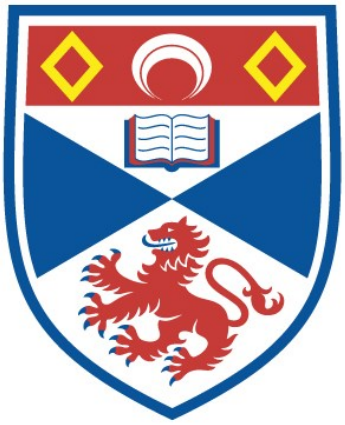
SOME STUDIES ON NYLON-SUPPORTED ENZYMES AND THEIR APPLICATIONS FOR USE IN AUTOMATED ANALYSIS

David James Inman

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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Some Studies on Nylon-Supported Enzymes and Their Applications for Use in Automated Analysis

by

David James Inman

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submitted to the University of St. Andrews
in application for the degree of
Doctor of Philosophy.

University of St. Andrews,
Department of Biochemistry,
North Street,
St. Andrews, Fife.

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Declaration

I hereby declare that the following thesis is based on work carried out by me, that the thesis is my own composition and that no part of it has been presented previously for a higher degree.

The research was carried out in the Department of Biochemistry of the University of St. Andrews, under the direction of Dr. William E. Hornby.

Certificate

I hereby certify that David James Inman has spent nine terms in research work under my direction, and that he has fulfilled the conditions of Ordinance No. 16 (St. Andrews) and that he is qualified to submit this thesis for the degree of Doctor of Philosophy.

Academic Record

I matriculated at the University of St. Andrews in October 1966, and graduated with the degree of Bachelor of Science, Upper Second Class Honours in Biochemistry in June 1970.

In October 1970, I matriculated as a research student at the University of St. Andrews.

Acknowledgements

I should like to thank my supervisor, Dr. W.E. Hornby for his advice and criticism throughout this work.

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Abbreviations & Symbols

DMAP	N,N-dimethyl-1,3-propanediamine
2,4-DNPH	2,4-dinitrophenylhydrazine
DNSA	3,5-dinitrosalicylic acid
DTT	dithiothreitol
Perid	2,2-diazo-(3-ethyl benzthiazoline-6-sulphonic acid)
Unit	IUB enzyme unit: 1 unit is equal to 1 micromole of substrate converted to product per min under the conditions specified.

Where possible, all other abbreviations and symbols conform with the recommendations in The Biochemical Journal, Instructions to Authors, 1973.

Contents

Introduction

A water-insoluble enzyme may be operationally described as an enzyme which has been rendered functionally insoluble by one of a variety of processes. For example, the enzyme may be either occluded within a gel matrix, the pores of which are too small to allow the escape of entrapped protein; adsorbed onto an inert insoluble polymeric support material; covalently bound to an insoluble polymeric support material or insolubilised by cross-linking of the enzyme protein molecules themselves through a bifunctional reagent. During the past twenty years numerous immobilized enzyme structures have been described and over 60 different enzymes have been immobilized onto one or more of 40 different structures (Goldman et al., 1971). Consequently, the literature to date contains a wealth of data describing the different coupling techniques and support structures, which have been used for the preparation of immobilized enzyme derivatives. This work has been comprehensively reviewed in a number of good review articles (Silman & Katchalski, 1966; Melrose, 1971; Goldman et al., 1971).

The impetus for the study of immobilized enzyme derivatives has arisen principally from the interesting and novel ways that these materials afford for the use of enzymes. For instance, it is possible to conceive of flow processes wherein the immobilised enzyme may be used to continuously effect conversion of its substrate into product. In this respect immobilised enzymes have been used in packed bed reactors and stirred tank reactors. Clearly, operations such as these are not feasible, with the same degree of facility, using the corresponding soluble enzymes. By virtue of their physical form, immobilised enzyme

derivatives can be easily recovered from reaction media; for example, by centrifugation or filtration. Hence they may be used repeatedly, consequently affording a considerable financial saving in terms of enzyme utilisation. Finally, since the immobilized enzyme derivatives can be removed from the reaction media, the reaction products themselves are not contaminated with enzyme protein.

