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The Immobilization of Enzymes on Nylon Structures and their Use in Automated Analysis

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1. Glucose oxidase (EC 1.1.3.4) and urease (EC 3.5.1.5) were covalently attached through glutaraldehyde to low-molecular-weight nylon powder. 2. Immobilized derivatives of glucose oxidase and urease were prepared by cross-linking the respective enzymes within the matrix of a nylon membrane. 3. An improved process is described for the immobilization of glucose oxidase and urease on the inside surface of partially hydrolysed nylon tube. 4. Automated analytical procedures are described for the determination of glucose with each of the three immobilized glucose oxidase derivatives and for the determination of urea with each of the three immobilized urease derivatives. 5. The efficiencies of the three immobilized enzyme structures as reagents for the automated determination of their substrates were compared.

In recent years many applications of immobilized enzymes have been suggested (Melrose, 1971). In particular it has been shown that these materials can be advantageously employed in automated analytical procedures, which are based on the continuous flow-through principle. For example, Hornby *et al.* (1970) showed that glucose oxidase covalently attached to polystyrene tubes could be used for the automated determination of glucose, and the same authors (Filippusson *et al.*, 1972) have since described a new method for the determination of uric acid by using a small packed bed of urate oxidase covalently attached to nylon powder. To date, however, no systematic attempt has been made to compare the efficiencies of different types of immobilized enzyme structures in automated analysis. The present work describes the preparation of three different immobilized enzyme structures, prepared by binding enzyme through glutaraldehyde to the inside surface of nylon tube, to the surface of low-molecular-weight nylon powder and by cross-linking the enzyme protein with glutaraldehyde within the matrix of a nylon membrane. All three enzyme structures were used in automated analysis and their efficiencies for the determination of their respective substrates compared.

Experimental

Enzymes

Urease from jack bean (type VI: Sigma Chemical Co., St. Louis, Mo., U.S.A.) was used without further purification in all experiments. Glucose oxidase from *Aspergillus niger* (type II; Sigma Chemical Co.) was treated in the following manner to minimize contaminating catalase activity. A 200mg batch of the enzyme was incubated for 3h at 37°C in 40ml of 0.1M-KH_2PO_4 buffer containing 4mmol of 3-amino-1,2,4-triazole (Ralph N. Emanuel Ltd., Alperton, Middx., U.K.) and 160μmol of H_2O_2 previously titrated to pH7.5 with 2M-NaOH. The solution was then dialysed at 4°C against water (4×2 litres) and 0.2M-KH_2PO_4 buffer, pH7.5 (1×2 litres). Any precipitate that formed during the dialysis was removed by centrifugation at 20000*g* ($r_{av.}$ 6cm) for 30min at 0°C. This preparation, which is referred to as the triazole-treated glucose oxidase, had a protein content of 3.5mg/ml, the glucose oxidase activity was 18μmol of glucose oxidized/min per mg and the catalase activity was 20μmol of H_2O_2 decomposed/min per mg.

Measurement of enzyme activities

Glucose oxidase activity was measured by the method described in the Seravac Catalogue [Seravac Laboratories (Pty.) Ltd., Holyport, Maidenhead, Berks., U.K.]. Catalase activity was measured by the method of Beers & Sizer (1952).

Preparation of nylon-tube-supported enzymes

Lengths (3m) of nylon tubing (1mm bore) made from type 6 nylon (obtained from Portex Ltd., Hythe, Kent, U.K.) were filled with a solution of 18.6% (w/w) $CaCl_2$ in 18.6% (w/w) water in methanol and incubated for 20min at 50°C. The amorphous nylon, which is removed by this treatment, was then purged from the tubes by washing through with 250ml of water at a flow rate of 10ml/min. The nylon tubes,

