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A Chemistry for the Immobilization of Enzymes on Nylon

THE PREPARATION OF NYLON-TUBE-SUPPORTED HEXOKINASE AND GLUCOSE 6-PHOSPHATE DEHYDROGENASE AND THE USE OF THE CO-IMMOBILIZED ENZYMES IN THE AUTOMATED DETERMINATION OF GLUCOSE

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Triethyloxonium tetrafluoroborate was used to *O*-alkylate nylon-tube thus producing the imidate salt of the nylon which was further made to react with 1,6-diaminohexane. 2. Hexokinase (EC 2.7.1.1) and glucose 6-phosphate dehydrogenase (EC 1.1.1.49) were immobilized on the amino-substituted nylon tube through glutaraldehyde and bisimidates. 3. The effect of varying the conditions of *O*-alkylation and the amount of enzyme immobilized on the activity of nylon tube–hexokinase derivatives was determined. 4. The effect of varying the amount of enzyme immobilized on the activity of nylon-tube–glucose 6-phosphate dehydrogenase derivatives was determined. 5. The thermal stability of nylon-tube–hexokinase and nylon-tube–glucose 6-phosphate dehydrogenase derivatives was studied. 6. Different ratios of hexokinase and glucose 6-phosphate dehydrogenase were co-immobilized on nylon tube, and the rate of conversion of glucose into 6-phosphogluconolactone was compared with the individual activities of the immobilized enzymes. 7. Hexokinase and glucose 6-phosphate dehydrogenase co-immobilized on nylon tube were used in the automated analysis of glucose.

Nylon-tube-supported enzymes have been prepared previously and shown to have potential applications in automated analysis (Sundaram & Hornby, 1970; Filippusson *et al.*, 1972; Hornby *et al.*, 1972). The chemistries used to activate the nylon invariably involved cleavage of the polyamide chain for the release of free amino groups to which the enzymes were covalently bound through glutaraldehyde. Sundaram & Hornby (1970) used partial acidic hydrolysis of the nylon to liberate equivalent amounts of free amino and carboxyl groups. Cleavage with *NN'*-dimethyl-1-,3-diaminopropane, which resulted in the release of free amino groups and amidated carboxyl groups, was introduced as an alternative to acidic hydrolysis when a positively charged tube was required (Hornby *et al.*, 1972). The same workers improved both methods through the prior incorporation of a 'pitting' procedure with $CaCl_2$–methanol solutions which removed amorphous nylon. However, all these methods are limited by the need to balance the degree of depolymerization required to release free amino groups with maintenance of the integrity of the nylon structure. Now it has been found to be possible to convert the secondary amide groups of nylon into secondary imidate groups by *O*-alkylation (Hornby & Morris, 1974). This process introduces a reactive functional group into the nylon without any accompanying depolymerization, and the functional group reacts very readily with amino compounds to produce a stable amidine link. Hence it is possible to build varied and flexible chemistries of enzyme immobilization based on an initial reaction of the nylon imidate with bifunctional compounds containing amino groups.

The application of this new chemistry is illustrated by the alkylation of nylon tube with triethyloxonium tetrafluoroborate and subsequent immobilization of hexokinase and glucose 6-phosphate dehydrogenase. These two enzymes were chosen because of their use as a linked enzyme system in a highly specific assay for serum or plasma D-glucose as described by Slein (1965).

$$\text{Glucose} + \text{ATP} \rightleftharpoons \text{glucose 6-phosphate} + \text{ADP}$$

$$\text{Glucose 6-phosphate} + \text{NADP}^+ \rightleftharpoons \text{6-phosphogluconolactone} + \text{NADPH}$$

Widdowson & Penton (1972) have shown how this assay can be applied to the automated determination of serum or plasma glucose in the Technicon 'Autoanalyser II'. It was therefore decided to prepare co-immobilized nylon-tube derivatives of hexokinase and glucose 6-phosphate dehydrogenase and study the feasibility of incorporating them in continuous-flow-analysis systems.

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Experimental

Materials

Grade I glucose phosphate dehydrogenase (D-glucose 6-phosphate–$NADP^+$ oxidoreductase, EC 1.1.1.49) from yeast and hexokinase (ATP–D-hexose 6-phosphotransferase, EC 2.7.1.1) from yeast were obtained from Boehringer Corp. (London) Ltd., London W.5, U.K. The $(NH_4)_2SO_4$ suspensions of these enzymes were centrifuged in a bench top centrifuge and the precipitates dissolved in 0.1M-*N*-ethylmorpholine adjusted to pH8.5 with HCl, immediately before the preparation of the nylon-tube-supported enzymes.

$NADP^+$, ATP and glucose 6-phosphate were obtained from Boehringer Corp., (London) Ltd., London W.5, U.K. Boron trifluoride diethyl etherate, 1-chloro-2:3-epoxypropane, 1,6-diaminohexane, 1,2-diaminoethane, *N*-ethylmorpholine and aq. 25% (w/v) glutaraldehyde were purchased from BDH Chemicals Ltd., Poole, Dorset, U.K. Adipamide was purchased from Cambrian Chemicals Ltd., Croydon CR9 3AL, Surrey, U.K.

Nylon tube with a 1mm internal diameter made from type-6 nylon was purchased from Portex Ltd., Hythe, Kent, U.K.

Ether and dichloromethane were redistilled and stored over $CaCl_2$. Methanol was redistilled and stored over $MgSO_4$.

Preparation of triethyloxonium tetrafluoroborate

The procedure used for preparing triethyloxonium tetrafluoroborate followed closely the method of Meerwein (1966). 1-Chloro-2:3-epoxypropane (12.5g) was added to a refluxing solution of 25g of boron trifluoride diethyletherate in 250ml of dry ether vigorously stirred with a magnetic stirrer. When all the former reagent had been added the mixture was refluxed for a further hour and then allowed to cool to room temperature with continuous stirring. The precipitate of triethyloxonium tetrafluoroborate was then washed thoroughly with ether, excess of ether was decanted off, and the compound dissolved in dichloromethane. The triethyloxonium tetrafluoroborate was always used within 24h of its preparation.