Furthermore, in many instances it has been observed that the stability of an enzyme is enhanced by its immobilization (Sundaram & Hornby, 1970; Marshall et al., 1972). This means that immobilized enzyme derivatives can be used under more taxing reaction conditions wherein the soluble enzyme might be unstable. Consequently, it can be argued that immobilized derivatives of enzymes provide a very practical means of utilizing more efficiently the catalytic potential of enzymes. As a result of this, several preparative processes, which involve the use of enzymes, have been described utilising immobilized enzyme derivatives. For instance, to name but a few, immobilized amyloglucosidase has been used for the continuous conversion of starch to glucose (Smiley, 1971). Mold aminoacylase has been adsorbed onto DEAE-cellulose and used in the form of a packed bed for the continuous resolution of synthetic amino acids (Tosa et al., 1967). Finally, Self et al. (1969) used an immobilized derivative of penicillin amidase for the continuous production of 6-aminopenicillanic acid, which is used for the formation of synthetic penicillins.

Analytical biochemistry is an area where enzymes have been successfully exploited. Their application in this field emanates from

the specificity which enzymes exercise over the reactions that they catalyse. Consequently, the inclusion of an enzyme as an analytical reagent for the determination of its substrate is an extremely facile way of introducing specificity into an analytical procedure. For example, the specific determination of glucose in the presence of other monosaccharides, such as fructose, is not feasible with a non-specific reducing sugar method such as the widely used alkaline ferricyanide procedure. However, an enzyme based assay where only the glucose is specifically modified can be used to determine glucose in the presence of other reducing sugars. For instance, glucose oxidase catalyses the conversion of glucose to gluconic acid and H_2O_2, the latter can be subsequently estimated by conventional chemical methods. Alternatively, the glucose can be converted into 6-phospho gluconic acid using the linked enzyme system of hexokinase and glucose-6-phosphate dehydrogenase and the reaction followed by the formation of NADPH.

Unfortunately, the use of enzymes in analysis is limited by a number of factors, principal among which are; their high cost, the cost of coenzymes, when these are required, and the limited stability of some enzymes under operational conditions. The first two points render some enzyme based assays unfavourable due to the high cost involved and the latter consideration means that the standardisation of some enzyme based assays is quite arduous. Clearly, there are many areas where the use of enzymes are involved, which could be improved. Consequently it would be profitable to consider the

application of immobilized enzyme derivatives to this particular field.

A number of reports describing the use of immobilized enzyme derivatives in analysis have already appeared in the literature. In 1966 Hicks & Updike described the use of immobilized glucose oxidase and immobilized lactate dehydrogenase in the form of columns for the automated determination of glucose and lactate respectively. Guilbault (1969, 1971) has utilised immobilized enzymes in the form of membranes around an electrode, for the specific determination of a metabolite. In this way he has prepared a specific electrode for urea with urease immobilized within a layer of polyacrylamide gel (Guilbault, 1969) and similarly a specific electrode for L-amino acids utilising L-amino acid oxidase (Guilbault, 1971) has likewise been constructed.

A third type of immobilized enzyme structure, where the enzyme is immobilized on the inside surface of a polystyrene tube has been considered for application in automated continuous flow analysis. Hornby et al. (1970) have used this method for the preparation of polystyrene tube-supported glucose oxidase and incorporated the derivative into a continuous automated flow system for the determination of glucose.

Continuous automated analysis is a very important technique, which is widely used in clinical laboratories and quality control laboratories. By its very nature this technique handles

large numbers of samples and consequently utilises large quantities of reagents. Thus the application of immobilized enzyme structures in this technique in order to alleviate some of the practical and economic problems associated with the use of enzymes is warranted. Unfortunately the general applicability of the structure described by Hornby et al. has limited applications due to the extremely hydrophobic nature of the material. A further drawback to the use of polystyrene is the very arduous activation procedure which is necessary for the preparation of immobilized enzyme derivatives. Therefore, further work is required on the application of new support materials, which are less hydrophobic and offer a more facile method for the preparation of immobilized enzyme derivatives, to be used in continuous automated analysis.

