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THE PREPARATION OF SEVERAL NEW NYLON TUBE-GLUCOSE OXIDASE DERIVATIVES AND THEIR INCORPORATION INTO THE 'REAGENTLESS' AUTOMATED ANALYSIS OF GLUCOSE

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Summary

Nylon tube was activated by alkylation with dimethyl sulfate and used for the immobilization of glucose oxidase. Lysine, hexamethylene diamine and polyethylene imine were also attached to activated nylon tube, and these nylon tube-spacer derivatives were reactivated with either glutaraldehyde or ethyl adipimidate for the subsequent coupling of glucose oxidase. The activities of all of the different nylon tube-glucose oxidase derivatives were compared by their incorporation into standard Technicon automated analysis systems. Activities were measured either spectrophotometrically, by following the production of hydrogen peroxide using an acid/KI assay, or polarographically by following the decrease in the dissolved oxygen concentration using a flow-through oxygen electrode assembly. The activity and stability of all of the nylon tube-glucose oxidase derivatives was such that their use in the routine estimation of glucose levels was an attractive proposition.

Introduction

The advantages of immobilized enzymes over their soluble counterparts are firmly established [1,2]. One of the most important assets of these artefacts is the fact that their insoluble nature makes them re-usable, and consequently economical to use.

The operational configuration of an immobilized enzyme reactor is largely determined by the type of process into which the immobilized enzyme is to be incorporated. For example, if it is required to use the immobilized enzyme to generate the product, or products of the enzymic reaction then a packed bed

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[3], or a stirred tank [4] may be the reactor configuration of choice. On the other hand, if it is required to use the immobilized enzyme to accurately detect levels of the enzyme's substrate(s), then it would be prudent perhaps to use the immobilized enzyme in the form of an enzyme electrode [5], or an open tubular reactor [6].

The use of immobilized enzymes in the latter mode is extremely attractive when applied to automated analysis, since tubular immobilized enzyme reactors can be readily incorporated into most standard analytical systems, without necessitating any gross change in either the existing technology or hardware. In fact, nylon tube-supported enzymes have been successfully employed in automated analysis to detect a variety of compounds including pyruvate, oxaloacetate, and glucose [6—9].

The enzyme glucose oxidase (β-D-glucose: oxygen 1-oxidoreductase EC 1.1.3.4) is highly specific for β-D-glucose. Thus, in situations where it may be desirable to either selectively remove glucose, (as in the food industry), or to accurately detect levels of glucose, (as in the clinical laboratory), the applications for glucose oxidase are obvious. In the light of these potential uses, glucose oxidase has been immobilized on many different water insoluble supports by a multitude of different methods [6,7,10—12]. For example, Inman and Hornby [6] immobilized glucose oxidase through glutaraldehyde on nylon tube which had been pitted and exposed to a cleaving reagent. The resulting nylon tube-glucose oxidase derivatives were employed to determine glucose levels using an automated acid/KI assay [6]. However, the preparation of these derivatives involved several lengthy incubation procedures and the assay employed was liable to interference by biologically occurring substances.

In the present study, a new method for the immobilization of glucose oxidase on nylon tubes is described. A superior and more convenient method for the activation of nylon tube is discussed, and the use of diamino and polyamino spacer molecules to increase the activity yield is considered. Furthermore, a new automated assay system for the determination of glucose using nylon tube-supported glucose oxidase is also described, which involves the incorporation of a flow-through oxygen electrode into a standard 'Technicon' continuous flow system.