Preparation of bisimidates

The preparation of bisimidates from adipamide and acetylated 1,2-diaminoethane is shown in Fig. 1.

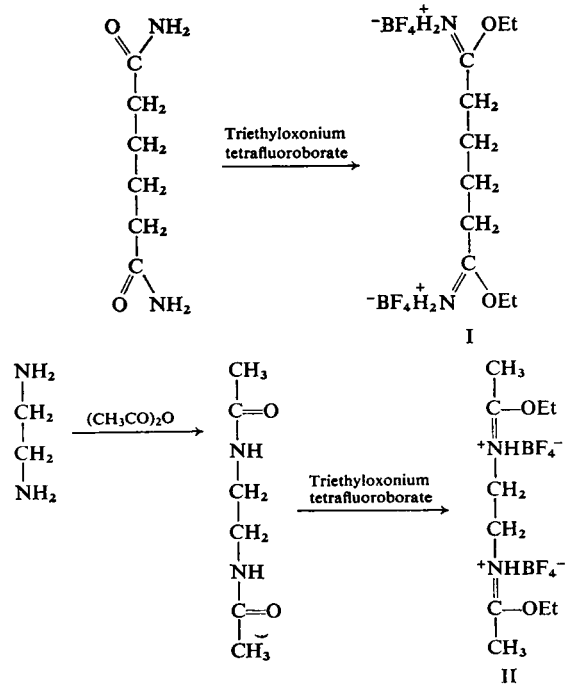

Fig. 1. *Preparation of bisimidates by O-alkylation of diamides with triethyloxonium tetrafluoroborate*

I, Ethyl adipimidate tetrafluoroborate; II, *NN*-bis(acetimidate tetrafluoroborate)1,2-diaminoethane.

Bisimidate I. Adipamide (10g) was suspended in 160ml of 10% (w/v) triethyloxonium tetrafluoroborate in dichloromethane and stirred vigorously at room temperature for 2h. Ethyl adipimidate tetrafluoroborate was formed as a viscous clear light-brown oil which was separated and washed with dichloromethane and ether. The excess of ether was removed by incubating the compound over concn. H_2SO_4 in an evacuated desiccator at room temperature. The final product was stored under these conditions.

Bisimidate II. 1,2-Diaminoethane was dissolved in ether and cooled in a salt–ice bath. Acetic anhydride was slowly added with continuous stirring until a sample portion of the resulting suspension gave no characteristic orange colour on addition to a solution of trinitrobenzenesulphonic acid in a saturated solution of sodium tetraborate. The precipitate was collected by filtration and thoroughly washed with ether. Typically 5g of the acetylated derivative was stirred in 80ml of 20% (w/v) triethyloxonium tetrafluoroborate in dichloromethane. A few minutes after addition the compound dissolved to give a clear solution which persisted for several minutes before an oily precipitate appeared. This oil was converted slowly into a solid by overnight stirring. The solid was collected by filtration, washed with dichloromethane and ether and stored under the same conditions as bisimidate I.

The i.r. spectra of the above compounds were obtained on a Unicam SP 200G grating i.r. spectrophotometer. The spectra of the solid compounds were obtained by using the KBr disc technique, whereas oils were measured by placing the material between rock-salt plates.

Adipamide gave a spectrum typical of an unsubstituted amide with the following strong absorption bands: 3380cm^{-1} and 3190cm^{-1}, symmetrical and asymmetrical NH_2 stretch; 1650cm^{-1}, C=O stretch, 1415cm^{-1}, C–N stretch; 665cm^{-1}, NH_2 wag.

Acetylated 1,2-diaminoethane gave a spectrum typical of an *N*-monosubstituted amide with the following strong absorption bands: 3366cm^{-1} and 3090cm^{-1} (weaker), NH stretch; 1660cm^{-1}, C=O stretch; 1567cm^{-1}, CNH vibration; 732cm^{-1}, NH_2 wag.

Bisimidate I had a spectrum with the following strong absorption bands: 3360cm^{-1} and 3124cm^{-1}, NH_2^+ stretch; 1710cm^{-1}, C=N stretch; 1590cm^{-1}, NH_2^+ deformation; 1250cm^{+1} to 900cm^{-1} (very broad), due to BF_4^- anion and C–O stretch.

Bisimidate II had a spectrum with the following strong absorption bands: 3250cm^{-1} and 2980cm^{-1}, NH^+ stretch; 1670cm^{-1}, C=N stretch; 1550cm^{-1}, NH^+ deformation; 1200cm^{-1} to 900cm^{-1} (very broad), due to BF_4^- anion and C–O stretch. No absorption appeared in the region from 900cm^{-1} to 600cm^{-1}.

Activation of nylon tube by alkylation

Nylon tube was activated by drawing a solution of triethyloxonium tetrafluoroborate in dichloromethane into the tube by using a vacuum pump, sealing its ends and incubating at room temperature. Alkylation was terminated by perfusing the tube with dichloromethane for 60s. The tube was then perfused with 1,6-diaminohexane for 60s, the ends of the tube were sealed with the tube full of the reagent and the tube was incubated at room temperature for 30min. Finally the tube was perfused with distilled water overnight. Nylon tube treated in this way is referred to as amine-substituted nylon tube (Fig. 2).

Immobilization of enzymes on modified nylon tube by the use of cross-linking reagents

Amine-substituted nylon was activated with either glutaraldehyde or bisimidate as illustrated in Fig. 2. In the first case 5% (w/v) glutaraldehyde in 0.1M-boric acid, adjusted to pH8.5 with NaOH, was pumped through the tube for 10min, The tube was then washed with 0.1M-*N*-ethylmorpholine–HCl, pH8.5, for 10min, filled with enzyme solution and incubated at 4°C for 2h. In the second case 4% (w/v) bisimidate in 20% (v/v) *N*-ethylmorpholine in methanol was pumped through the tube for 10min, the tube was washed with methanol for 5min and filled immediately with enzyme solution. The tube was then incubated at 4°C for 2h. In both methods enzyme remaining free in solution in the tube after incubation was collected by washing out the post-coupling supernatant with a known volume of 0.1M-Tris, adjusted to pH8.0 with HCl, containing 0.25M-NaCl. The tube was then perfused for 2h with the same buffer.