Materials

1.1. Buffers

The pH of all buffers was checked using either a Type TTTlc Titrator (Radiometer, Copenhagen, Denmark) or an EIL Model 7030 pH meter (Electronic Instruments Ltd., London, U.K.).

In each case the instrument was calibrated using a standard reference buffer of pH6.86 at 25°C (Beckman Instruments Inc., Fullerton, California, U.S.A.).

Constant molarity buffers were prepared by dissolving the appropriate amount of buffer salt or free acid in distilled water, titrating to the required pH and making up to the required volume.

1.2. Inorganic and Organic Reagents

Wherever possible analytical grade reagents were used without further purification.

1.2.1. N,N-Dimethyl-1,3-Propanediamine

DMAP was obtained from Ralph N. Emanuel Ltd., 264 Water Road, Alperton, Middlesex. U.K.

This reagent was distilled before use and stored in a sealed container at room temperature in the dark.

1.2.2. Glutaraldehyde

Glutaraldehyde (electron microscopy grade) was obtained as a 25%(v/v) solution in water from BDH Chemicals Ltd., Poole, Dorset, U.K. This reagent was stored at 4°C.

1.2.3. 3-Amino-1,2,4-Triazole

3-amino-1,2,4-triazole was obtained from Ralph Emanuel Ltd., and stored as a powder at room temperature.

1.2.4. Bovine Serum Albumin

Bovine serum albumin (crystallised and lyophilised, Sigma Chemical Co., London, U.K.) was stored desiccated at 4°C. Solutions in distilled water were made up immediately prior to use.

1.2.5 Haemoglobin

Bovine haemoglobin (Type II, Sigma Chemical Co.) was stored desiccated at 4°C. Solutions were made up immediately prior to use.

1.3. Enzymes

1.3.1. Glucose Oxidase (EC 1.1.3.4)

Type II glucose oxidase from Aspergillus niger (Sigma Chemical Co.) was treated in the following manner to minimise contaminating catalase activity (Margoliash et al., 1960).

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 and 160μmol of H_2O_2 previously titrated to pH7.5 with 2M-NaOH. The solution was then dialysed at 4°C against water (4x2l) and 0.2M-KH_2PO_4 buffer, pH7.5 (1x2l). Any precipitate that formed during dialysis was removed by centrifugation at 20000g$_{av.}$ 6cm) for 30min at 0°C. This preparation, which is referred to as the triazole-treated glucose

oxidase, had a protein concentration of 3.5mg/ml (the determination of protein is described in section 2.8); a glucose oxidase activity corresponding to 18μmol of glucose oxidised/min per mg protein; and catalase activity corresponding to 20μmol of H_2O_2 decomposed/min per mg protein. Enzymic activities were determined by the methods described in section 2.3. This preparation was stored at 4°C and showed no significant loss in activity over a period of 14 days.

Grade I glucose oxidase from Aspergillus niger (Boehringer Corporation (London) Ltd.) was used without further purification. This preparation was essentially catalase free.

1.3.2. Urease (EC 3.5.1.5)

Jack bean urease (Type VI, Sigma Chemical Co.) was used without further purification.

1.3.3. Invertase (EC 3.2.1.26)

Grade X invertase from Candida utilis (Sigma Chemical Co.) was used without further purification.

1.3.4. Amyloglucosidase (EC 3.2.1.3)

Fungal amyloglucosidase (BDH Chemicals Ltd.) was used without further purification.

1.3.5. Lactate Dehydrogenase (EC 1.1.1.27)

Beef heart lactate dehydrogenase was obtained as a crystalline suspension in 2.1M-$(NH_4)_2SO_4$ (TypeIII, Sigma Chemical Co.) and was treated in the following manner to remove the $(NH_4)_2SO_4$.

5mg of enzyme were collected by centrifugation, dissolved in 1ml of 0.1M-KH_2PO_4,1mM-EDTA,0.1mM-DTT,pH7.8 and dialysed at 4°C for 1 h against 5l of the same buffer. After dialysis the enzyme solution was made up to 2.50ml with the buffer and immediately used for the preparation of nylon tube-supported lactate dehydrogenase, as described in section 2.2.