Materials and Methods

Activation of nylon tube

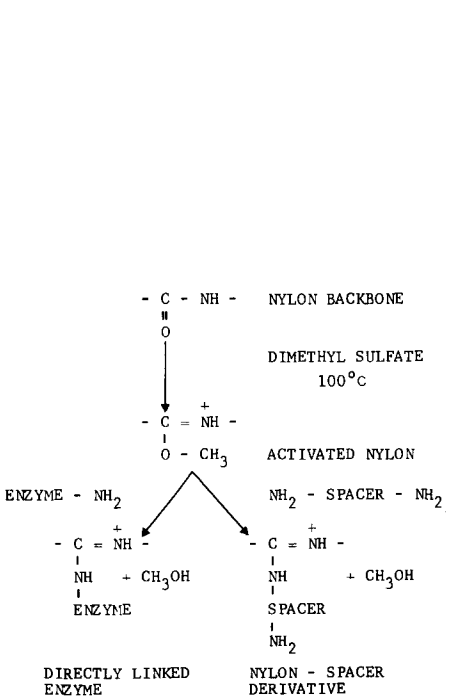
Glucose oxidase (grade I from *Aspergillus niger*) was obtained from Boehringer Corporation (London) Ltd, and was used without further purification. Type '6' nylon tube (1 mm internal diameter) was obtained from Portex Ltd, Hythe, Kent, U.K., and was activated by alkylation using dimethyl sulphate (B.D.H. Chemicals Ltd, Poole, Dorset, U.K.) [13]. The appropriate length of nylon tube was filled with 100% dimethyl sulphate, and the ends of the tube were sealed. The tube was immersed in a boiling water bath for exactly 3 min, at which time it was plunged into an ice bath to stop the reaction. The tube was then purged free of excess alkylating agent by washing through with 100 ml of absolute ethanol.

The active imidate groups thus introduced into the nylon backbone are

reactive towards free amino groups, and consequently were used to couple glucose oxidase directly to the nylon (Fig. 1). Immediately after alkylation and washing, the activated nylon tube was pumped dry and then filled with a 0.3 mg/ml glucose oxidase solution in 0.1 M *N*-ethylmorpholine, pH 8.5. The tube was sealed, and incubated for 2 h at 4° C, after which, unbound protein was removed by washing through at 4° C with 500 ml of 0.5 M NaCl, and then 500 ml of 50 mM phosphate buffer, pH 5.5. Finally any unreacted imidate groups were blocked by flushing through the tube with 50 ml of 0.1 M Tris, pH 8.5.

Introduction of spacers

Alternatively, diamino spacers (such as lysine and hexamethylene diamine) or polyamino spacers (such as polyethyleneimine) were coupled to activated nylon tube (Fig. 1). After the tube had been activated with dimethyl sulphate, washed, and pumped dry, it was filled with either 0.5 M lysine, pH 9.5 or 0.5 M hexamethylene diamine, pH 9.5, or 3% (w/v) polyethyleneimine, pH 8.5. The tube was then sealed and incubated at room temperature for 2 h. Excess spacer was removed by washing through with 500 ml of 0.5 M NaCl. The nylon tube-spacer derivatives which were stable at room temperature, subsequently were reactivated for the immobilization of glucose oxidase with either glutaraldehyde (25% w/v solution in water from B.D.H. Chemicals Ltd, Poole, Dorset, U.K.) or diethyl adipimidate dihydrochloride (prepared by the synthesis of Pinner [14,15] (Fig. 2).



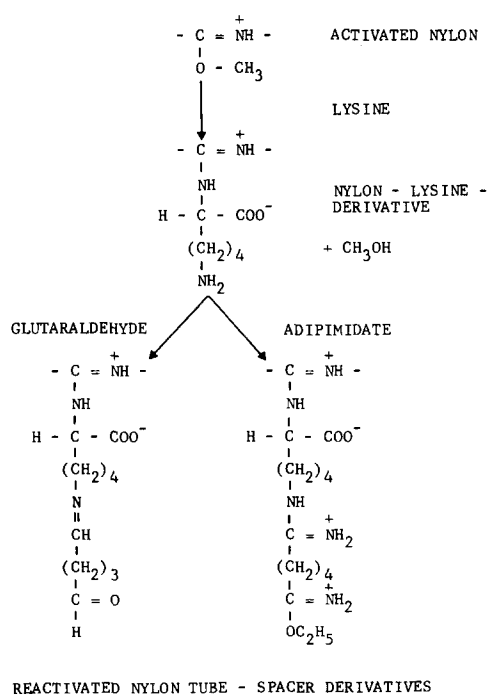


Fig. 1. Activation of nylon tube by alkylation with dimethyl sulphate and the subsequent direct coupling of enzyme or the introduction of a spacer molecule.