etched in this way, were then partially hydrolysed on their inside surface only by perfusing with 3.65M-HCl at 45°C for 40min at a flow rate of 5ml/min. The tubes were then washed with 250ml of water at a flow rate of 10ml/min and then perfused for 20min at 0°C at a flow rate of 2.5ml/min with a solution of 12.5% (w/v) glutaraldehyde (BDH Chemicals Ltd., Poole, Dorset, U.K.) in 0.1M-sodium borate buffer, pH8.5. After being washed through for 5min with 50ml of 0.1M-sodium borate buffer, pH8.5, the tubes were immediately used for the preparation of immobilized enzyme derivatives. Urease was immobilized on the inside surface of a 3m length of nylon tube, treated as described above, by perfusing in a closed loop for 4h at 0°C at a flow rate of 2.5ml/min with 5ml of 0.05M-KH_2PO_4 buffer containing 10mg of enzyme, 25μmol of EDTA and 5μmol of mercaptoethanol, previously adjusted to pH7.0 with dil. NaOH. Finally the tube was washed free of non-covalently attached enzyme by perfusion with 500ml of 0.2M-NaCl at a flow rate of 10ml/min. Nylon-tube glucose oxidase was similarly prepared by perfusing a 3m length of treated nylon tube in a closed loop with 10ml of the triazole-treated glucose oxidase solution for 4h at 0°C at a flow rate of 2.5ml/min. Physically adsorbed enzyme was then removed by washing through the tubes with 500ml of 0.2M-NaCl at a flow rate of 10ml/min.

Preparation of nylon-powder-supported enzymes

Low-molecular-weight type 6 nylon powder (120–150 mesh) was used. This material, which was specially prepared for Dr. M. D. Lilly, University College London, by Imperial Chemical Industries Ltd., Dyestuffs Division, Blackley, Manchester, U.K., contained 240μequiv. of free amino groups and 240μequiv. of free carboxyl groups/g. A 250mg portion of the nylon powder was suspended with rapid stirring in 10ml of 12.5% (w/v) glutaraldehyde in 0.1M-sodium borate buffer, pH8.5, at 0°C for 20min. The nylon powder was then washed on a sintered-glass funnel with 500ml of 0.2M-sodium borate buffer, pH8.5, and immediately used for the preparation of immobilized enzyme derivatives. Urease was immobilized on this material by suspending the powder for 16h at 1°C in 5ml of 0.05M-KH_2PO_4 buffer containing 10mg of urease, 25μmol of EDTA and 5μmol of mercaptoethanol, all previously adjusted to pH7.0 with dil. NaOH. The powder was then packed under gravity into a glass column (4.0cm × 0.2cm bore) and washed free of physically adsorbed enzyme by perfusion with 500ml of 0.2M-NaCl at a flow rate of 2ml/min. Nylon-powder-supported glucose oxidase was prepared by suspending 250mg of the glutaraldehyde-treated powder for 16h at 4°C in 10ml of the triazole-treated glucose oxidase solution. The powder was then packed under gravity into a glass column (4.0 × 0.2cm bore) and purged of non-covalently bound enzyme by washing through with 250ml of 0.2M-NaCl at a flow rate of 2.0ml/min.

Preparation of nylon-membrane-supported enzymes

A type 6 nylon membrane (NRWP 293; Millipore Ltd., Alperton, Middx., U.K.) was mounted in a Technicon dialyser module (Technicon Instruments Co. Ltd., Chertsey, Surrey, U.K.). The upper channel was then perfused in a closed loop at 1°C at a flow rate of 0.23ml/min with 5ml of 0.05M-KH_2PO_4 buffer containing 10mg of urease, 25μmol of EDTA and 5μmol of mercaptoethanol, all previously adjusted to pH7.0 with dil. NaOH, while the lower channel was similarly perfused with 5ml of 12.5% (w/v) glutaraldehyde in 0.1M-sodium borate buffer, pH8.5. After 2h, the membrane was washed free of excess of enzyme and glutaraldehyde by perfusion through both channels of the dialyser module with 250ml of 0.2M-NaCl at a flow rate of 5ml/min. For the preparation of nylon-membrane-supported glucose oxidase the upper channel of the dialyser module was perfused in a closed loop at 1ml/min at 1°C with 10ml of the triazole-treated glucose oxidase solution, while the lower channel was similarly perfused with 12.5% (w/v) glutaraldehyde in 0.1M-sodium borate buffer, pH8.5. After 2h, the system was washed free of excess of enzyme and glutaraldehyde by perfusion of both channels of the dialyser module with 250ml of 0.2M-NaCl at a flow rate of 5ml/min.

