THE PREPARATION OF SOME IMMOBILISED DEHYDROGENASES AND THEIR USE IN AUTOMATED ANALYSIS

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1. Introduction

The preparation of enzymes supported by nylon tubing and their use in automated analysis has been described [1, 2]. Until now the enzymes used have been robust and no attempt has been made to describe comparable techniques using less stable enzymes, such as dehydrogenases. Clearly this group of enzymes has important clinical applications and, therefore, it is important to consider methods whereby their use in automated analysis may be facilitated.

Previous methods used for the preparation of enzymes supported by nylon tubing have involved a prior partial hydrolysis of the inside surface of the nylon tube [1], and covalent attachment of the enzyme through glutaraldehyde to the liberated amino groups. This method, which leaves a residual ionised carboxyl group on the surface of the support, is unsuitable for the immobilisation of dehydrogenases. This paper describes a new process for coupling enzymes to nylon whereby a residual positive charge is left on the surface of the support. Using this method, lactate dehydrogenase (EC 1.1.1.27), malate dehydrogenase (EC 1.1.1.37) and yeast alcohol dehydrogenase (EC 1.1.1.1) have been coupled to nylon tube and successfully exploited in automated analysis.

2. Experimental

In order to increase the available surface area on the inside of the nylon tube, the material was first "pitted" to remove the regions of amorphous nylon. 3 m lengths of "Type 6" nylon tube, 1 mm bore, (Portex, Hythe, Kent) were filled with a mixture of 18.6% (w/w) $CaCl_2$, 18.6% (w/w) water in methanol and incubated for 20 min at 50°, after which they were purged free of precipitated amorphous nylon by perfusing with water. The tubes were then filled with *N,N*-dimethyl-1,3-propanediamine (Ralph N. Emanuel Ltd., 264 Water Road, Alperton, Middlesex, England) and incubated for 12 hr at 70°. This process breaks some of the peptide bonds on the surface of the nylon, liberating free amino groups and amidating the carboxyl groups with the amine, thus leaving cationic dimethylamino groups on the nylon surface. The tubes were then perfused at 1° for 90 min with a mixture of 12.5% (w/v) glutaraldehyde in 0.1 M borate, pH 8.0, washed through with 0.1 M phosphate, 1.0 mM EDTA, 0.1 mM dithiothreitol, pH 7.8 (referred to as the coupling buffer) and used immediately for the preparation of immobilised enzyme derivatives.

Yeast alcohol dehydrogenase (salt-free, crystallised and lyophilised, Sigma Chemical Co.) was coupled to the inside surface of a 3 m length of treated nylon tube by filling the tube with 2.25 ml of the coupling buffer containing 10 mg of enzyme and 2.5 μmole NAD and incubating for 12 hr at 4°. The tube was then perfused at 1° for 30 min with 100 ml of the coupling buffer containing 2 mmoles sodium borohydride and finally with 100 ml of the coupling buffer.

25 mg of crystalline beef heart lactate dehydrogenase in ammonium sulphate (Type III, Sigma Chemical Co.) were collected by centrifugation, dissolved in 1.0 ml of the coupling buffer and dialysed for 1 hr against 5 l of the same buffer. The enzyme

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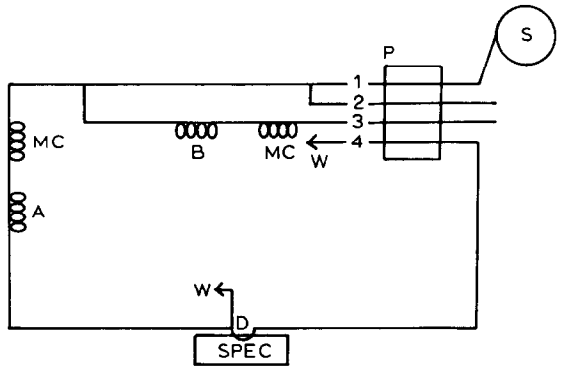

Fig. 1. Flow system for the automated determination of pyruvate, oxalacetate and ethanol using nylon tube-supported lactate, malate and alcohol dehydrogenases, respectively. The sampler (S) and pump (P) were standard Technicon equipment. Other symbols used are as follows: MC, mixing coil; D, debubbler; SPEC, spectrophotometer and W, waste. The pump tubing lines 1, 2, 3 and 4 gave flow rates of 2.00, 0.42, 0.42 and 2.00 ml min^{-1}, respectively. The reagent solution was pumped through line 3 and air through line 2. The enzyme coils were maintained at 25°, a 2:1 wash–sample ratio was used and analyses were performed at the rate of 30 samples per hr.

solution was then made up to 2.25 ml with coupling buffer and used for the preparation of nylon tube-supported lactate dehydrogenase as previously described. Nylon tube-supported malate dehydrogenase was prepared from 10 mg of pig heart malate dehydrogenase (suspension in 2.8 M $(NH_4)_2SO_4$, Sigma Chemical Co.) by the same method used for the lactate dehydrogenase.

Fig. 1 shows the flow system used for the automated determination of pyruvate, oxalacetate and ethanol using nylon tube-supported lactate, malate and alcohol dehydrogenases, respectively. In each case samples were assayed by recording either the decrease or increase of the absorbance at 340 nm due to the utilisation or production of NADH using a Beckman DBGT spectrophotometer fitted with 1 cm light path flow-through cuvettes.