Fig. 2. The coupling of a lysine spacer to nylon tube and the subsequent reactivation of the nylon tube-spacer derivative with either glutaraldehyde or adipimidate.

In the case of glutaraldehyde-reactivation, activation of the nylon tube-spacer derivatives was achieved by perfusion in a closed loop with a 2.5% (w/v) solution of glutaraldehyde in 0.1 M sodium borate, pH 8.5 at room temperature for 10 min at a flow rate of 5 ml · min^{-1}.

Alternatively, nylon tube-spacer derivatives were reactivated using ethyl adipimidate by filling the tube with a solution containing 3.0 ml of absolute alcohol, 2.0 ml of 100% *N*-ethylmorpholine, and 250 mg of ethyl adipimidate. The tube was sealed and incubated at room temperature for 2 h.

In both cases, the reactivated nylon tube-spacer derivatives were washed free of excess bifunctional reagent by perfusion with absolute alcohol, pumped dry, and immediately filled with a 0.3 mg/ml glucose oxidase solution in 0.1 M *N*-ethylmorpholine, pH 8.5. The time of coupling, and the washing free of unbound protein were identical to those described previously for the direct coupling of glucose oxidase to nylon tube.

Unless otherwise stated, all nylon tube-glucose oxidase derivatives were stored, filled with 0.1 M phosphate buffer, pH 5.5 at 4°C.

Automated analysis using nylon tube-glucose oxidase

Standard glucose solutions were made up in the appropriate buffer at least 24 h before use to ensure complete mutarotation. They were stored at 4°C and used within 2 days of their preparation.

The nylon tube-glucose oxidase derivatives were used to determine glucose by their incorporation into Technicon automated continuous flow systems. The glucose oxidase derivatives were incorporated into the flow systems in the form of coils, and unless stated otherwise, were maintained at 25°C by their immersion in a water bath.

Glucose concentrations were determined spectrophotometrically using the acid/KI method described by Inman and Hornby [6]. Alternatively, glucose was determined polarographically by measuring the consumption of dissolved oxygen during the glucose oxidase reaction by employing a flow-through oxygen electrode incorporated into the standard Technicon flow system shown in

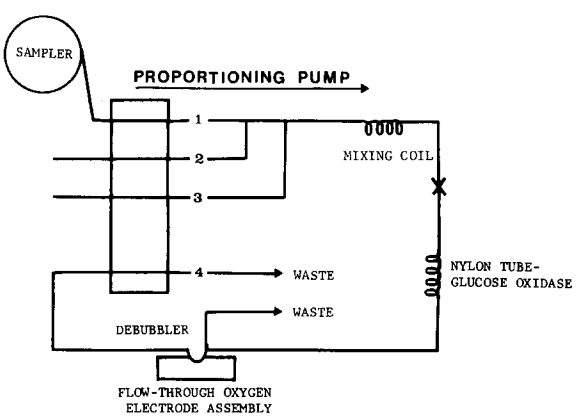


Fig. 3. Flow circuit for the use of nylon tube-glucose oxidase in automated analysis using the flow-through oxygen electrode assay. Pump tubing lines 1, 2, 3, and 4 gave flow rates of 0.6, 0.6, 1.6, and 1.6 ml · min^{-1} respectively. Lines 1, 2, and 3 carried sample, air and 50 mM acetate buffer, pH 5.5 respectively. The sampler, proportioning pump and mixing coil were standard Technicon equipment.

