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Preparation of some Immobilized Linked Enzyme Systems and their Use in the Automated Determination of Disaccharides

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1. Glucose oxidase (EC 1.1.3.4), amyloglucosidase (EC 3.2.1.3), invertase (EC 3.2.1.26) and β-galactosidase (EC 3.2.1.23) were covalently attached via glutaraldehyde to the inside surface of nylon tube. 2. The linked enzyme system, comprising invertase immobilized within a nylon tube acting in series with glucose oxidase immobilized in a similar way, was used for the automated determination of sucrose. 3. The linked enzyme system, comprising β-galactosidase immobilized within a nylon tube acting in series with glucose oxidase immobilized in a similar way, was used for the automated determination of lactose. 4. The linked enzyme system, comprising amyloglucosidase immobilized within a nylon tube acting in series with glucose oxidase immobilized in a similar way, was used for the automated determination of maltose. 5. Mixtures of glucose oxidase and amyloglucosidase were immobilized within the same piece of nylon tube and used for the automated determination of maltose. 6. Mixtures of glucose oxidase and invertase were immobilized within the same piece of nylon tube and used for the automated determination of sucrose.

The usefulness of enzymes as analytical reagents derives principally from the specificity that they exercise over the reactions that they catalyse. However, their general application in routine analysis is limited by the high cost of purified enzymes. Therefore the application of immobilized enzyme derivatives as enzyme substitutes in analytical procedures has been considered in order to exercise a greater economy over the utilization of enzyme protein. Thus Updike & Hicks (1967) and Guilbault & Montalvo (1969) have described the construction and application of enzyme-based electrodes for the specific determination of glucose and urea respectively and Hornby *et al.* (1970) have described the preparation of tube-supported enzyme derivatives and their successful application in automated analytical procedures of the continuous-flow type. To date, however, no significant attempt has been made to evaluate the potential of immobilized linked enzyme systems as analytical materials for the determination of biological compounds.

The specific determination of the disaccharides sucrose, maltose and lactose is very important in, for example, the food industry. These substances can be determined specifically by means of the linked enzyme systems invertase–glucose oxidase, amyloglucosidase–glucose oxidase and β-galactosidase–glucose oxidase respectively. However, the cost of the component enzymes in these assays detracts from their routine usefulness. Therefore the present work describes the preparation of the corresponding immobilized linked enzyme systems and their application in the automated analysis of these disaccharides. In this way it is possible to exercise a greater economy in enzyme utilization, since in contrast with the free enzymes, the corresponding immobilized enzymes are reusable.

Experimental

Enzymes

Grade X invertase (β-fructofuranosidase, EC 3.2.1.26) from *Candida utilis* (Sigma Chemical Co., St. Louis, Mo., U.S.A.), glucose oxidase (EC 1.1.3.4) from *Aspergillus niger* [Grade 1; Boehringer Corporation (London) Ltd., London W5 2TZ, U.K.] and amyloglucosidase (exo-1,4-α-glucosidase; EC 3.2.1.3) of fungal origin (BDH Chemicals Ltd., Poole, Dorset, U.K.) were used without further purification. *Escherichia coli* β-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) was obtained from Boehringer Corporation Ltd. and was treated as follows to remove $(NH_4)_2SO_4$. The $(NH_4)_2SO_4$ suspension (0.35ml) containing 1.75mg of β-galactosidase was added to 1.65ml of 0.2M-KH_2PO_4–0.1mM-dithiothreitol, adjusted to pH7.8 with NaOH, and dialysed against the same buffer (3 × 3 litres). After dialysis the solution was made up to 2.5ml with the buffer and immediately used for the preparation of β-galactosidase supported in nylon tubes.