For the determination of pyruvate, 3 m of nylon tube-supported lactate dehydrogenase was inserted at position A in the flow system and the reagent solution consisted of 0.1 M phosphate, 1 mM EDTA, 0.1 mM dithiothreitol, 1.0 mM NADH, pH 7.5. Oxalacetate was determined by inserting 3 m of nylon tube-

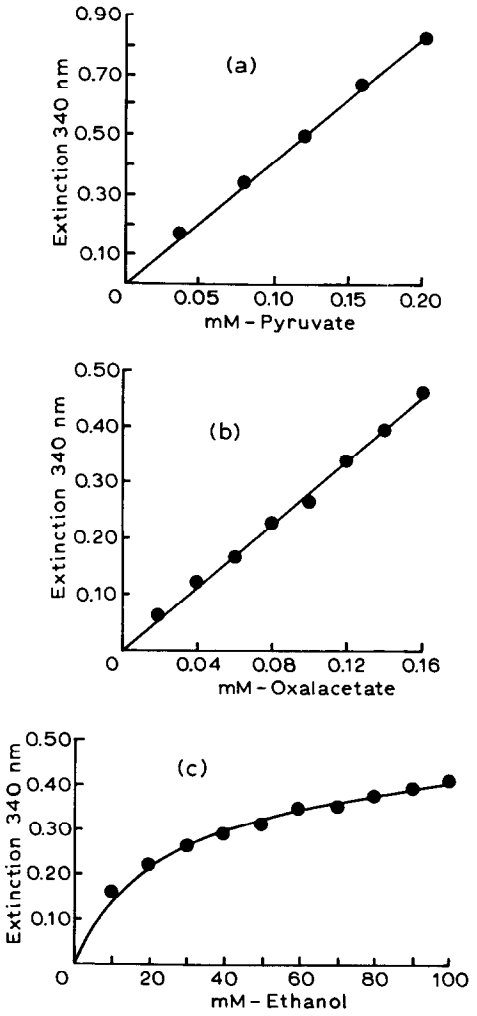

Fig. 2. Standard curves for the automated determination of pyruvate using 3 m of nylon tube-supported lactate dehydrogenase (a), oxalacetate using 3 m of nylon tube-supported malate dehydrogenase (b), and ethanol using 3 m of nylon tube-supported alcohol dehydrogenase (c).

supported malate dehydrogenase at position A in the flow system and using the same reagent solution as above. Finally, ethanol was determined using 3 m of nylon type-supported alcohol dehydrogenase inserted at position A in the flow system with a reagent solution similar to that used previously except 1.0 mM NAD replaced the 1.0 mM NADH.

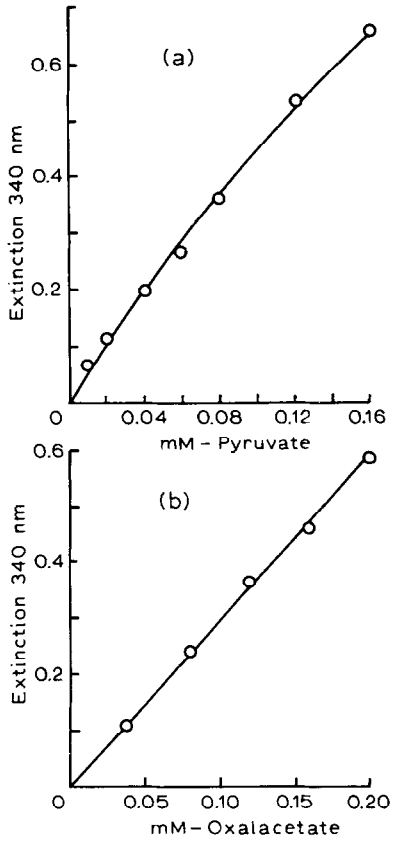

Fig. 3. Standard curves for the automated determination of pyruvate using 3 m of nylon tube-supported lactate dehydrogenase (a) and oxalacetate using 3 m of nylon tube-supported malate dehydrogenase (b). These results were obtained with 3 m of nylon tube-supported alcohol dehydrogenase at position B in the flow system and with NAD in the reagent solution.

3. Results and discussion

Fig. 2 (a–c) shows the standard curves obtained for the assay of pyruvate, oxalacetate and ethanol using nylon tube-supported derivatives of lactate, malate and alcohol dehydrogenases, respectively. The data demonstrate that these systems can be used for the determination of pyruvate, oxalacetate and ethanol in the concentration ranges 0.04–0.20 mM, 0.02–0.16 mM and 10–100 mM, respectively.

So far tube-supported enzymes have been used in automated analysis exclusively for the determination of their substrates. However, it is possible to conceive of other ways of exploiting their potential in this field. For example, it is possible to use them for the production of an expensive analytical reagent from a cheaper material. This has been demonstrated using the present systems. By inserting nylon tube-supported alcohol dehydrogenase at position B in the flow system shown in fig. 1 it has been possible to generate continuously NADH from NAD for the assay of both pyruvate and oxalacetate using nylon tube-supported lactate and malate dehydrogenases, respectively, inserted at position A in the flow system. The results of these experiments, presented in fig. 3, show that these systems can be used for the determination of pyruvate and oxalacetate in the concentration ranges 0.01–0.16 mM and 0.04–0.20 mM, respectively.

This work demonstrates how immobilised dehydrogenases can be used in automated analysis. Due to the enhanced stability of the immobilised enzymes and their physical form it is possible to use them repetitively for the automated estimation of their substrates, thereby effecting a considerable economy in enzyme utilisation. All three nylon tube-supported dehydrogenases were filled with reagent solution, less the pyridine nucleotide, and stored at 4° when not in use. The three derivatives have been used over a period of twenty days without incurring any loss in their activity and during this time each derivative was used for at least 1000 analyses. Solutions of the three enzymes, stored under comparable conditions, however, lost at least 90% of their activity over the same period. Furthermore, by using immobilised alcohol dehydrogenase for the continuous generation of NADH from NAD, it is possible to realise at least a two-fold saving in the cost of pyridine nucleotide, for analytical systems using NADH.

Acknowledgements

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References

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