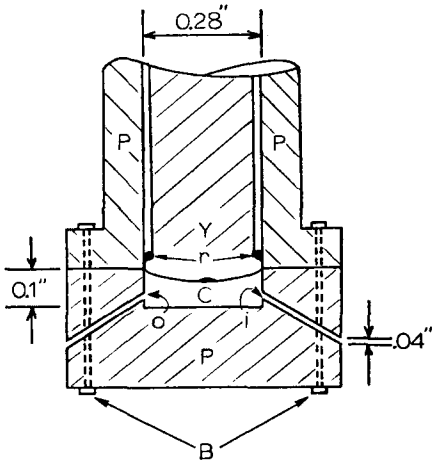


Fig. 4. Flow-through oxygen electrode design. (Y) Yellow Springs oxygen electrode; (P) perspex housing; (B) clamping bolts; (i) inlet port; (o) outlet port; (C) flow-through assay chamber; (r) sealing rubber ring.

Fig. 3. In this system sample stream is first air segmented, and then diluted with 50 mM acetate buffer, pH 5.5, before mixing and perfusion through the nylon tube-glucose oxidase derivative. The emergent stream is debubbled, and the oxygen concentration is monitored by an oxygen sensor (Yellow Springs Instrument Co., Yellow Springs, Ohio. U.S.A.) fitted with a flow-through perspex housing (Fig. 4). The internal volume of the flow-through chamber was about 0.1 ml, thus the residence time in the chamber was less than 4 s.

Results and Discussion

Effect of spacers

Nylon tube extruded from high molecular weight nylon contains relatively few sites for the attachment of protein. Thus, if it is required to immobilize a reasonable quantity of an enzyme on the inside surface of this type of nylon tube, then either the amount of potential binding sites has to be increased, or new active centres have to be introduced. Inman and Hornby [6] increased the number of free amino groups on the inside of nylon tube by partially hydrolysing the inside surface with HCl. Dimethylaminopropylamine has also been employed to produce a controlled non-hydrolytic cleavage of some of the amide bonds in the backbone of nylon tube [8], again resulting in an increase in the amount of free amino groups. Both of these methods have been successfully employed to facilitate the immobilization of enzymes [6,8]. However, both of these techniques require the cleavage of some of the bonds in the nylon backbone, and consequently result in a weakening of the nylon's structure. The activation of nylon tube described here does not involve the cleavage of any bonds, and thus activates the nylon without destroying the integrity of the polymeric structure (Fig. 1).

Enzymes coupled directly onto activated nylon tube as described above, are held close to the surface of the support material (Fig. 1). Some enzymes may be sensitive to such a close exposure to the fairly hydrophobic nylon

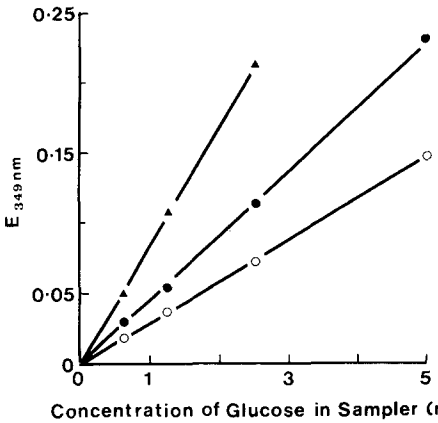


Fig. 5. Comparison between the direct coupling of glucose oxidase to nylon tube and between glutaraldehyde and adipimidate for the binding of glucose oxidase to nylon tube-lysine derivatives. In each case, activity was measured by compiling the standard curve for the automated determination of glucose using 0.5-m lengths of the different nylon tube-glucose oxidase derivatives in the acid/KI assay system. Samples were assayed at the rate of 20/h. ●, glucose oxidase coupled directly onto nylon tube; ▲, lysine spacer and subsequent reactivation with adipimidate; ○, lysine spacer and subsequent reactivation with glutaraldehyde.

surface. Furthermore, the active site of the enzyme may be sterically hindered from binding the substrate, especially if the substrate is a large molecule. Consequently, both of these factors may reduce the observed activity of a bound enzyme. Thus, it may be advantageous to introduce a spacer molecule, which can couple to the activated nylon, and which itself can be readily activated for the subsequent coupling of enzyme. In this way, the enzyme would be removed from the surface of the support material, and the above factors alleviated.