The amount of enzyme immobilized by the above methods was estimated by equating it to the amount of enzyme activity which disappeared from the coupling solution. This was assumed to be valid for coupling conditions in which the enzymes were stable over the coupling times used. This assumption was checked by correlating the disappearance of enzyme activity with decrease in the E_{280} of the coupling solution.

Soluble-enzyme assays

Soluble enzymes were assayed by using a Beckmann DBGT spectrophotometer to measure the initial rate of increase in E_{340} which was recorded on a Kipp and Zonen BD8 recorder.

Hexokinase was assayed by the addition of 0.01ml of enzyme solution to 3.00ml of a solution of 43mM-Tris–HCl, pH7.6, 40mM-glucose, 6.7mM-$MgCl_2$, 0.45mM-$NADP^+$ and 2.75mM-ATP containing 5ng of glucose 6-phosphate dehydrogenase. Glucose

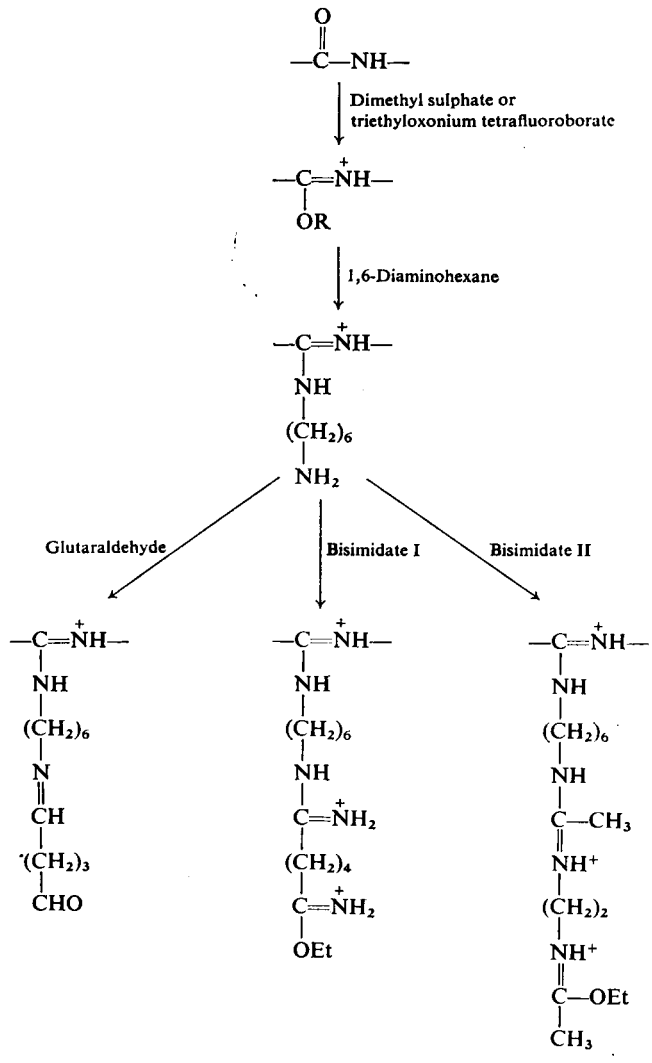

Fig. 2. *Preparation and activation of amine-substituted nylon by O-alkylation with triethyloxoniun tetrafluoroborate*

6-phosphate dehydrogenase was assayed by the addition of 0.01 ml of enzyme solution to 3.00 ml of a solution of 86 mM-Tris–HCl, pH 7.6, 6.7 mM-$MgCl_2$, 0.22 mM-$NADP^+$ and 0.55 mM-glucose 6-phosphate. Both assays were performed at 25°C.

Nylon-tube-supported-enzyme assays

A gradientless recirculation reactor based on the system described by Ford *et al.* (1972) was used to assay the activity of nylon-tube-supported enzyme derivatives. The reservoir was fitted with a water jacket connected to a water bath maintained at 25°C. A flow of 25 ml/min was achieved in the system with a Watson–Marlow peristaltic pump. The initial rate of increase in E_{340} was measured with a Beckmann DBGT spectrophotometer fitted with a flow cell of 1 cm light-path and the output was recorded with a Kipp and Zonen BD8 recorder.

To assay the activity of hexokinase, 12 ml of a solution of 83 mM-Tris–HCl, pH 7.6, 8.3 mM-$MgCl_2$, 2.75 mM-ATP, 0.45 mM-$NADP^+$, 33 mM-glucose containing 20 ng of glucose 6-phosphate dehydrogenase was used. The reaction was started by the addition of the glucose (0.2 ml, 2.0 M).

To assay the activity of glucose 6-phosphate dehydrogenase, 12 ml of a solution of 83 mM-Tris–HCl, pH 7.6, 8.3 mM-$MgCl_2$, 0.45 mM-$NADP^+$ and

0.55mM-glucose 6-phosphate was used. The reaction was started by the addition of the glucose 6-phosphate (0.4ml, 16.5mM).

In both assays the length of the nylon-tube-supported enzyme incorporated in the recirculation system was chosen such that the amount of substrate converted into product per passage through the tube was less than 2% of the total amount of substrate initially present. In practice the lengths of tube were 3, 5 and 10cm.

The activity of the nylon-tube-supported enzyme was calculated from the formula:

$$\text{Tube activity} = \frac{V_0 \cdot V_f}{6.2\,L}\ \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{m}^{-1}$$

where V_0 is the initial increase in E_{340}/min, V_f is the fluid volume of the system in ml, 6.2 is the extinction coefficient of $NADP^+$ at 340nm in litre·mmol^{-1}·cm^{-1} (Siegel *et al.*, 1959) and L is the length of the nylon tube in metres.