Enzymes supported in nylon tubes

Lengths (3m) of nylon tube (1mm bore) made from type 6 nylon (obtained from Portex Ltd., Hythe, Kent, U.K.) were pretreated with 18.6% (w/v) $CaCl_2$ in

18.6% (w/v) water in methanol to increase their internal surface area (Inman & Hornby, 1972). The inside surface of the pretreated nylon tubes was then subjected to one of two processes to liberate free amino groups. Either the tubes were partially hydrolysed on their inside surface by perfusion with 3.65M-HCl at 45°C (Inman & Hornby, 1972) or they were filled with and incubated at 70°C with *NN*-dimethyl-1,3-propanediamine (Hornby *et al.*, 1972). Tubes prepared by the former process are referred to as 'hydrolytically cleaved nylon tube' and those prepared by the latter process are referred to as 'non-hydrolytically cleaved nylon tube'.

The 3m lengths of either hydrolytically cleaved or non-hydrolytically cleaved nylon tube were prepared for coupling by perfusion for 1h at 0°C with a solution of 12.5% (w/v) glutaraldehyde (electron-microscopy grade; BDH Chemicals Ltd.) in 0.1M-sodium borate buffer, pH8.5, at a flow rate of 2ml/min. After being washed through for 5min with 50ml of 0.2M-sodium borate buffer, pH8.5, the tubes were immediately used for the preparation of immobilized enzyme derivatives.

The enzyme was dissolved in 2.5ml of 0.2M-KH_2PO_4 buffer, pH7.8, and drawn into a 3m length of the glutaraldehyde derivative of the appropriate nylon tube with a syringe. The ends of the tube were then sealed and the tube was incubated at 4°C for 16h. Protein that was non-covalently bound was then purged from the tube by perfusion with the buffer at 20°C for 1h at a flow rate of 2.5ml/min. The experimental details for the preparation of each of the enzyme derivatives are summarized in Table 1.

Mixed enzyme derivatives were prepared by dissolving the required amount of the disaccharidase and glucose oxidase in 2.5ml of 0.2M-KH_2PO_4 buffer, pH7.8, and drawing the solution into a 3m length of hydrolytically cleaved nylon tube. The ends of the tube were then sealed and the tube incubated at 4°C for 16h. Protein that was non-covalently bound was then purged from the tube by perfusion with buffer at 20°C for 1h at a flow rate of 2.5ml/min.

Two different mixed derivatives of invertase and glucose oxidase were prepared, one from an enzyme solution containing equal amounts of invertase and glucose oxidase and the second from an enzyme solution containing a 2:1 (w/w) ratio of invertase to glucose oxidase. In both cases the tubes were washed with 0.1M-sodium acetate buffer, pH5.0, after the coupling.

A 3m length of a mixed derivative of amyloglucosidase and glucose oxidase was prepared from 2mg of amyloglucosidase and 2mg of glucose oxidase and the tube was washed with 0.1M-sodium acetate buffer, pH5.5, after the coupling.

Automated analysis

The nylon-tube derivatives of single and mixed enzymes were used for the automated determination of their respective substrates by inclusion of the tubes in Technicon autoanalyser flow systems (Figs. 1 and 2). The sampler (S), pump (P) and heating bath (HB) were all standard Technicon equipment. Extinction was measured by using a Beckman DBGT spectrophotometer (SPEC) fitted with flow-through cuvettes of 1cm light-path. Other symbols used in the figures describing the flow systems are as follows: MC, mixing coil: DB, de-bubbler; W, waste.

Two methods were used for the determination of disaccharides by using linked enzyme systems. (1) The appropriate disaccharidase and glucose oxidase were immobilized on to separate lengths of nylon tube and inserted at positions A and B respectively in the flow system shown in Fig. 1. (2) The appropriate disaccharidase and glucose oxidase were immobilized on to the same length of nylon tube and inserted at position A in the flow system shown in Fig. 1. In each case the glucose that was produced by the immobilized disaccharidase was measured by its oxidation with the immobilized glucose oxidase as described by Hornby *et al.* (1970).

The sample stream containing the disaccharide was air-segmented and then mixed with the appropriate buffer before being perfused through the enzyme tube or tubes, which were maintained at 37°C in a thermostatically controlled water bath. After leaving the tube or tubes the sample stream was acidified with 1.25M-HCl, mixed with 0.25M-KI and passed through

Table 1. *Experimental details for the preparation of enzymes supported on nylon tubes*

The general procedure adopted for the preparation of each of these derivatives is described in the Experimental section.