Automated analysis

Nylon-supported enzyme derivatives were used for the automated determination of their respective substrates by incorporation into Technicon Auto-Analyzer flow systems. The sampler (S), pump (P), heating bath (HB) and dialyser module (D) were all standard Technicon equipment. Extinction was measured by using a Beckman DBGT spectrophotometer (SPEC) fitted with 1cm-light-path flow-through cuvettes. Other symbols used in the figures describing the flow systems are as follows: MC, mixing coil; DB, debubbler; W, waste.

Glucose was determined spectrophotometrically by measuring the extinction at 349nm of I_2 formed by the reaction of the H_2O_2, produced in the enzymic reaction, with acid KI. The KI reagent was always used within 12h of its preparation and glucose solutions were prepared 24h before their use to ensure complete mutarotation. Fig. 1 shows the flow system used for the determination of glucose with nylon-tube-supported glucose oxidase, and Fig. 2 shows the flow system used for the determination of glucose by using a small packed bed of nylon-powder-supported glucose oxidase. Since it is not possible to perfuse a packed bed with an air-segmented stream, it was

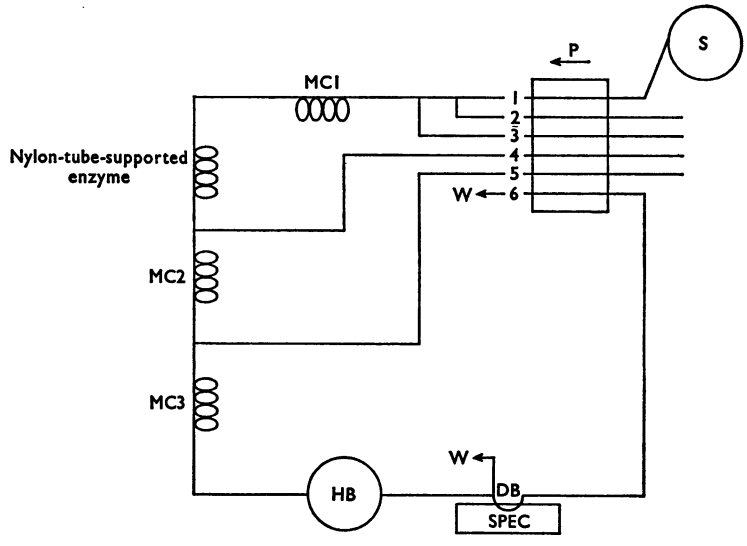

Fig. 1. *Flow system for the use of nylon-tube-supported enzymes in automated analysis*

For the determination of glucose with nylon-tube-supported glucose oxidase, the pump tubing lines 1, 2, 3, 4, 5 and 6 gave flow rates of 1.20, 0.80, 0.23, 1.20, 1.20 and 2.50ml/min respectively. A 2:1 (w/w) wash/sample ratio was used. The nylon-tube-supported enzyme was maintained at 37°C. The mixing coil (MC 2) and the heating bath (HB) were not included in the flow system. Substrate, air, 1.0M-sodium acetate buffer, pH5.0, 1.25M-HCl and 0.25M-KI were pumped through the pump tubing lines 1, 2, 3, 4 and 5 respectively. For the determination of urea with nylon-tube-supported urease the pump tubing lines 1, 2, 3, 4, 5 and 6 gave flow rates of 1.00, 0.80, 0.23, 2.00, 2.00 and 2.00ml/min respectively. The heating bath (HB) and the nylon-tube-supported enzyme were maintained at 37°C. Substrate, air, 5mM-EDTA–0.1M-KH_2PO_4 buffer, pH7.0, reagent A and reagent B were pumped through the pump tubing lines 1, 2, 3, 4 and 5 respectively. For the meaning of the symbols see the Experimental section.

necessary to de-gas the sample stream before perfusing it through the packed bed of nylon-powder-supported glucose oxidase. The sample stream is air-segmented again immediately after leaving the packed bed. Fig. 3 shows the flow system used for the determination of glucose with nylon-membrane-supported glucose oxidase. To ensure equilibration of substrate across the membrane-supported enzyme, the sample or donor stream is pumped into the dialyser module at a flow rate greater than it is pumped out to the exit stream. This maintains a continuous flow of sample from the donor stream to the recipient stream.