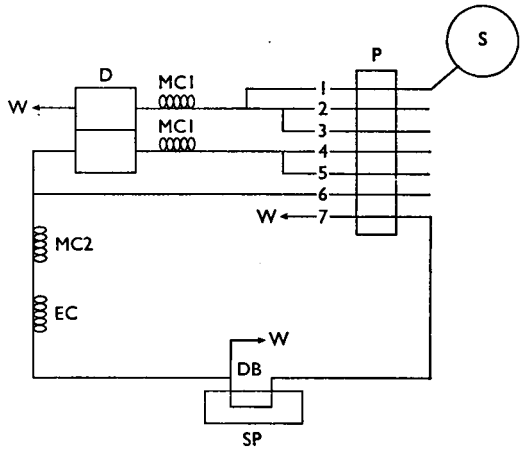

Fig. 3. *Flow system used for the study of the thermal stability of nylon-tube-supported enzyme derivatives*

This sytem was used for the determination of nylon-tube-supported hexokinase and nylon-tube-supported glucose 6-phosphate dehydrogenase activity. The pump tubing lines 1,2,3,4,5 and 6 gave flow rates of 0.16, 1.20, 0.60, 1.20, 0.05 and 2.00ml/min respectively. For the determination of nylon-tube-supported hexokinase activity, 4.5mM-ATP in 4.5mM-$NADP^+$, 0.2M-Tris–HCl, pH7.6, (containing 13.4mM-$MgCl_2$), air, 2mM-glucose in 0.25mM-NaCl, and glucose 6-phosphate dehydrogenase (50μg/ml) were pumped through the pump tubing lines 1, 2, 3, 4 and 5 respectively. The same system was used for the determination of glucose 6-phosphate dehydrogenase activity with the following alterations: 4.5mM-$NADP^+$ in 4.5mM-glucose- 6-phosphate; 0.25M-NaCl and water were pumped through the pump tubing lines 1, 4 and 5 respectively. Samples were assayed at the rate of 40 per h, and a 2:1 (v/v) wash/sample ratio was used throughout. For the meaning of the symbols see the Experimental section.

Automated analysis

Nylon-tube derivatives of hexokinase and glucose 6-phosphate dehydrogenase were assayed by using the flow system described in Fig. 3, and co-immobilized nylon-tube derivatives of hexokinase and glucose 6-phosphate dehydrogenase were assayed by using the flow system shown in Fig. 4. The sampler (S) dialyser (D) and pump (P) were standard Technicon equipment. Absorbance was measured by using a Beckmann DBGT spectrophotometer fitted with a flow-through cuvette of 1cm light-path. The mixing coil MC2 and the enzyme coil were contained in a thermostatically controlled water bath. The mixing coil MC3 used in the system described in Fig. 4 was contained in a separate thermostatically controlled bath. All automated analyses were performed at the rate of 40 samples per h, a 2:1 sample/wash ratio being used. Other symbols used in the Figures describing the flow systems are as follows: MC, mixing coil; DB, de-bubbler; EC, enzyme coil; W, waste.

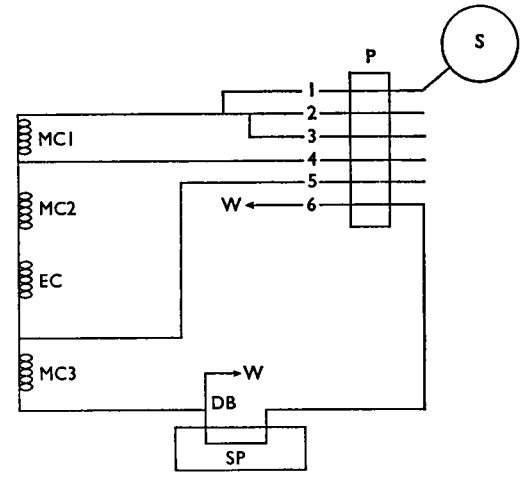

Fig. 4. *Flow system for the use of nylon-tube-co-immobilized hexokinase and glucose 6-phosphate dehydrogenase in the automated analysis of glucose*

The pump tubing lines 1, 2, 3, 4, 5, 6 and 7 gave flow rates of 0.23, 1.40, 0.32, 1.60, 0.32, 0.32, and 1.00ml/min respectively. Sample + 0.1M-Tris–HCl, pH7.6 (containing 8.0mM-$MgCl_2$ and 0.25M-NaCl) + air + 0.1M-Tris–HCl, pH7.6 (containing 8.0mM-$MgCl_2$ and 0.25M-NaCl) + air and 1.0mM-$NADP^+$ in 1.7mM-ATP was pumped through the pump tubing lines 1, 2, 3, 4, 5 and 6 respectively. Samples were assayed at the rate of 40 per h, and a 2:1 (v/v) sample/wash ratio was used throughout.

Results

Homogeneity of alkylation

Two 1m lengths of amine-substituted nylon tube were prepared by alkylation with triethyloxonium tetrafluoroborate [20% (w/v) in dichloromethane for 30min] followed by reaction with diaminohexane.

Hexokinase (0.5mg) was immobilized on one tube with glutaraldehyde and on the other with bisimidate I. Each tube was then cut into 5cm lengths, ten of which were selected at random and assayed. The glutaraldehyde derivative was found to have an activity of $5.38\pm0.35\,\mu mol\cdot min^{-1}\cdot m^{-1}$ (10) and the bisimidate derivative an activity of $5.38\pm0.60\,\mu mol\cdot min^{-1}\cdot m^{-1}$ (10). These results show that the methods used for the preparation of nylon-tube-supported enzymes yield products in which the enzyme is uniformly coated along the entire length of the tube. Henceforth the activity of nylon-tube derivatives was determined by assaying a short segment cut out of the centre portion of the tube. Such a segment was considered representative of the entire tube.

Variation of the time of alkylation

About 8m of nylon tube was rapidly filled with 20% (w/v) triethyloxonium tetrafluoroborate in dichloromethane. At various times 1m lengths of tube were cut off and washed with dichloromethane and treated with 1,6-diaminohexane as described above. The tubes were activated with glutaraldehyde and filled with 2.8mg of hexokinase dissolved in 0.1M-*N*-ethylmorpholine, pH8.5. After being washed, the derivatives were assayed for hexokinase activity. The results of this experiment are shown in Table 1. From 10min onwards the time of alkylation with triethyloxonium tetrafluoroborate had no effect on the amount of hexokinase immobilized, which corresponded to 100% of the enzyme present in the coupling solution. The activity of the tubes was maximal at 10min and remained constant till after 60min when it appears to diminish. The activity of soluble hexokinase is 140 units$\cdot mg^{-1}$, whereas the highest activity retained after immobilization was 4.7 units$\cdot mg^{-1}$ (1 unit is 1μmol of substrate transformed/min). Thus more than 96% of hexokinase activity was lost on immobilization. Although the amount of enzyme immobilized remained constant with alkylation times of 10min and longer there were indications that the degree of alkylation was still increasing. The colour produced by the reaction of glutaraldehyde with the modified tubes varied from a pale yellow with the 5min tube, to orange-brown obtained with the 90min tube.