Enzyme	Amount of enzyme used in coupling (mg)	Nylon tube	Washing buffer
Invertase	7.5	Hydrolytically cleaved	0.1M-Sodium acetate, pH5.0
Glucose oxidase	7.5	Hydrolytically cleaved	0.1M-Sodium acetate, pH5.0
Amyloglucosidase	10.0	Hydrolytically cleaved	0.1M-Sodium acetate, pH5.5
β-Galactosidase	1.75	Non-hydrolytically cleaved	1mM-$MgCl_2$, 0.05mM-EDTA in 0.1M-KH_2PO_4, pH6.5

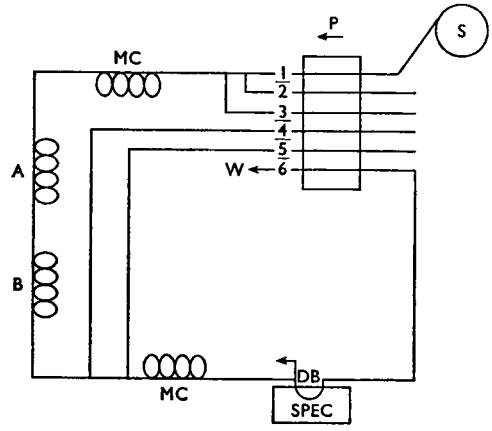

Fig. 1. *Flow system for the use of nylon-tube derivatives of linked enzyme systems in automated analysis*

This system was used for (*a*) the determination of sucrose, maltose and lactose by using derivatives of invertase, amyloglucosidase and β-galactosidase, placed in series with nylon tube that carried glucose oxidase, and (*b*) for the determination of sucrose and maltose by using mixed derivatives of invertase and glucose oxidase and mixed derivatives of amyloglucosidase and glucose oxidase. The pump tubing lines 1, 2, 3, 4, 5 and 6 gave flow rates of 1.20, 0.60, 0.32, 1.20, 1.20 and 2.50ml/min respectively. Substrate, air, buffer, 1.25M-HCl and 0.25M-KI were pumped through the pump tubing lines 1, 2, 3, 4 and 5 respectively. The tube-supported enzymes were maintained at 37°C and a 2:1 (v/v) wash/sample ratio was used. For the meaning of the symbols see the Experimental section.

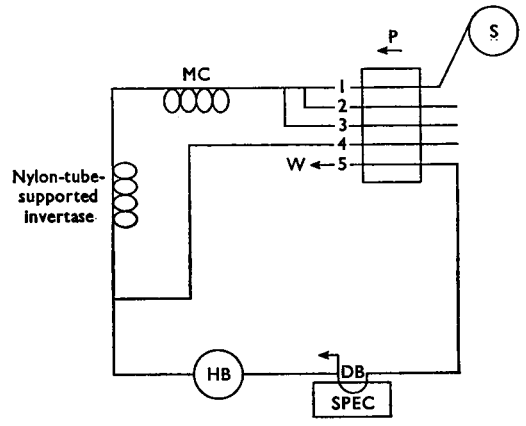

Fig. 2. *Flow system for the use of nylon-tube derivatives of invertase in automated analysis*

For the determination of sucrose the pump tubing lines 1, 2, 3, 4 and 5 gave flow rates of 1.20, 0.60, 0.32, 2.50 and 2.90ml/min respectively. The tube-supported invertase was maintained at 37°C and the heating bath (HB) was maintained at 65°C. A 2:1 (v/v) wash/sample ratio was used. Substrate, air, 0.5M-sodium acetate buffer, pH5.0, and 0.6% (w/v) 3,5-dinitrosalicylic acid in 1M-KOH were pumped through the pump tubing lines 1, 2, 3 and 4 respectively. For the meaning of the symbols see the Experimental section.

a standard mixing coil. Finally, the stream was degassed and its extinction at 349nm recorded.