A crystalline suspension of rabbit muscle lactate dehydrogenase in 2.1M-$(NH_4)_2SO_4$ (Type II, Sigma Chemical Co.) was prepared for use in an identical manner to the beef heart lactate dehydrogenase, except that no dilution was made after dialysis.

For studies on the soluble rabbit muscle enzyme, the $(NH_4)_2SO_4$ suspension was dissolved in 0.1%(w/v) bovine serum albumin. These solutions were stored at 0°C and always used within 12h of their preparation.

1.3.6. Malate Dehydrogenase (EC 1.1.1.37)

Malate dehydrogenase from pig heart was obtained as a suspension in 2.8M-$(NH_4)_2SO_4$(1000 units/mg) from Sigma Chemical Co., and pretreated in an identical manner to the beef heart lactate dehydrogenase.

1.3.7. Pyruvate Kinase (EC 2.7.1.40)

A suspension of rabbit skeletal muscle pyruvate kinase in 2.6M-$(NH_4)_2SO_4$ (Type II, Sigma Chemical Co.) was used. 2mg batches were prepared for use in the same way as the beef heart lactate dehydrogenase. After the dialysis stage the volume was adjusted to 1.5ml.

For studies on the soluble enzyme the $(NH_4)_2SO_4$ suspension was dissolved in 0.1%(w/v) bovine serum albumin. Solutions of this preparation were stored at 0^oC and used within 12h.

1.3.8. Alcohol Dehydrogenase (EC 1.1.1.1)

Yeast alcohol dehydrogenase (salt free, crystallized and lyophilized, Sigma Chemical Co.) was used without further purification.

1.3.9. Peroxidase (EC 1.11.1.7)

Horseradish peroxidase (Type II, Sigma Chemical Co.) was used without further purification.

1.3.10. Penicillinase (EC 3.5.2.6)

Penicillinase (BDH Chemicals Ltd.) was used without further purification. The contents of one sealed vial containing 900 enzyme units (where one unit of penicillinase activity corresponds to 1μmol of penicillin G hydrolysed/min at pH 7.5 at 25^oC) were dissolved in 3ml of 0.1M-KH_2PO_4, pH7.8 and the solution stored at 4^oC.

1.3.11. β-Galactosidase (EC 3.2.1.23)

Escherichia coli β-galactosidase was obtained as a crystalline suspension in 2.2M-$(NH_4)_2SO_4$ (Boehringer Corporation Ltd.) and was treated in the following manner to remove $(NH_4)_2SO_4$.

0.35ml of $(NH_4)_2SO_4$ suspension containing 1.75mg of β-galactosidase were added to 1.65ml of 0.2M-KH_2PO_4, 0.1mM-DTT, pH7.8 and dialysed against the same buffer (3x3l). After dialysis the enzyme solution was

made up to 2.5ml with the buffer and immediately used for the preparation of nylon tube-supported β-galactosidase as described in section 2.2.

1.4. Substrates

1.4.1. Coenzymes

NADH (Grade III, Sigma Chemical Co.), NAD^+ (Grade III, Sigma Chemical Co.) and ADP (Fermentation grade, Sigma Chemical Co.) were stored desiccated at $4^{\circ}C$. Solutions of these coenzymes were always used within 12h of their preparation.

1.4.2. Disaccharides

Solutions of sucrose, maltose and lactose were stored at $4^{\circ}C$ and used within 7 days of their preparation.

1.4.3. α-Keto Acids

Sodium pyruvate (Type II, Sigma Chemical Co.) and cis-oxalacetic acid (Grade I, Sigma Chemical Co.) were stored desiccated at $4^{\circ}C$. Solutions of these α-keto acids were used within 12h of their preparation.

1.4.4. Glucose

Solutions of glucose were made up in distilled water at least 24h before use to ensure complete mutarotation. They were stored at $4^{\circ}C$ and used within 3 days of their preparation.

1.4.5. Urea

Urea solutions in distilled water were stored at $4^{\circ}C$ and used within 7 days of their preparation.

1.4.6. 2,2-Diazo-(3-Ethyl Benzthiazoline-6-Sulphonic Acid)

Solutions of this reagent were stored in the dark at room temperature and used within 12 h of their preparation.