Variation of the concentration of triethyloxonium tetrafluoroborate

Lengths (1m) of nylon tube were alkylated for 30min with different triethyloxonium tetrafluoroborate concentrations, made to react with 1,6-diaminohexane and glutaraldehyde as described above and filled with 3.5mg of hexokinase in 1ml of 0.1M-*N*-ethylmorpholine, pH8.7. Table 2 shows that maximum enzyme immobilization was achieved with 10% (w/v) triethyloxonium tetrafluoroborate in dichloromethane.

Effect of enzyme concentration in the coupling mixture

The effect of the enzyme concentration in the coupling mixture on the activity of the resulting nylon-tube-supported enzymes was investigated. Amine-substituted nylon tube was prepared with triethyloxonium tetrafluoroborate [17% (w/v) in dichloromethane; 30min] and 1,6-diaminohexane. The activity of these derivatives are presented in Table 3 (hexokinase) and Table 4 (glucose 6-phosphate dehydrogenase).

Table 1. *Effect of the time of alkylation with triethyloxonium tetrafluoroborate on the immobilization of hexokinase on nylon tube*

Experimental details are given in the text. Coupling conditions for hexokinase were 2.8mg of hexokinase/m of tube.

Alkylation time (min)	Hexokinase immobilized (mg/m)	Tube activity (μmol/min per m)	Activity of immobilized hexokinase (μmol/min per mg)
5	1.8	8.7	4.3
10	2.7	12.6	4.7
20	2.6	10.3	4.0
30	2.5	8.9	3.5
40	2.8	10.8	3.9
60	2.8	10.3	3.7
90	2.8	6.2	2.2

Table 2. *Effect of the variation of the concentration of triethyloxonium tetrafluoroborate on the immobilization of hexokinase on nylon tube*

Nylon tubes (1.0m) were alkylated for 30min; 3.5mg of hexokinase per tube was used in the couplings. Further details are given in the text.

Triethyloxonium tetrafluoroborate (%, w/v)	Hexokinase immobilized (mg/m)	Tube activity (μmol/min per m)	Activity of immobilized hexokinase (μmol/min per mg)
4	0.8	5.3	6.8
10	2.7	7.7	2.9
20	2.7	8.5	3.2

Table 3. *Variation of hexokinase concentration in the coupling conditions*

Nylon tube was activated with triethyloxonium tetrafluoroborate [17% (w/v) in dichloromethane] for 30min. The alkylated tube was caused to react with 1,6-diaminohexane and activated with either glutaraldehyde (A) or bisimidate I (B).

Hexokinase in coupling (mg/m)	Hexokinase immobilized (mg/m)	Tube activity (μmol/min per m)	Activity of immobilized hexokinase (μmol/min per mg)
(A)			
3.00	1.86	15.3	8.2
1.00	1.00	17.5	17.5
0.33	0.33	8.3	25.0
0.11	0.11	3.5	31.6
(B)			
3.00	2.58	15.0	5.8
1.00	1.00	19.8	19.8
0.33	0.33	3.3	10.0
0.11	0.11	1.8	16.3

The nature of the cross-linking reagent used to immobilize hexokinase did not produce any significant difference in the activity of the tubes although with bisimidate I the maximum amount of enzyme immobilized was 40% greater than that with glutaraldehyde. In both cases the maximum tube activity was realized with 1mg of hexokinase per metre.

An important feature of the results was the dependence of the retention of enzymic activity on the amount of enzyme immobilized. In general the greater the amount of enzyme immobilized the smaller the retention of activity. This is clearly illustrated by the results for hexokinase immobilized with glutaraldehyde, and glucose 6-phosphate dehydrogenase immobilized with bisimidate I. Tube activities for glucose 6-phosphate dehydrogenase were lower than those for hexokinase, and the variation in activity was not so pronounced.

Although free glucose 6-phosphate dehydrogenase (350 units·mg^{-1}) has over twice the activity of free hexokinase (140 units·mg^{-1}), the highest tube activity obtained for the former is 8-fold less than that for hexokinase. The percentage retention of hexokinase activity varied from 23 to 3% and that of glucose 6-phosphate dehydrogenase from 3.6 to 0.2%.

Since it was intended to use co-immobilized nylon-tube derivatives of the enzymes in a linked assay for glucose the lower activity of the indicator enzyme would detract from the efficiency of the system. An investigation of different coupling conditions for glucose 6-phosphate dehydrogenase was made in an attempt to obtain more active derivatives. Lengths (1m) of nylon tube were alkylated with 17% (w/v) triethyloxonium tetrafluoroborate in dichloromethane, a 1,6-diaminohexane spacer group was inserted and the amine-substituted tube activated with glutaraldehyde. The tubes were filled with 0.25mg of enzyme in 0.1M-*N*-ethylmorpholine–HCl, pH8.5, containing various substrates, inhibitors and activators. The concentration of compounds added was as follows: (*a*) $MgCl_2$ (6.7mM); (*b*) $MgCl_2$ (6.7mM) and $NADP^+$ (2.7mM); (*c*) $MgCl_2$ (6.7mM) and glucose 6-phosphate (9.2mM); (*d*) $MgCl_2$ (6.7mM) and ATP (3.2mM); (*e*) $MgCl_2$ (6.7mM), $NADP^+$ (2.7mM) and glucose 6-phosphate (9.2mM); (*f*) dithiothreitol (5.4mM). In all these cases 100% of the supernatant enzyme activity disappeared and the activity of the tubes was 0.8, 1.0, 1.3, 0.6, 1.4 and 1.4μmol·min^{-1}·m^{-1} respectively. The tube activity of a derivative prepared without any added substance in the coupling conditions was 1.4μmol·min^{-1}·m^{-1}. Thus none of these conditions improved the activity of immobilized glucose 6-phosphate dehydrogenase.