Sucrose was also determined by the dinitrosalicylic acid method of Bruner (1964) by using the flow system shown in Fig. 2. The sample stream, after being air-segmented, was mixed with 0.5M-sodium acetate buffer, pH5.0, before being perfused through a nylon tube to which invertase was bound. The tube was maintained at 37°C. The effluent from the tube, containing glucose and fructose as reaction products, was then mixed with 0.6% (w/v) dinitrosalicylic acid in 1M-KOH and passed through a standard delay coil at 65°C. It was subsequently degassed and its extinction at 540nm recorded.

Results

Determination of disaccharides by method 1

Fig. 3(*a*) shows the calibration curve obtained when standard solutions of sucrose were assayed by using a 3m length of the invertase derivative and a 3m length of the glucose oxidase derivative, inserted at positions A and B respectively in the flow system shown in Fig. 1. These results show that sucrose in the concentration range 5–30mM may be determined by this method. The system was further calibrated by subjecting H_2O_2 solutions of known concentration to the assay procedure. In this way the results are also presented in terms of the H_2O_2 produced, which is a measure of the overall activity of the linked enzyme system. Fig. 4 shows the effect of the delay time between the two enzyme tubes on the sensitivity of the assay and the overall activity of the linked enzyme system. These results indicate that a fourfold increase in sensitivity can be obtained by increasing the delay time between the two coils from 1.51min to 26.5min. In this way the sensitivity of the assay system is improved and sucrose can be measured in the concentration range 0.5–2.5mM.

Fig. 5(*a*) shows the calibration curve obtained when standard solutions of maltose were assayed by using a 3m length of nylon tube that carried amyloglucosidase and a 3m length of nylon tube that carried glucose oxidase, inserted at positions A and B respectively in the flow system shown in Fig. 1. These results show that maltose in the concentration range 5–30mM may be determined by this method.

Fig. 6 shows the effect of sampling rate on the calibration curves obtained when standard solutions of

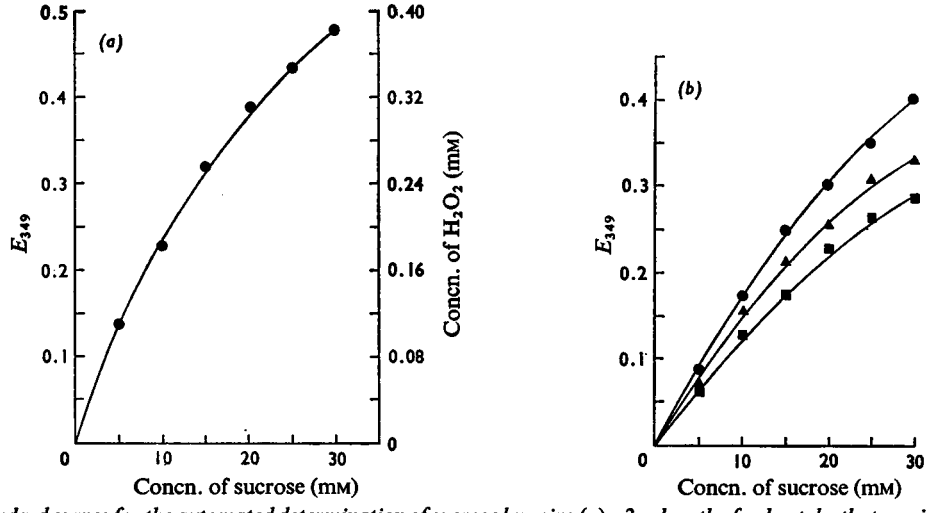

Fig. 3. *Standard curves for the automated determination of sucrose by using (a) a 3 m length of nylon tube that carried invertase in series with a 3 m length of nylon tube that carried glucose oxidase and (b) a 3 m length of a mixed derivative of invertase and glucose oxidase*

●, 20 samples/h; ▲, 30 samples/h; ■, 40 samples/h. The flow system described in Fig. 1 was used and full experimental details are given in the text.