Urea was determined by measurement of the ammonia formed in the enzymic reaction by the method of Chaney & Marbach (1962). Reagent A was a mixture of 0.006% (w/v) sodium nitroprusside in aq. 4.7% (w/v) phenol, and reagent B was a solution of NaOCl in 0.5M-NaOH, containing 0.10–0.15% available chlorine. Both reagents were stored in dark bottles. Fig. 1 shows the flow system used for the determination of urea with nylon-tube-supported urease and Fig. 2 shows the corresponding flow system for the determination of urea with a small packed bed of nylon-powder-supported urease. In the latter system the air segmentation was temporarily removed while the sample stream was pumped through the packed bed of immobilized urease. Fig. 3 shows the flow system used for the determination of urea with nylon-membrane-supported urease. As in the case of nylon-membrane-supported glucose oxidase, differential pumping of the donor stream into and out of the dialyser module was employed.

Results

The effect of added enzymically inert protein on the preparation of membrane-supported glucose oxidase was studied. The triazole-treated glucose oxidase solution, containing 3.5mg of enzyme/ml, was used to prepare a membrane that gave an absorbance change of 0.04 when a 20mM-glucose sample was assayed by using the flow system shown in Fig. 3. A second membrane was prepared by using an identical solution of glucose oxidase but with the further addition of 10mg of haemoglobin/ml. This

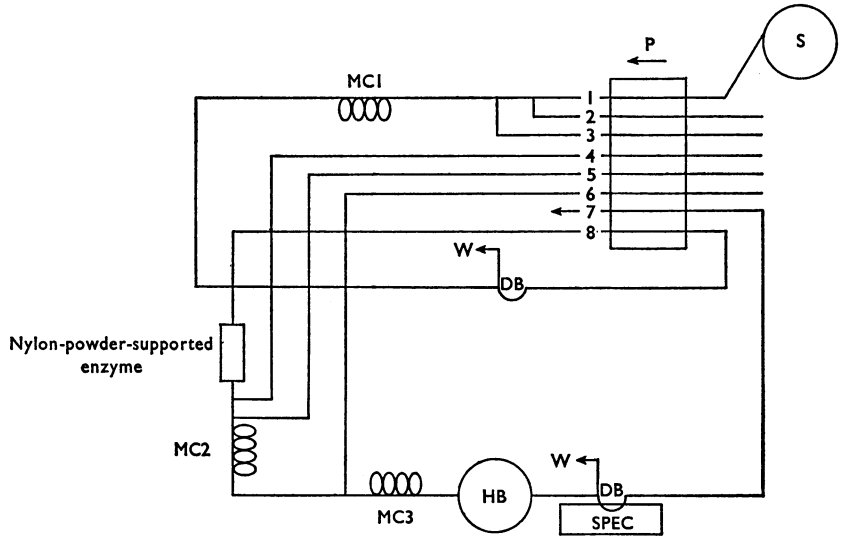

Fig. 2. *Flow system for the use of nylon-powder-supported enzymes in the form of small packed beds in automated analysis*

For the determination of glucose with a small packed bed of nylon-powder-supported glucose oxidase the pump tubing lines 1, 2, 3, 4, 5, 6, 7 and 8 gave flow rates of 2.00, 0.42, 0.42, 0.60, 1.20, 1.20, 2.50 and 1.20ml/min respectively. A 2:1 (w/w) wash/sample ratio was used. The packed bed of nylon-powder-supported enzyme was maintained at 37°C. The mixing coil (MC 2) and the heating bath (HB) were not included in the flow system. Substrate, air, 0.2M-sodium acetate buffer, pH5.0, air, 1.25M-HCl and 0.25M-KI were pumped through the pump tubing lines 1, 2, 3, 4, 5 and 6 respectively. For the determination of urea with a small packed bed of nylon-powder-supported urease the pump tubing lines 1, 2, 3, 4, 5, 6, 7 and 8 gave flow rates of 1.60, 0.60, 0.80, 0.80, 2.00, 2.00, 2.00 and 1.60ml/min respectively. A 2:1 (w/w) wash/sample ratio was used. The heating bath (HB) and the packed bed of nylon-powder-supported enzyme were maintained at 37°C. Substrate, air, 5mM-EDTA–0.1M-KH_2PO_4 buffer, pH7.0, air, reagent A and reagent B were pumped through the pump tubing lines 1, 2, 3, 4, 5 and 6 respectively. For the meaning of symbols see the Experimental section.

membrane gave a change in absorbance of 0.275 when a 5mM-glucose sample was assayed in the prescribed manner and was used in subsequent experiments.