Thermal stability of nylon-tube-supported hexokinase

The stability of nylon-tube-supported hexokinase was determined by using the automated flow system shown in Fig. 3. The sample pots contained alternatively 4.5mM-$NADP^+$, 4.5mM-ATP and water. Tris–HCl (0.2M; pH7.6), containing 13.4mM-$MgCl_2$, 2.0mM-glucose in 0.25M-NaCl and 50μg of glucose 6-phosphate dehydrogenase/ml were pumped through lines 2,4 and 5 respectively. The length of the nylon-tube–hexokinase derivative was chosen such that when the tube was incorporated into the system the conversion of substrate into product was less than 15%. This condition was necessary so that the absorbance changes due to enzyme activity would be an approximation to the initial velocity of the immobilized enzyme. Allowance was made for the intrinsic absorbance of $NADP^+$ and ATP in the samples when the absorbance change due to enzyme activity was measured by subtracting the absorbance changes obtained by pumping samples through the system in the absence of nylon-tube–hexokinase derivative. These 'blank' absorbance changes were of the order of 0.10 whereas the absorbance changes due to enzyme activity were in the range 0.1–0.2 at the beginning of a stability determination. The thermal-stability experiments were performed at 58°C. To measure the tube activity simultaneously with heating the enzyme derivative to this temperature the following procedure was used. Samples were pumped through the system before the enzyme derivative was inserted, and the pump was stopped a few seconds before the first sample reached the position at which

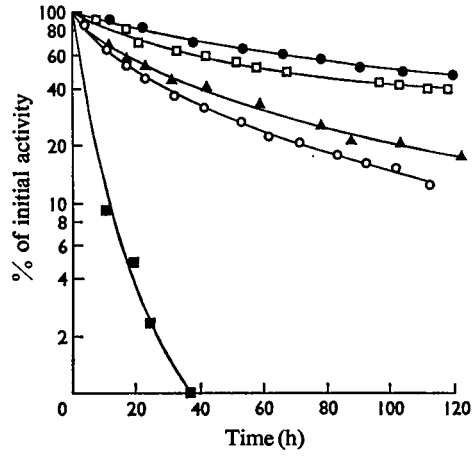

Fig. 5. *Stability of nylon-tube-supported hexokinase at 58°C*

The flow system described in Fig. 3 was used and full experimental details are given in the text. The tubes were alkylated with 20% triethyloxonium tetrafluoroborate for 5min (○), 20min (▲), 40min (□) and 90min (●). The stability of free hexokinase was also determined (■).

the nylon tube was to be inserted. The hexokinase derivative was then inserted into the system and the pump immediately restarted. The first activity measurement was then taken to be 100% activity at zero time in the stability determination.

The stability of nylon-tube-supported hexokinase at 58°C is illustrated in Fig. 5. The derivatives used were tubes that had been alkylated for 5, 20, 40 and 90min respectively, and are described in Table 1. All four derivatives were considerably more stable than free hexokinase, which had no detectable activity after less than 40min. Free hexokinase ($40\mu g \cdot ml^{-1}$) was dissolved in 0.1M-Tris–HCl, pH7.6, incubated at 58°C and 0.01ml samples were withdrawn and assayed at intervals. To test if the difference in stability between the free and nylon-supported enzymes was caused by the intermittent presence of $NADP^+$ and ATP in the immobilized enzyme assays, free hexokinase was incubated at 58°C in the presence of 0.25mM-$NADP^+$, 0.25mM-ATP and 6.7mM-$MgCl_2$. However, this had no significant effect on the stability of the free enzyme. A similar experiment performed in the presence of 1.0mM-glucose produced the same result.

Different alkylation times with triethyloxonium tetrafluoroborate had a significant effect on the thermal stability of the hexokinase derivatives. The longer the alkylation time the more stable was the derivative. After 100min the 5min tube retained 15% of its activity, whereas the 90min tube retained 50%.

Table 4. *Variation of glucose 6-phosphate dehydrogenase concentration in the coupling conditions*

Nylon tube was activated with triethyloxonium tetrafluoroborate [17% (w/v) in dichloromethane] for 30min. The alkylated tube was caused to react with 1,6-diaminohexane and activated with either glutaraldehyde (A) or bisimidate I (B).

	Glucose 6-phosphate dehydrogenase in coupling (mg/m)	Glucose 6-phosphate dehydrogenase immobilized (mg/m)	Tube activity (μmol/min per m)	Activity of immobilized glucose 6-phosphate dehydrogenase (μmol/min per mg)
(A)				
	1.50	1.50	2.0	1.3
	0.75	0.75	2.4	3.1
	0.25	0.25	1.4	5.5
	0.10	0.10	0.2	2.1
(B)				
	3.00	2.16	1.3	0.6
	0.86	0.86	1.6	1.8
	0.43	0.43	2.4	5.6
	0.09	0.09	1.1	12.8

Thermal stability of nylon-tube-supported glucose 6-phosphate dehydrogenase

The flow system used for measurement of the activity of nylon-tube-supported hexokinase was adapted for measurement of the dehydrogenase activity as described in Fig. 3. Otherwise the experiments were performed exactly as described for hexokinase.

The nylon-tube–enzyme derivatives listed in Table 4 were incubated at 58°C and their stabilities compared with that of the free enzyme. The free enzyme ($20\mu g \cdot ml^{-1}$) was dissolved in 0.1M-Tris–HCl, pH7.6, incubated at 58°C, and 0.01ml samples were withdrawn and assayed at intervals. After 10min 99% of the free enzyme activity had been lost. The presence of either $NADP^+$ (0.25mM) and $MgCl_2$ (6.7mM) or glucose 6-phosphate (0.25mM) and $MgCl_2$ (6.7mM) failed to make any significant difference to the stability of the enzyme at this temperature. The stability of nylon-tube-supported glucose 6-phosphate dehydrogenase was much greater than that of the free enzyme for all the derivatives tested.