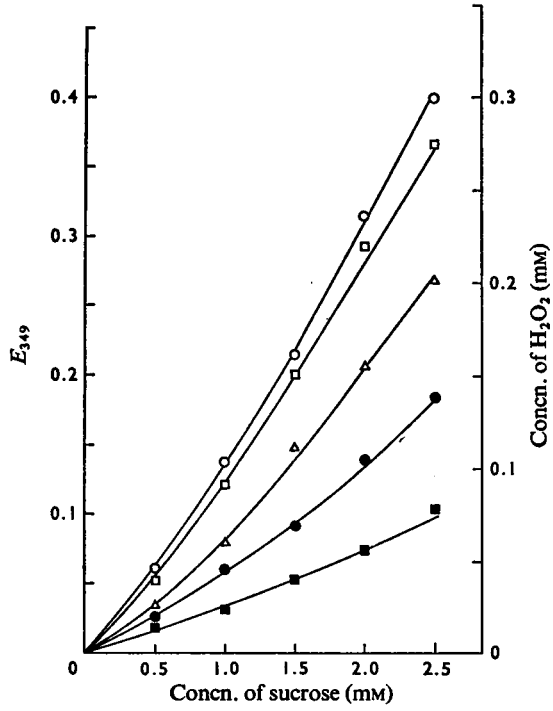

Fig. 4. *Standard curves for the automated determination of sucrose by using a 3 m length of nylon tube that carried invertase in series with a 3 m length of nylon tube that carried glucose oxidase*

Sample delay between the tube-supported enzymes: ○, 26.5 min; □, 13.25 min; △, 6.9 min; ●, 3.45 min; ■, 1.51 min. The flow system shown in Fig. 1 was used and full experimental details are given in the text.

lactose were assayed by using a 3 m length of nylon tube that carried β-galactosidase, placed in series with a 3 m length of nylon tube that carried glucose oxidase. By this method lactose in the concentration range 0.1–0.6 mM may be determined.

Determination of sucrose and maltose by method 2

Fig. 3(*b*) shows the effect of sampling rate on the calibration curves obtained when standard solutions of sucrose were assayed with a 3 m length of a mixed derivative of invertase and glucose oxidase [which was prepared from an enzyme solution containing a 2:1 (w/w) ratio of invertase to glucose oxidase] inserted at position A in the flow system shown in Fig. 1. These data show that this linked enzyme structure can be used for the determination of sucrose in the concentration range 5–30 mM.

Fig. 5(*b*) shows the calibration curve obtained when standard solutions of maltose were assayed by using a 3 m length of a mixed derivative of amyloglucosidase and glucose oxidase, inserted at position A in the flow system shown in Fig. 1. These results show that this system is twice as sensitive as that with the two enzymes carried on separate lengths of nylon tube, and may be used for the determination of maltose in the concentration range 1–6 mM.

The activity of the 3 m length of nylon tube that carried invertase was determined separately by measurement of the total reducing sugar produced by using the flow system shown in Fig. 2. The results of this experiment are shown in Fig. 7(*a*). By concurrently subjecting standard solutions containing equimolar concentrations of fructose and glucose to