The activities of nylon-tube-supported enzyme derivatives, prepared by the method of Sundaram & Hornby (1970), were compared with those of comparable derivatives prepared from nylon tube that had been previously etched by the process described above. When samples containing 5mM-glucose were assayed as described above activities corresponding to 0.021 and 0.170 increase in extinction at 349nm/m length of nylon-tube-supported glucose oxidase were obtained for the derivatives prepared from the non-etched and etched nylon tube respectively. Similarly when samples containing 0.1mM-urea were assayed in the prescribed manner activities corresponding to 0.028 and 0.250 increase in extinction at 630nm/m length of nylon-tube-supported urease were obtained for the derivatives from the non-etched and etched nylon tubes respectively. These results show that etching increases the activity of nylon-tube-supported urease ninefold and increases the activity of nylon-tube-supported glucose oxidase eightfold.

Fig. 4 shows the effect of sampling rate on the calibration curves obtained when standard solutions of glucose were assayed by using the three nylon-supported glucose oxidase derivatives, nylon-tube-supported glucose oxidase (Fig. 4*a*), nylon-powder-supported glucose oxidase (Fig. 4*b*) and nylon-membrane-supported glucose oxidase (Fig. 4*c*). In all cases increasing the sampling rate decreased the sensitivity of the assay. By reference to standard curves, concurrently compiled by subjecting known concentrations of H_2O_2 to each of the three assay procedures, the efficiencies of the nylon-supported glucose oxidase preparations were evaluated by measuring the percentage reaction of 5mM-glucose samples. When assays were performed at the rate of 20 samples/h, percentage reactions of 3.7, 2.9 and

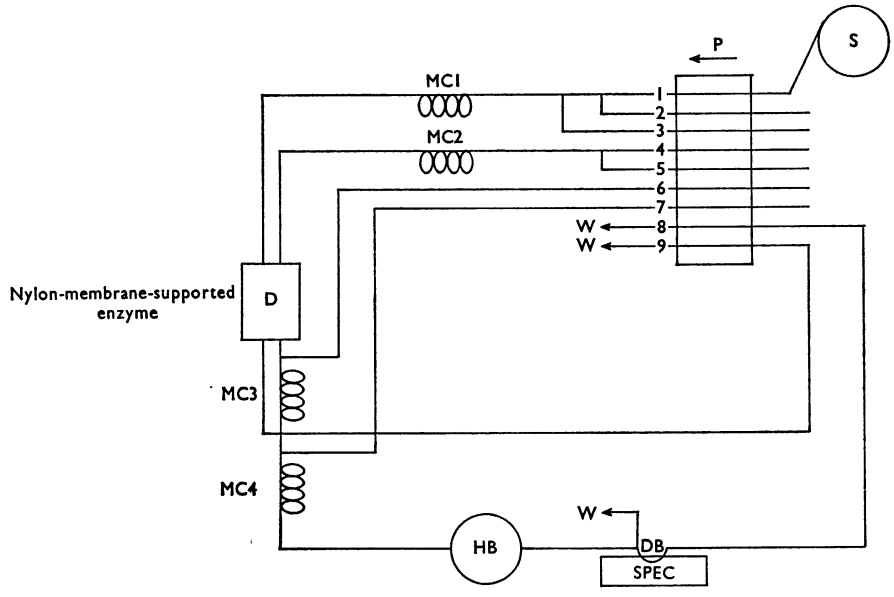

Fig. 3. *Flow system for the use of nylon-membrane-supported enzymes in automated analysis*