1.4.7. Penicillin G

The sodium salt of penicillin G was obtained from Sigma Chemical Co. and stored desiccated at $4^{\circ}C$. Solutions of this salt in distilled water were always used within 12h of their preparation.

1.4.8. Hydrogen Peroxide

Analar grade H_2O_2 (BDH Chemicals Ltd.) was stored in a dark bottle at $4^{\circ}C$. Immediately prior to use the normality of the H_2O_2 was determined by titration against standard acidified solutions of potassium permanganate (Vogel, 1953).

1.4.9. Phosphoenolpyruvate

Phosphoenolpyruvate (potassium salt, Sigma Chemical Co.) was stored desiccated at $4^{\circ}C$. Solutions in distilled water were used within 12h of their preparation.

Methods

2.1. Structural Material

2.1.1. Nylon Tube

Nylon tube (Type 6 nylon, 1.34mm external diameter, 1.0mm internal diameter) was obtained from Portex Ltd., Hythe, Kent.

Pretreatment of Nylon Tube

Removal of Amorphous Nylon

In order to increase the internal surface area of the tube available for the attachment of protein, the tubes were pitted by the process described by Du Pont de Nemours & Co., (1970).

A solution of 18.6% (w/v) $CaCl_2$, 18.6% (w/v) water in methanol was pumped through 3m lengths of nylon tube for 20min at 50°C at a flow rate of 3ml/min. The amorphous nylon, which is removed by this process, was then purged from the tubes by washing through with 250ml of water at a flow rate of 10ml/min.

Liberation of Free Amino Groups

Nylon tube was hydrolytically cleaved with HCl or non-hydrolytically cleaved with DMAP to liberate free amino groups on the inside surface.

Hydrolytically cleaved nylon tube was prepared by perfusing 3m lengths of nylon tube 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 (Fig.1). This treatment cleaves some of the peptide bonds on the inside surface of the nylon tube, thereby liberating free