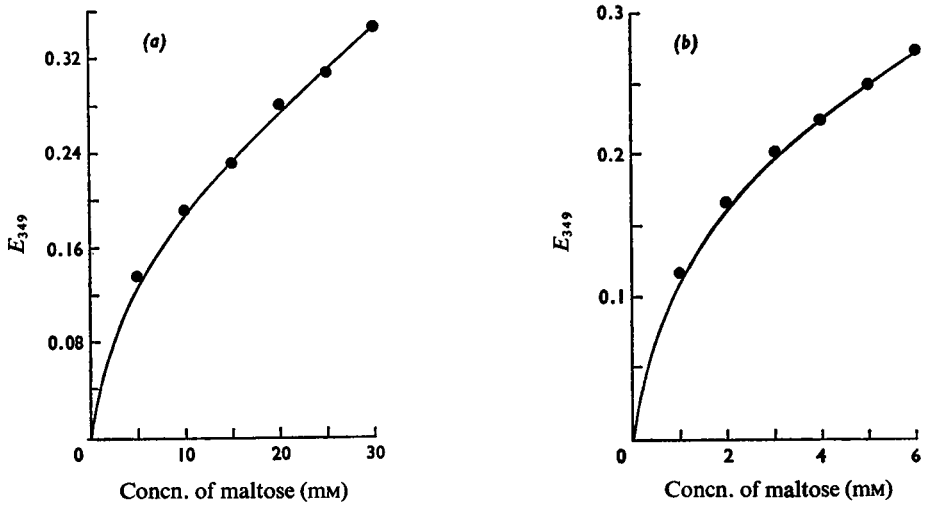

Fig. 5. *Standard curves for the automated determination of maltose with (a) a 3 m length of nylon tube that carried amyloglucosidase in series with a 3 m length of nylon tube that carried glucose oxidase and (b) a 3 m length of a mixed derivative of amyloglucosidase and glucose oxidase*

Samples were assayed at the rate of 20/h. The flow system shown in Fig. 1 was used and full experimental details are given in the text.

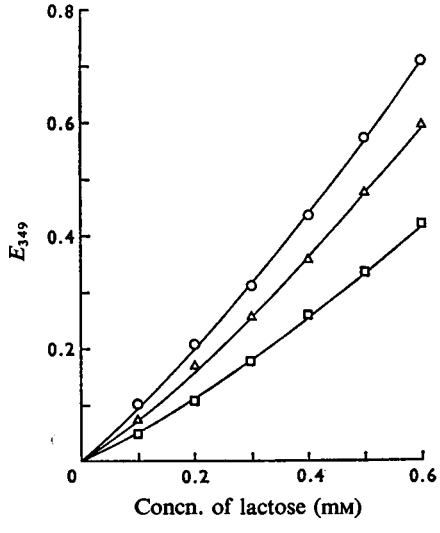

Fig. 6. *Standard curves for the automated determination of lactose with a 3 m length of nylon tube that carried β-galactosidase in series with a 3 m length of nylon tube that carried glucose oxidase*

The flow system shown in Fig. 1 was used. Full experimental details are given in the text. ○, 20 samples/h; △, 30 samples/h; □, 40 samples/h.

the assay procedure, the results are also expressed in terms of the total equivalents of reducing sugar produced. Likewise the activity of the 3 m nylon tube that carried glucose oxidase was determined separately by subjecting standard solutions of glucose to the assay procedure described in Fig. 1. The results of this experiment are shown in Fig. 7(*b*). By concurrently subjecting standard solutions of H_2O_2 to the assay procedure the results are also expressed in terms of the H_2O_2 produced. The overall activity of the two nylon-tube derivatives acting in sequence for the hydrolysis of sucrose and subsequent oxidation of the glucose produced was shown in Fig. 3(*a*).

The stability of the derivatives was tested by subjecting them to the operational conditions used in the automated determination of their respective substrates. To do this the linked enzyme systems were used intermittently over prolonged periods. In all cases there was no significant loss of catalytic activity when the materials were used over a period of 30 days, during which each structure was used for at least 500 determinations.

Discussion

In this work three different linked enzyme systems were investigated as analytical reagents for the deter-