Fig. 5 shows the effect of introducing a lysine spacer on the activity of nylon tube-glucose oxidase. The nylon tube-glucose oxidase derivative prepared by coupling the enzyme directly to the activated nylon exhibited only about half the activity of the derivative prepared by coupling the glucose oxidase to a nylon tube-lysine derivative, which had been activated using ethyl adipimidate. The introduction of the lysine spacer, and the subsequent reactivation with ethyl adipimidate both tended to remove the glucose oxidase from the surface of the nylon support (Fig. 2), and this may account for the increased activity of the nylon tube-lysine-enzyme derivative. However, the nylon tube-lysine derivative which was coupled to glucose oxidase via glutaraldehyde was the least active of the three structures. In this case, the enzyme was also removed from the surface of the support material and thus it appears that the immobilization via glutaraldehyde is less efficient in terms of the resultant activity than the imidate methods. Furthermore, although both bifunctional reagents are thought to react with free amino groups (Fig. 2) the chemistry of the glutaraldehyde reaction is not well established [16], and may also involve other amino acid residues in proteins. Consequently, enzymes immobilized using glutaraldehyde may be bound to the support material in a random manner, and thus the possibility of the coupling involving an 'essential' amino acid residue is increased. This again emphasises the superiority of the imidate coupling methods, as these are thought to involve only free amino groups in the protein.

Many other compounds could be used as spacer molecules, and Fig. 6 compares lysine, hexamethylene diamine and polyethyleneimine as spacers for the preparation of nylon tube-glucose oxidase derivatives. In each case, the reactivation of the nylon tube-spacer derivative was achieved using ethyl adipimidate, and the results show that under the conditions employed, hexamethylene diamine produced the most active nylon tube-glucose oxidase derivative. The use of polyamino spacers is very attractive, since the attachment of the polyamino compound to one activated group in the nylon backbone, in principle yields a large number of potentially active amino groups. Hence, by introducing a polyamino spacer the number of potential protein coupling sites can be increased. Furthermore, the introduction of polyamino spacers may also provide means of immobilizing multi subunit enzymes, which tend to dissociate on immobilization, by presenting an environment which would tend to maintain the integrity of the enzyme. In the case of the polyamino spacer used here, polyethyleneimine, no such beneficial effect was observed. Polyethyleneimine is labile at alkaline pH [17], and as the coupling procedures are carried out at pH 8.5, this may account for the observed low activity. However, the use of other polyamino compounds (especially inert proteins such as albumin), as spacer molecules, has proved more successful [18]. Consequently, the use of proteins as spacers may provide a convenient method for increasing the amount of protein which can be bound to a given support material, and at the same time provide a more acceptable microenvironment for the immobilized enzyme.

Flow-through oxygen electrode

All the nylon tube glucose oxidase derivatives were sufficiently active to detect glucose levels down to 1.0 mM using the acid/KI assay method (Figs 5 and 6). Resting blood glucose levels are in the range 3.3—5.5 mM [19], hence all the nylon tube-glucose oxidase derivatives were sufficiently active to be of use in the clinical estimation of blood glucose. However, the acid/KI assay

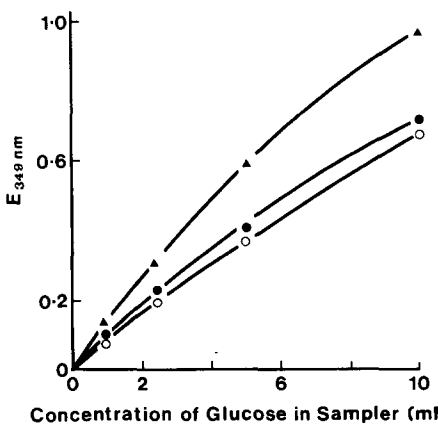


Fig. 6. The effect of interposing different spacer molecules between glucose oxidase and nylon tube on the activity of the nylon tube-supported glucose oxidase. In each case activity was measured as in Fig. 5. ▲, hexamethylene diamine spacer; ●, lysine spacer; ○, polyethyleneimine spacer.

