a satisfactory system for analysis.

CONCLUSIONS

The investigation reported in this paper involves a system that has complicated kinetics. Simpler systems involving two or three enzymes acting on a single substrate such as, for example, cyclic AMP which is broken down in several steps by the system phosphodiesterase-alkaline phosphatase-adenosine deaminase, Sundaram (21), are possible.

It is concluded that a general prerequisite to obtaining an immobilized coupled or multienzyme system that retains most of the native properties of the enzymes are (1) the conditions of coupling, especially if the enzymes are simultaneously coupled, should be acceptable to all the enzymes and (2) the conditions of assay such as the choice of pH, temperature, buffer species, concentrations of cofactors, salts, and substrates should be not only optimal but also experimentally so arranged that the rate limiting step in the chain reaction will not be the last step. This ensures that there is no build up of intermediates which can further complicate the kinetics of the system. If it is desired to use the system to catalyze reactions in both directions then it is worthwhile having both the enzymes in a coupled-enzyme system or the first and the last enzyme in a multienzyme system functioning with equal efficiency.

It is often necessary to compromise in selecting a pH suitable for the functioning of a coupled or multienzyme system. Thus, even though the overall turnover rate could be improved if the different enzymes were to function consecutively in distinct separate compartments, each with its conditions optimized, when they are coimmobilized on the same polymer the best set of mutually compatible conditions, including pH, must be chosen for a satisfactory routine operation.

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NOTE ADDED IN PROOF

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