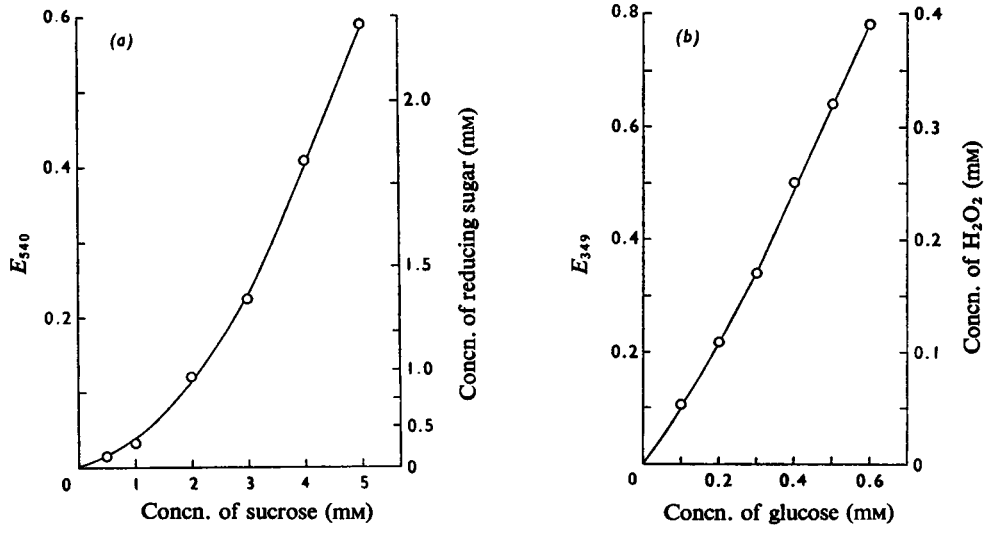

Fig. 7. *Standard curves for the automated determinations of (a) sucrose, by using a 3 m length of nylon tube that carried invertase and the flow system shown in Fig. 2 and (b) glucose, by using a 3 m length of nylon tube that carried glucose oxidase and the flow system shown in Fig. 1*

In both experiments samples were assayed at the rate of 20/h and full experimental details are given in the text.

Table 2. *Separate enzymic activities of the mixed derivative of invertase and glucose oxidase, the derivative of invertase and the derivative of glucose oxidase*

For details see the text.

Derivative	Conversion of sucrose into H_2O_2 (%)	Conversion of sucrose into glucose (%)	Conversion of glucose into H_2O_2 (%)
Separate tubes of invertase and glucose oxidase (system 1)	1.3	22.5	65.0
System 1 with a 1.51 min delay between the coils	3.0	22.5	65.0
System 1 with a 26.5 min delay between the coils	11.8	22.5	65.0
Mixed derivative of invertase and glucose oxidase (system 2)	0.6	10.1	66.5
Mixed derivative of invertase and glucose oxidase (system 3)	1.06	17.6	66.0

mination of sucrose. These were; invertase and glucose oxidase immobilized on separate lengths of nylon tube and used in series (system 1); two immobilized enzyme structures both with the two enzymes co-immobilized on the same tube, one prepared from equal amounts of the two enzymes (system 2) and the other prepared with a 2:1 ratio of invertase to glucose oxidase in the coupling mixture (system 3). Table 2 summarizes the separate enzymic activities of the enzyme in each structure together with the overall activity of the respective structures for the complete linked enzyme reaction from the data shown in Fig. 3. In each case the glucose oxidase activity, in terms of the overall percentage oxidation of glucose in each system, was in excess of 64%. However, since glucose in solution exists as an equilibrium mixture of 64% β-D-glucose and 36% α-D-glucose and only the β-anomer is a substrate for *Aspergillus niger* glucose oxidase (Barman, 1969), then the percentage conversion of available substrate to product is very high (this assumes that the amount of α-D-glucose mutarotating to β-D-glucose is small during the residence time of the sample in the enzyme tube).

On the other hand the invertase activity of the structures in terms of the overall percentage hydrolysis of sucrose to glucose and fructose was 10.1, 17.6

and 22.5% for the systems 2, 3 and 1 respectively. These results show that increasing the ratio of invertase to glucose oxidase in the coupling mixture from 1:1 to 2:1 had no effect on the glucose oxidase activity of systems 2 and 3. However, the invertase activity was increased by almost 80% at the same time as was the overall percentage conversion of sucrose for the complete linked system. From the separate glucose oxidase and invertase activities of the three systems the overall percentage conversion of sucrose might have been expected to be greater than that observed. However, the product of the invertase reaction, α-D-glucose, has to undergo a relatively slow spontaneous mutarotation before it becomes the substrate of the glucose oxidase reaction, β-D-glucose, in which case the rate-limiting step in the overall reaction may be controlled by neither of the enzyme-catalysed steps, but by the spontaneous mutarotation. This proposal is strengthened by the observation that increasing the delay time between the enzyme-catalysed steps in system 1 from 1.51 min to 26.5 min increased the overall conversion of the sucrose fourfold.