When glutaraldehyde was used to immobilize the enzyme the stability of the derivatives increased as the amount of enzyme immobilized increased (Fig. 6). When bisimidate I was used quantitatively similar results were obtained. However, in this case no definite relationship was observed between the stability of the derivatives and the amount of enzyme immobilized.

A feature of the semi-log plots of percentage activity against time for the thermal denaturation of the nylon-tube-supported enzymes (Figs. 5 and 6) is that they are non-linear. This was to be expected if the systems studied comprised populations of enzyme molecules with different stabilities. It was perhaps inevitable that the immobilized enzyme molecules existed in several different environments. The number of covalent bonds between the enzyme and the support, the amino acid residues in the enzyme which are involved in covalent bonding and the geometry of the binding site are just a few of the factors which may contribute to make the microenvironment of every enzyme molecule unique.

Co-immobilization of hexokinase and glucose 6-phosphate dehydrogenase

The properties of nylon-tube derivatives with different ratios of hexokinase to glucose 6-phosphate dehydrogenase immobilized were investigated and the results are presented in Table 5. As expected the activity of the dehydrogenase was lower than that of hexokinase in all three derivatives. However, the efficiency of the system was good since the overall linked activity was over 90% of the maximal dehydrogenase activity for tubes 1 and 2 and 66% for tube 3. Thus the linked co-immobilized system appeared to operate satisfactorily when the activity of the hexokinase was at least twice that of the glucose 6-phosphate dehydrogenase. It was concluded that a tube with 1mg of each enzyme immobilized/m was the best derivative to use in a study of the application of the system in automated analysis.

Use of co-immobilized derivatives of hexokinase and glucose 6-phosphate dehydrogenase in automated analysis

Fig. 7 shows the calibration curve obtained when aqueous glucose standards were assayed with a 1.5m length of nylon-tube-co-immobilized hexokinase and glucose 6-phosphate dehydrogenase (which was prepared with 1mg of each enzyme immobilized/m through glutaraldehyde), inserted at position EC in the flow system shown in Fig. 4. These data show that this linked enzyme derivative can be used for the determination of glucose in the concentration range 1–10mM.

The stabilities of two 1.5m lengths of the same derivative were determined, one at 25°C and the other at 37°C. Water instead of $NADP^+$ and ATP was pumped through line 6, and bovine plasma instead of the aqueous glucose standards was continuously pumped through line 7. At approximately daily intervals the system was washed clear of plasma;

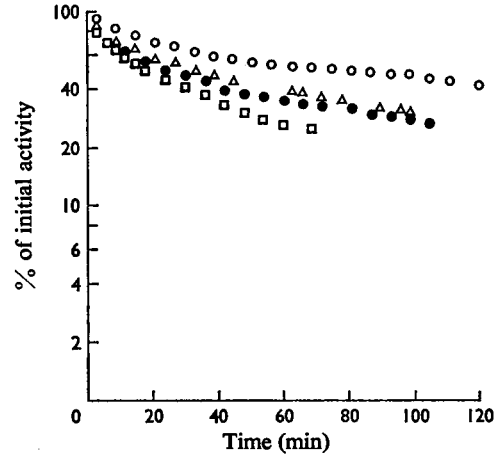

Fig. 6. *Stability of nylon-tube-supported glucose 6-phosphate dehydrogenase at 58°C*

The flow system described in Fig. 3 was used and full experimental details are given in the text. The tubes carried different amounts of enzyme immobilized through glutaraldehyde as described in Table 4. ○, 1.50mg/m; △, 0.75mg/m; ●, 0.25mg/m; □, 0.10mg/m.

Table 5. *Hexokinase and glucose 6-phosphate dehydrogenase co-immobilized on nylon tube*

Nylon tube was activated with triethyloxonium tetrafluoroborate for 30min. The alkylated tube was caused to react with 1,6-diaminohexane and activated with glutaraldehyde. Linked enzyme activity as expressed as μmol of glucose converted into 6-phosphogluconolactone/min per m.

	Enzyme immobilized (mg/m)		Ratio hexokinase/ glucose 6-phosphate dehydrogenase	Tube activity (μmol/min per m)		Activity of immobilized enzyme (μmol/min per mg)		Linked enzyme activity of tube
	Hexokinase	Glucose 6-phosphate dehydrogenase		Hexokinase	Glucose 6-phosphate dehydrogenase	Hexokinase	Glucose 6-phosphate dehydrogenase	
1.	1.16	0.58	2:1	7.5	1.6	6.5	2.7	1.5
2.	0.58	1.16	1:2	3.9	1.5	6.8	1.3	1.4
3.	0.29	1.45	1:5	3.3	1.8	11.4	1.2	1.2

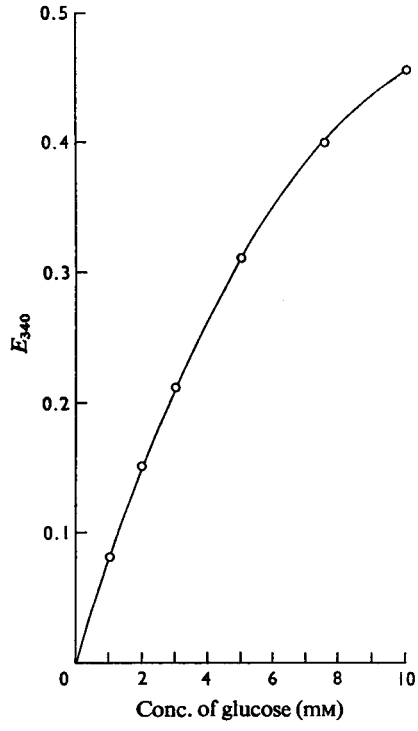

Fig. 7. *Standard curve for the automated determination of glucose by using a 1.5 m length of nylon-tube-co-immobilized hexokinase and glucose 6-phosphate dehydrogenase*

The flow system described in Fig. 4 was used. The glucose concentration denotes the concentration of glucose standards in the sample pots. Full experimental details are given in the text.