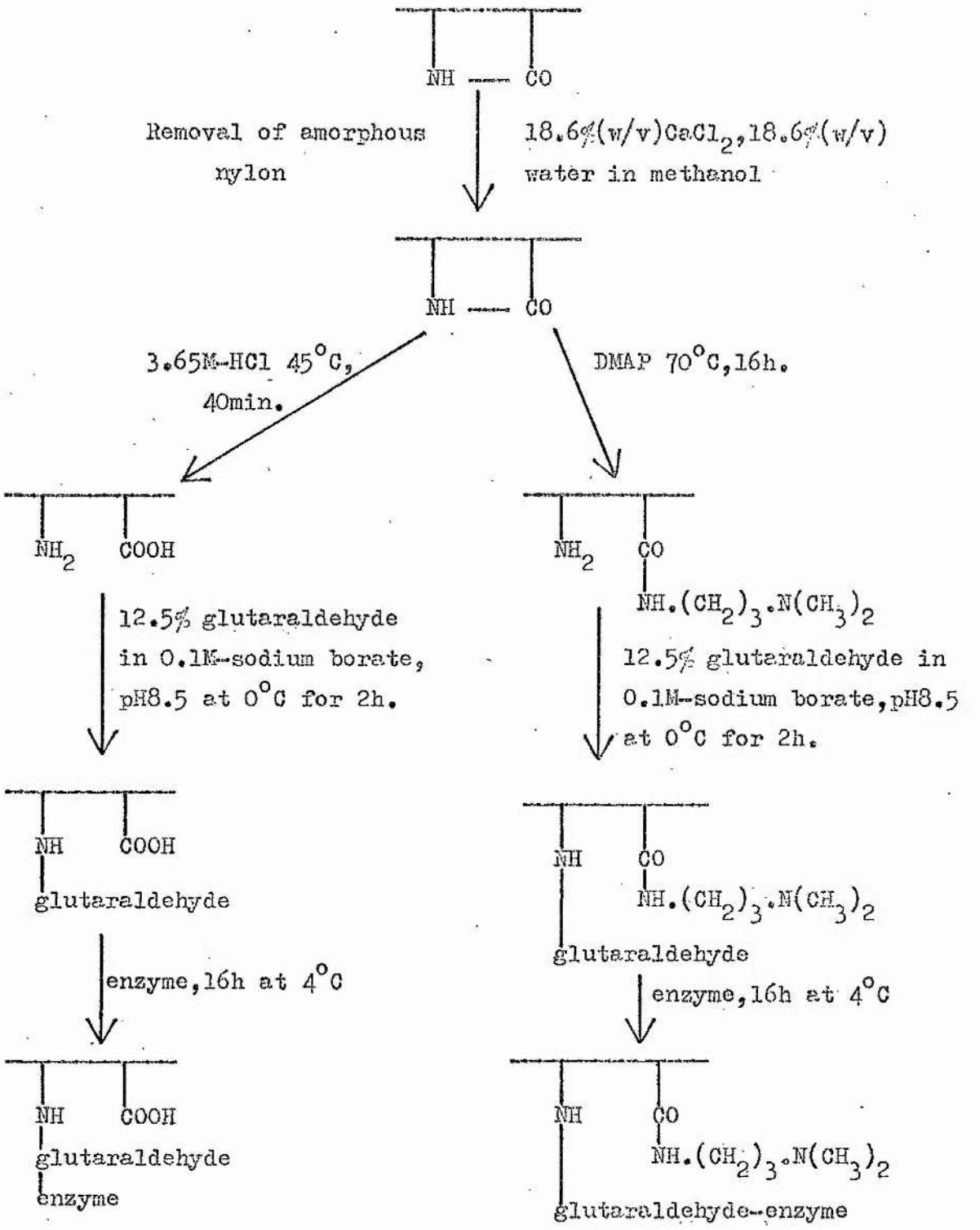


Fig.1. The preparation of nylon tube-supported enzymes.

amino groups and a complement of free carboxyl groups. Under all the experimental conditions used the carboxyl groups will dissociate, thus leaving a residual negative charge on the inner surface of the nylon tube. Nylon tube treated in this manner is referred to as "hydrolytically cleaved" nylon tube.

Non-hydrolytically cleaved tube was prepared as follows: 3m lengths of nylon tube were perfused with dioxan at room temperature for 1h at a flow rate of 1ml/min. The tubes were then filled with DMAP and incubated at 70°C for 16h. After this the tubes were washed with 250ml of water at a flow rate of 10ml/min. Some of the peptide bonds on the inside surface of the nylon tube were cleaved by this process liberating free amino groups and a complement of amidated carboxyl groups. Under all the operating conditions used for the nylon tube derivative the DMAP derivative of the carboxyl groups carries a positive charge, thus the nylon tube had a resultant positive charge. Nylon tube treated in this manner is referred to as "non-hydrolytically cleaved" nylon tube.

Preparation of Gluteraldehyde-Nylon Tube Derivatives

Gluteraldehyde-nylon tube derivatives were prepared by perfusing 3m lengths of either hydrolytically or non-hydrolytically cleaved nylon tube with a solution of 12.5% (v/v) gluteraldehyde in 0.1M-sodium borate buffer, pH8.5 for 60min at 0°C. 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 (Fig. 1).

2.1.2. Nylon Powder

Type 6 low molecular weight 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.

Preparation of Glutaraldehyde-Nylon Powder Derivatives

A 250mg portion of the nylon powder was suspended with rapid stirring in 10ml of 12.5% (v/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 0.2 M-sodium borate buffer, pH8.5 and immediately used for the preparation of immobilized enzyme derivatives.

2.1.3. Nylon Membranes

Type 6 nylon membranes (NRWP 293) were obtained from Millipore Ltd., Alperton, Middlesex, U.K. The membranes were first soaked in distilled water for 10 min and then mounted in a standard Technicon dialyser module (Technicon Instruments Co. Ltd., Chertsey, Surrey, U.K.). Nylon membrane-supported enzymes were then prepared in a one step process as described in section 2.2.

2.2. Preparation of Immobilized Enzyme Derivatives

2.2.1. Nylon Powder-Supported Glucose Oxidase

Triazole-treated glucose oxidase was immobilized onto glutaraldehyde treated nylon powder by suspending 250mg of the powder for 16h at 4°C in 10ml of the enzyme solution. The powder was then packed under

gravity into a glass column (4.0x0.2cm 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.