For the determination of glucose with nylon-membrane-supported glucose oxidase the pump tubing lines 1, 2, 3, 4, 5, 6, 7, 8 and 9 gave flow rates of 1.20, 0.42, 0.23, 0.80, 0.42, 1.20, 1.20, 2.50 and 1.20ml/min respectively. A 2:1 (w/w) wash/sample ratio was used. The nylon-membrane-supported enzyme was maintained at 37°C. The mixing coil (MC 3) and the heating bath (HB) were not included in the circuit. Substrate, air, 0.20M-sodium acetate buffer, pH5.0, 0.20M-sodium acetate buffer, pH5.0, air, 1.25M-HCl and 0.25M-KI were pumped through the pump tubing lines 1, 2, 3, 4, 5, 6 and 7 respectively. For the determination of urea with nylon-membrane-supported urease the pump tubing lines 1, 2, 3, 4, 5, 6, 7, 8 and 9 gave flow rates of 1.00, 0.42, 0.23, 0.80, 0.42, 2.00, 2.00, 2.00 and 1.20ml/min respectively. A 2:1 (w/w) wash/sample ratio was used. The heating bath (HB) and the dialyser module (D) were maintained at 37°C. Substrate, air, 5mM-EDTA–0.1M-KH_2PO_4 buffer, pH7.0, 5mM-EDTA–0.1M-KH_2PO_4 buffer, pH7.0, air, reagent A and reagent B were pumped through the pump tubing lines 1, 2, 3, 4, 5, 6 and 7 respectively. For the meanings of the symbols see the Experimental section.

0.5 were obtained for the tube, membrane and powder derivatives respectively.

Standard solutions of urea were assayed at several sampling rates with the three nylon-supported urease derivatives. These results are shown in Figs. 5(*a*), 5(*b*) and 5(*c*) for the nylon-tube-, nylon-powder- and nylon-membrane-supported derivatives respectively. For both nylon-tube- and nylon-powder-supported urease an increase in the sampling rate caused a decrease in the amount of colour produced. Each of the three flow systems was calibrated in terms of the amount of ammonia produced by subjecting standard solutions of NH_4Cl to the various assay procedures. In this way the efficiencies of the three derivatives for the assay of urea were compared. When 0.1mM-urea samples were assayed at the rate of 20 samples/h, values for the percentage hydrolysis of 81.3, 16.0 and 7.5 were obtained for the tube, powder and membrane derivatives respectively.

The stabilities of nylon-tube-supported glucose oxidase and nylon-tube-supported urease were studied by continuously subjecting standard solutions of the respective substrates to the assay procedures. On average about 150 samples of each of the substrates were assayed/day over a period of 30 days. During this time a total of 3500 glucose samples was assayed with the nylon-tube-supported glucose oxidase derivative without incurring any loss in the catalytic activity of the preparation, and 5000 urea samples were assayed with the nylon-tube-supported urease derivative without incurring any loss of its activity. The amount of glucose oxidase used for the preparation of the nylon-tube-supported glucose oxidase was sufficient for the determination of not more than 1500 glucose samples if used free in solution, and the corresponding amount of urease was sufficient for the determination of not more than 1100 urea samples when used free in solution.