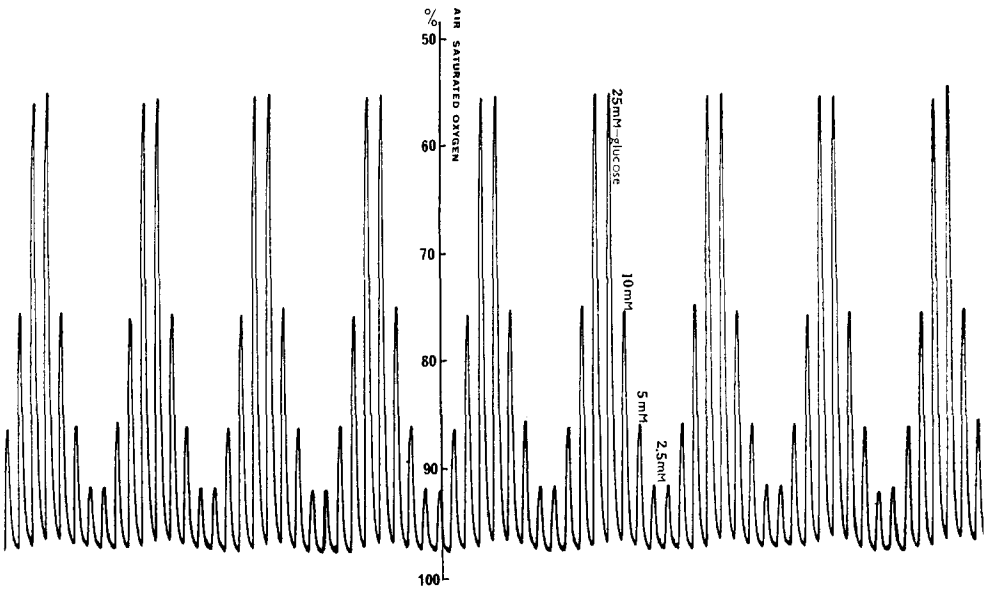


Fig. 7. Continuous recording of glucose analysis using nylon tube-supported glucose oxidase in the flow-through oxygen electrode assay system. Samples were assayed at the rate of 20/h, using the flow circuit shown in Fig. 3.

system may not be suitable for use under all circumstances, since other oxidising substances, which may be present in biological fluids, could also cause oxidation of the acid/KI. The use of the flow-through oxygen electrode (Fig. 4) in the flow circuit shown in Fig. 3, overcomes this problem. In this assay system, only the disappearance of dissolved oxygen is monitored, thus oxidising agents in blood, etc. do not interfere. However, the presence of catalase would interfere, as it would catalyse the conversion of hydrogen peroxide to water, with the simultaneous production of oxygen. This difficulty could be easily eliminated by incorporating a standard Technicon dialyser unit at position X in the flow system shown in Fig. 3.