2.2.2. Nylon Powder-Supported Urease

250mg of glutaraldehyde-treated nylon powder was suspended for 16h at 4°C in 5ml of 0.05M-KH_2PO_4 buffer containing 10mg 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 (4.0x0.2cm bore) and washed free of physically adsorbed enzyme by perfusion with 500ml of 0.2M-NaCl at a flow rate of 2ml/min.

2.2.3. Nylon Membrane-Supported Glucose Oxidase

The upper channel of the dialyser module was perfused in a closed loop at 1°C at a flow rate of 1ml/min 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 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.

2.2.4. Nylon Membrane-Supported Urease

For the preparation of nylon membrane-supported urease the upper channel of the dialyser module was 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 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) gluteraldehyde in 0.1M-sodium borate buffer, pH8.5. After 2h, the membrane was washed free of excess 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.

2.2.5. Nylon Tube-Supported Glucose Oxidase

Nylon tube-supported glucose oxidase was prepared by perfusing a 3m length of hydrolytically cleaved 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 tube with 500ml of 0.2M-NaCl at a flow rate of 10ml/min.

Alternatively, 7.5mg of glucose oxidase (Grade 1, Boehringer Corporation Ltd.) were dissolved in 2.5ml of 0.2M-KH_2PO_4 buffer, pH7.8 and drawn into a 3m length of hydrolytically cleaved nylon tube with a syringe. The ends of the tube were then sealed and the tube incubated at 4°C for 16h. The tube was then washed with 125ml of 0.1M-sodium acetate buffer, pH5.0 at a flow rate of 2.5ml/min.

2.2.6. Nylon Tube-Supported Urease

Urease was immobilised on the inside surface of a 3m length of hydrolytically cleaved nylon tube by perfusing in a closed loop for 4h at 0°C at a flow rate of 2.5ml/min, 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.

2.2.7. Nylon Tube-Supported Enzymes

General Procedure

A general procedure was adopted for the preparation of nylon tube-supported enzymes, which was applicable to both hydrolytically cleaved and non-hydrolytically cleaved nylon tubes.

The enzyme to be immobilised was dissolved in 2.5ml of the coupling buffer and drawn into a 3m length of the appropriate nylon tube derivative with a syringe. The ends of the tube were then sealed and the tube incubated at 4°C for 16h. Physically adsorbed protein was then purged from the nylon tube by perfusion with buffer at 20°C for 1h at a flow rate of 2.5ml/min. The experimental details for the preparation of nylon tube-supported derivatives are presented in Table 1.

2.2.8. Nylon Tube-Co-Supported Enzymes

General Procedure

A general procedure was adopted for the preparation of nylon tube-co-supported enzymes. The procedure used was similar to the method described for the preparation of single enzyme tubes. The experimental details for the preparation of nylon tube-co-supported enzymes are presented in Table 2.