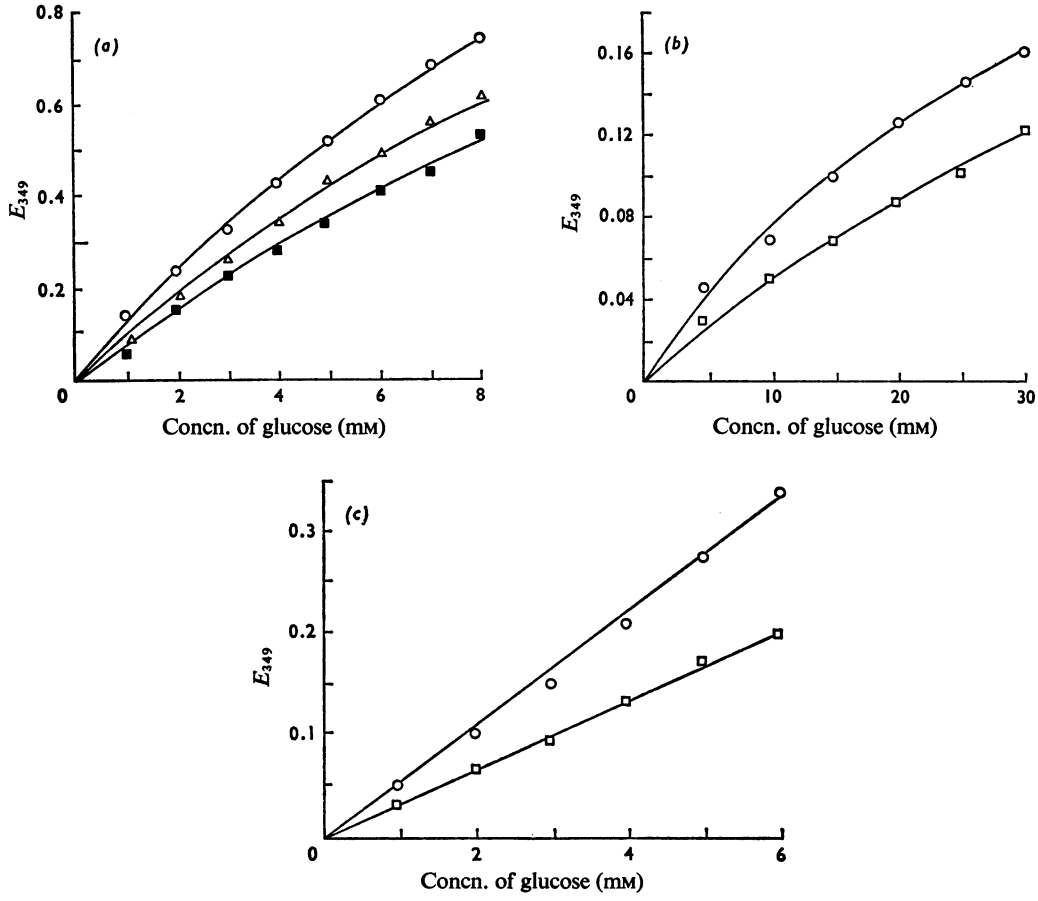

Fig. 4. *Standard curves for the automated determination of glucose with a 3 m length of nylon-tube-supported glucose oxidase (a), 250 mg of nylon-powder-supported glucose oxidase in the form of a small packed bed (b) and nylon-membrane-supported glucose oxidase (c)*

Experimental details are given in the text. o, 20 samples/h; □, 30 samples/h; △, 40 samples/h; ■, 60 samples/h.

Discussion

The application of the three types of immobilized enzyme structures described to automated analysis by using conventional equipment has been established. It is most likely that enzyme protein is immobilized to both nylon tube and nylon powder through covalent linkages formed between the glutaraldehyde and free amino groups on the nylon and free amino groups on the surface of the enzyme protein molecule (Sundaram & Hornby, 1970). However, since the nylon membranes could not be partially hydrolysed without destroying their structural integrity, it was not possible to use the same method of immobilization for the preparation of their insoluble enzyme derivatives. In view of this and since the nylon membranes contain relatively few free amino groups, it is unlikely that the enzyme is immobilized to this structure by a process similar to that described for the nylon tube and the nylon powder. Further, since the membrane derivatives are prepared with reaction mixtures containing both enzyme and glutaraldehyde, it is likely that the enzymic activity is 'trapped' in the membrane via the cross-linking through glutaraldehyde of the enzyme protein around and within the matrix of the membrane. Jansen *et al.* (1971) showed that an immobilized derivative of mercuripapain–chymotrypsin could be prepared by cross-linking the proteins together with glutaraldehyde. Therefore it is possible to explain the increased activity of the