Fig. 7 shows a typical set of traces obtained using a 3 metre length of nylon tube-glucose oxidase in the flow-through oxygen electrode assay system (Fig. 3). These results show that the assays were reproducible, and that the electrode exhibited acceptable flow characteristics. Furthermore, using this assay technique, all of the nylon tube-glucose oxidase derivatives were sufficiently active to detect glucose levels down to 2.5 mM (Fig. 8). Again this afforded sufficient sensitivity to make the routine use of nylon tube-glucose oxidase a viable proposition when used in conjunction with the flow-through oxygen electrode. Furthermore, the standard curve obtained using the flow-through oxygen electrode system was linear (for the glucose concentrations used here) (Fig. 8), whereas the standard curve obtained using the acid/KI assay system was curved (Fig. 6). This may have been due to the fact that the former assay system relied solely on the disappearance of dissolved oxygen, while the latter involved the interaction of H_2O_2 with a colour reagent (acid/KI). In any event, the linearity obtained using the flow-through oxygen electrode assay system is preferable, and emphasises the potential of this technique. In addi-

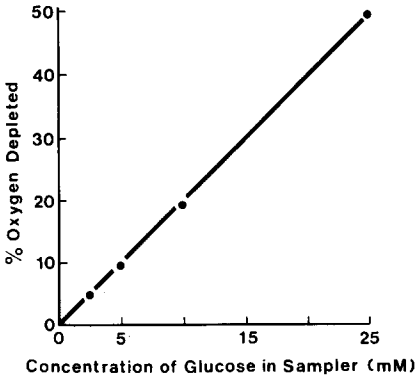


Fig. 8. The effect of glucose concentration on the extent of oxygen depletion using nylon tube-glucose oxidase in the flow-through oxygen electrode assay system. Samples were assayed at the rate of 20/h, using the flow circuit shown in Fig. 3.

tion, this assay system was virtually reagentless, and thus lent itself perfectly to the study of the long-term operational stabilities of the various nylon tube-glucose oxidase derivatives.

Operational stabilities of nylon tube-glucose oxidase

In most cases, the stability of an immobilized enzyme is assessed by studing the susceptibility of the derivative to processes such as thermal inactivation, urea denaturation or proteolytic digestion. Although such methods do give an indication of the stability of the immobilized enzyme, relative to the corresponding soluble enzyme, the only real test of stability is to actually subject the derivatives to their 'working' conditions. Hence, the nylon tube-glucose oxidase derivatives were used to assay glucose standards continuously over a period of time using the flow-through oxygen electrode assay. The results are shown in Table I. From Table I it is clear that all of the nylon tube-glucose oxidase derivatives were stable under the conditions employed. Each of the glucose oxidase derivatives was used to perform at least 200 sepa-

TABLE I

OPERATIONAL STABILITIES OF THE NYLON TUBE-GLUCOSE OXIDASE DERIVATIVES

1, glucose oxidase coupled directly onto nylon tube activated with dimethyl sulphate (Fig. 1). 2, glucose oxidase coupled to nylon tube-spacer derivatives which had been reactivated with adipimidate (Fig. 2). 3, glucose oxidase coupled to a nylon tube-spacer derivative which had been reactivated with glutaraldehyde (Fig. 2).

Derivative type	Temperature (°C)	No. of glucose estimations at 20/h	Decrease in activity (%)
Direct link[1]	25	340	0
Lysine spacer[2]	25	850	0
Lysine spacer[2]	37	220	0
Lysine spacer[3]	25	320	2
Hexamethylene diamine spacer[2]	25	390	5
Polyethyleneimine spacer[2]	25	470	0

rate glucose estimations. More recently, nylon tube-glucose oxidase derivatives with lysine spacers have been used to perform 10 000 glucose estimations with no significant decrease in their activity. In addition, preliminary studies involving the use of these nylon tube-glucose oxidase derivatives to estimate glucose levels of plasma samples also confirm the excellent operational stability of these immobilized glucose oxidase derivatives when exposed to plasma.

Thus, it is obvious that these new methods for the preparation of nylon tube-glucose oxidase produce immobilized enzyme derivatives which are both sufficiently active and stable to make their routine use for the determination of glucose levels an attractive proposition. Furthermore, the incorporation of the flow-through oxygen electrode into the Technicon autoanalyser systems suggests an ideal, reagentless assay system which can be readily applied to all reactions involving oxidases.

Acknowledgement

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