Mattiasson & Mosbach (1971) showed that the overall catalytic efficiency of the linked enzyme system β-galactosidase–hexokinase–glucose 6-phosphate dehydrogenase was enhanced when the enzymes were simultaneously immobilized on Sephadex G-50C. Mosbach & Mattiasson (1970) also reported an enhancement in the overall conversion of glucose into gluconolactone 6-phosphate when the enzymes affecting the reaction, hexokinase and glucose 6-phosphate dehydrogenase, were immobilized on the same support. These observations were attributed to an overall enhancement in the rate of the reaction of the systems in the initial phase, which, they argued, was caused by the proximity of the bound enzymes producing an increased concentration of the intermediate product(s) in the micro-environment of the linked enzyme system. Clearly, an effect such as this would not contribute significantly to the overall reaction rate of a linked enzyme system such as that described here, in view of the fact that mutarotation of the α-D-glucose must occur between the two stages.

The calibration curves shown in Fig. 3 for the automated determination of sucrose were concave downwards, whereas the corresponding calibration curves shown in Fig. 4 were concave upwards at the lower range of sucrose concentrations. The standard sucrose solutions used for the construction of the former curves were considerably more concentrated than those used for the construction of the latter curves. Consequently any upwards concavity would not be obvious in the standard curves shown in Fig. 3. Likewise, the lack of upwards concavity displayed by the standard curves for the automated determination of maltose shown in Fig. 5 could be accounted for by the relatively high concentrations of the maltose standard solutions that were used for their construction.

The preparation, storage and operating conditions for a co-supported linked enzyme system must always be a compromise between the ideal conditions of preparation, storage and operation for the individual immobilized enzyme systems. For instance, it is easier to define the optimum coupling conditions for a single immobilized enzyme than for a linked system, because the conditions that favour the immobilization of one enzyme will not necessarily favour the immobilization of a second enzyme. The *E. coli* β-galactosidase, for example, could only be used for the preparation of nylon-tube-supported β-galactosidase on non-hydrolytically cleaved nylon tube, whereas the glucose oxidase preferred the hydrolytically cleaved nylon tube. Therefore a mixed derivative of these two enzymes was not feasible, since each preferred a different support. A support with no residual charge might have proved successful for the mixed derivative, but again this would have represented a compromise because each preferred a differently charged support.

The optimum storage conditions for immobilized enzyme preparations will differ between enzymes with respect to pH, ionic strength, temperature and solvent composition. Therefore immobilizing enzymes on the same support might decrease the overall stability of the system, because each enzyme is not being stored under its own optimum conditions. Finally, the pH optima of the composite enzymes in a mixed derivative may be quite different, in which case the system would be assayed at a compromise pH and so neither enzyme would be operating under ideal conditions. However, this problem does not arise when the two enzymes are immobilized on separate coils, because the solvent composition of the sample stream can be readily altered after leaving the first coil and before being perfused through the second coil.

Therefore the major advantage derived from the immobilization of two enzymes each on a separate tube compared with their immobilization on the same tube is the increased versatility of the system. For example, a disaccharide may be determined in the presence of glucose. The glucose oxidase tube is inserted at position B in Fig. 1 and the glucose content of the sample determined. After this the appropriate disaccharidase tube is inserted at position A and the total disaccharide and glucose content determined. Subtraction of the former value from the latter gives a measure of the disaccharide concentration in the sample and by reference to the appropriate standard curve the disaccharide concentration can be determined.

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