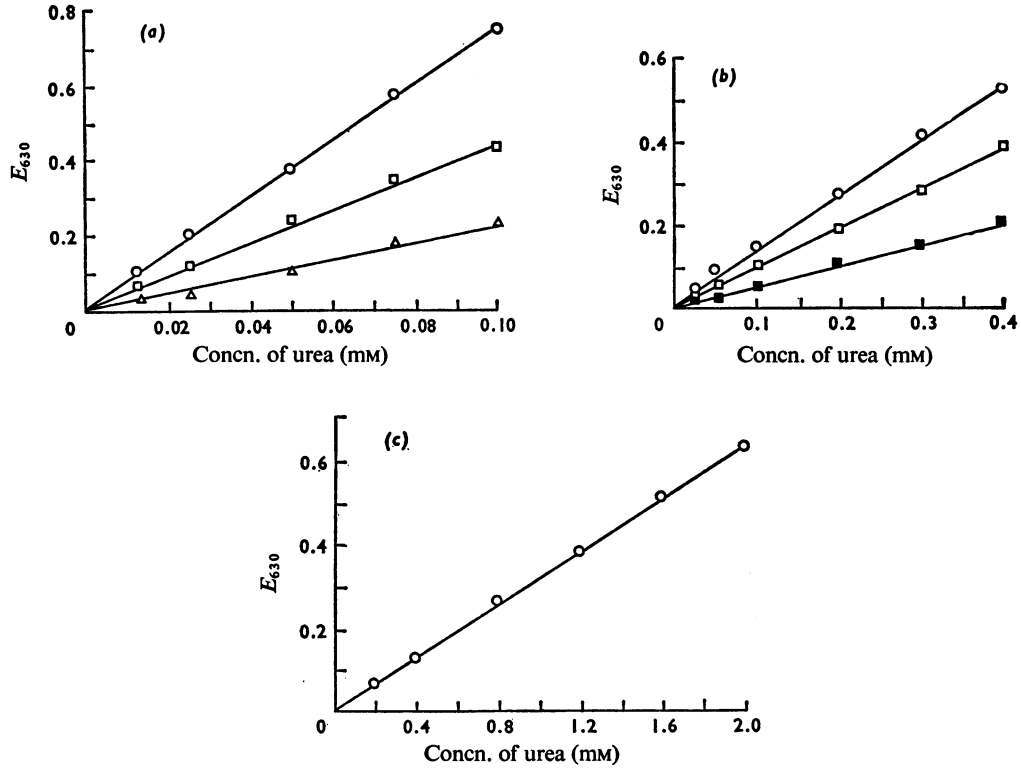

Fig. 5. *Standard curves for the automated determination of urea with a 3m length of nylon-tube-supported urease (a), 250mg of nylon-powder-supported urease in the form of a small packed bed (b), and nylon-membrane-supported urease (c)*

Experimental details are given in the text. o, 20 samples/h; □, 30 samples/h; ■, 40 samples/h; △, 60 samples/h.

nylon-membrane-supported glucose oxidase prepared in the presence of an excess of a catalytically inert protein, such as haemoglobin, in terms of an increased yield of cross-linked protein caused by the overall increase in the initial amount of protein present.

It was shown that pretreatment of nylon tubes with 18.6% (w/v) $CaCl_2$ in 18.6% (w/v) water in methanol increases the activity of the nylon-tube-supported enzyme derivatives. This process removes the regions of amorphous nylon from the inside of the nylon tube and therefore would be expected to increase the available surface area on the inside surface (Du Pont de Nemours and Co., 1970). The greater activity of nylon-tube enzymes, prepared in this way, can therefore be accounted for by the immobilization of more enzyme protein on the increased surface area of the etched tube.

The overall sensitivity of the assay procedures with tube-supported enzymes was always greater than the sensitivity of the corresponding procedures with the membrane-supported derivatives and the powder-supported derivatives in the form of small packed beds. Further, of the three immobilized enzyme structures studied, the tube-supported derivatives were most easily incorporated into Technicon Auto-Analyzer flow systems. The use of the nylon-powder-supported derivatives in the form of small packed beds was complicated by the necessity of removing the air segmentation while the sample stream was perfused through the packed bed, and the use of the nylon-membrane-supported derivatives was complicated by the need for differential pumping across the dialyser module to ensure passage of substrate across the membrane. Further, it was possible to use higher sampling rates with the tube-supported derivatives than with the corresponding powder- and membrane-supported derivatives. This is presumably due, with the powder-supported derivatives, to mixing in the sampling stream when the air segmentation is

removed, and with the membrane-supported derivatives, to diffusion in the enzyme membrane. Therefore, in terms of ease of application, sensitivity of assay and the number of samples assayed in unit time, the nylon-tube-supported enzyme preparations are the preferred structures for use in automated analysis.

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