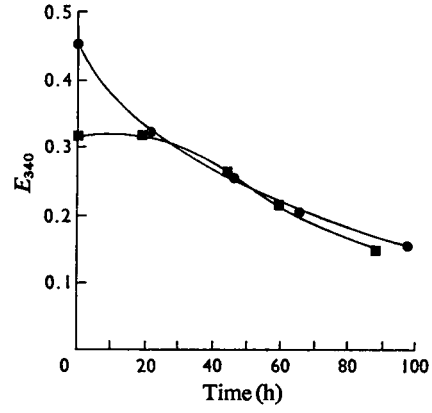

Fig. 8. *Comparison of the stability of nylon-tube-co-immobilized hexokinase and glucose 6-phosphate dehydrogenase at 25°C and 37°C in the presence of plasma*

The flow system shown in Fig. 6 was used. Full experimental details are given in the text. The points represent the steady-state absorbance change obtained when 10mM-glucose was pumped through the sample pump tubing. The water bath was maintained at 25°C (■) and 37°C (●).

$NADP^+$ and ATP were pumped through line 6, 10mM-glucose standards were sampled through line 7 and the steady-state absorbance change was recorded. The results of this experiment are shown in Fig. 8. Although after 90h (25°C) and 100h (37°C) the absorbance change produced by 10mM-glucose had decreased to about 0.15, this was still sufficient for the accurate determination of glucose.

Further, 90h of continuous usage permits over 3500 separate glucose determinations with this system.

The glucose concentration of serum samples from ten individuals was determined by using the flow system shown in Fig. 6. In the first instance a nylon-tube-co-immobilized hexokinase and glucose 6-phosphate dehydrogenase derivative was incorporated and in the second instance the nylon tube was omitted and soluble enzymes were included in the solution pumped through line 6 (1.4 units of hexokinase/ml and 3.6 units of glucose 6-phosphate dehydrogenase/ml). Values in the range 90.3–280.4mg of glucose/100ml were obtained for the samples in the second system and the first system gave values which agreed with these, with a standard error of 2.69%.

Discussion

Previous work on the activation of nylon tube by *O*-alkylation used dimethyl sulphate as the alkylating reagent (Hornby & Morris, 1974). For several reasons triethyloxonium tetrafluoroborate is a preferable reagent for this operation as it is more efficient and does not have the disadvantages associated with dimethyl sulphate. First, *O*-alkylation of nylon tube with the latter reagent was carried out with undiluted reagent for 6min at 100°C. On the other hand, dilute solutions of triethyloxonium tetrafluoroborate in dichloromethane *O*-alkylates nylon tube smoothly at room temperature, and the extent of alkylation can be controlled to give a more reproducible product. Secondly, it has been consistently found with a variety of enzymes that about eight times as much protein can be immobilized on amine-substituted nylon tube prepared with triethyloxonium tetrafluoroborate instead of dimethyl sulphate. Thirdly, although triethyloxonium tetrafluoroborate must be treated as poisonous it is a safer reagent than dimethyl sulphate since it does not give off poisonous vapours and does not cause burns.

It is recommended that the imidate salt of the nylon is treated with a suitable bifunctional amine immediately after *O*-alkylation for two reasons. First, imidate salts are susceptible to hydrolysis, which causes cleavage of the polymer chain, and reaction with an amine results in formation of a stable amidine. Secondly, it has been generally found that although active enzyme derivatives can be prepared by direct reaction of enzyme with the *O*-alkylated nylon tube they are consistently less active than those in which the enzyme is coupled through a spacer molecule and a cross-linking reagent.

The activation of nylon tube by *O*-alkylation provides the starting point for a flexible chemistry of enzyme immobilization. Thus a wide range of derivated nylons can be produced by causing the imidate salt of nylon to react with a variety of different amines such as amino acids, bis alkyl amines, bis aromatic amines, aminothiols, etc.

The different functional groups introduced on to the nylon in this way allow different chemistries to be used for the immobilization of the enzyme.

For a given chemistry the degree of alkylation allows the amount of functional groups on the nylon to be controlled, which in turn may govern the number of covalent bonds between the nylon and the immobilized enzyme. This may affect both the activity and the stability of the immobilized enzyme. Thus increasing the time of alkylation with triethyloxonium tetrafluoroborate yielded hexokinase derivatives with increasing thermal stability.

Despite the low retention of glucose 6-phosphate dehydrogenase activity on immobilization, hexokinase and the dehydrogenase co-immobilized on nylon tube provided excellent sensitivity and accuracy when used to determine glucose. This was due to the high efficiency of the co-immobilized linked enzyme system. Mosbach & Mattiasson (1970) previously reported an enhancement in the overall conversion of glucose into 6-phosphogluconolactone when hexokinase and glucose 6-phosphate dehydrogenase were immobilized on the same support. Comparison of the dehydrogenase activity of tubes with the dehydrogenase immobilized alone and in the presence of hexokinase reveals that they are very similar (Tables 4 and 5]. Therefore it was considered to be of no practical advantage to investigate the performance of the linked system with the enzymes immobilized on separate tubes and incorporated into the auto-analyser flow system. Clearly the overall performance of the co-immobilized system for the automated assay of glucose will be increased if the activity retained on immobilization is increased, particularly for glucose 6-phosphate dehydrogenase. A rational approach to realizing this aim would be to attempt the co-immobilization of the enzymes to various modified nylons with different methods of attachment. This approach is rendered possible owing to the flexible chemistry afforded by *O*-alkylation.

The loss of activity of the co-immobilized linked enzyme system in the continuous presence of plasma is the major drawback in the application of the system at the present stage of development. However, the method used to test the long-term stability differed from operational conditions in several ways. When $NADP^+$ and ATP are also continuously present they may stabilize the enzyme against denaturation, and the introduction of a wash between plasma samples may also be beneficial. Moreover, the pH used may be optimal for determination of glucose, but not with regard to stability of the immobilized enzymes. The use of another buffer system and the presence of a stabilizing substance such as mercaptoethanol may enhance the stability. Finally, manipulation of the chemistry of immobilization may be also advantageous in this report. The application of a stable derivative of this co-immobilized system in the analysis of glucose is an attractive proposition for reasons of specificity, sensitivity, convenience and economics.

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