TABLE I

THE PREPARATION OF NYLON-TUBE-SUPPORTED ENZYMES

Enzyme	Quantity of Enzyme used in Coupling (mg)	Concentration of Enzyme in Coupling Mixture (mg/ml)	Method of Nylon tube Cleavage	Length of Nylon tube (m)	Coupling Buffer	Coupling Conditions	Washing Conditions
Amylo-glucosidase	10.0	4	Hydrolytic 3.65M-HCl 45°C for 40min	3	0.2M-KH_2PO_4, pH 7.8	16h, 4°C	0.1M-sodium acetate, pH 5.0 for 1h at 20°C at a flow rate of 2.5ml/min
Beef Heart Lactate Dehydrogenase	5	2	Non-hydrolytic DMAP at 70°C for 16h	3	0.1M-Na_2HPO_4, 1.0mM-EDTA, 0.1mM-DTT, pH 7.8	16h, 4°C	(i) 0.1M-Na_2HPO_4, 1.0mM-EDTA, 0.1mM-DTT, 20.0mM-$NaBH_4$, pH 7.8 for 1h at a flow rate of 3.3ml/min at 1°C (ii) 0.1M-Na_2HPO_4, 1.0mM-EDTA, 0.1mM-DTT, pH 7.8 for 1h at a flow rate of 2.5ml/min at 20°C
β-Galactosidase	1.75	0.7	Non-hydrolytic DMAP at 70°C for 16h	3	0.1M-Na_2HPO_4, pH 7.8	16h, 4°C	0.1M-Na_2HPO_4, 1mM-$MgCl_2$, 0.05mM-EDTA, pH6.5 for 1 h at a flow rate of 2.5ml/min at 20°C
Glucose oxidase (Grade I, Boehringer)	7.5	3	Hydrolytic 3.65M-HCl at 45°C for 40min	3	0.2M-KH_2PO_4, pH 7.8	16h, 4°C	0.1M-CH_2COONa, pH5.0 for 1h at a flow rate of 2.5ml/min at 20°C
Invertase	7.5	3	Hydrolytic 3.65M-HCl 45°C for 40min	3	0.2M-KH_2PO_4, pH 7.8	16h, 4°C	0.1M-sodium acetate, pH 5.0 for 1h at 20°C at a flow rate of 2.5ml/min

TABLE I (CONTD.)

THE PREPARATION OF NYLON TUBE-SUPPORTED ENZYMES

Enzyme	Quantity of Enzyme used in Coupling (mg)	Concentration of Enzyme in Coupling Mixture (mg/ml)	Method of Nylon Tube Cleavage	Length of Nylon Tube (m)	Coupling Buffer	Coupling Conditions	Washing Conditions
Malate Dehydrogenase	5	2	Non-hydrolytic DMAP at 70°C for 16h	3	0.1M-Na_2HPO_4, 1.0mM-EDTA 0.1mM-DTT, pH 2.8	16h, 4°C	(i) 0.1M-Na_2HPO_4, 1.0mM-EDTA, 0.1mM-DTT, 20.0mM-$NaBH_4$, pH7.8 for 1h at a flow rate of 3.3ml/min at 1°C (ii) 0.1M-Na_2HPO_4, 1.0mM-EDTA, 0.1mM-DTT, ph7.8 for 1 h at a flow rate of 2.5ml/min at 20°C
Penicillinase	750 units	300 units/ml	Non-hydrolytic DMAP at 70°C for 16h	3	0.2M-KH_2PO_4, pH 7.8	16h, 4°C	75mM-KH_2PO_4, pH6.5 for 1 h at a flow rate of 2.5ml/min at 20°C
Yeast Alcohol Dehydrogenase	10	4	Non-hydrolytic DMAP at 70°C for 16h	3	0.1M-Na_2HPO_4, 1.0mM-EDTA 0.1mM-DTT, pH 7.8	16h, 4°C	(i) 0.1M-Na_2HPO_4, 1.0mM-EDTA, 0.1mM-DTT, 20.0mM-$NaBH_4$, pH 7.8 for 1 h at a flow rate of 3.3 ml/min at 1°C (ii) 0.1M-Na_2HPO_4, 1.0mM-EDTA, 0.1mM-DTT, pH7.8 for 1 h at a flow rate of 3.3ml/min at 20°C

TABLE II

THE PREPARATION OF NYLON TUBE-CO-SUPPORTED ENZYMES

Enzyme	Quantity of Enzyme used in Coupling (mg)	Concentration of Enzyme in Coupling Mixture (mg/ml)	Method of Nylon tube Cleavage	Length of Nylon tube (m)	Coupling Buffer	Coupling Conditions	Washing Conditions
Amyloglucosidase and Glucose Oxidase (Grade 1, Boehringer)	2 2	1.6	Hydrolytic 3.65M-HCl at 45°C for 40 min	3	0.2M-KH_2PO_4, pH 7.8	16h, 4°C	0.1M-CH_3COONa, pH 5.5 for 1h at a flow rate of 2.5ml/min at 20°C
Glucose Oxidase (Grade 1, Boehringer) and Peroxidase	3 2	2.0	Hydrolytic 3.65M-HCl at 45°C for 40 min	3	0.2M-sodium borate, pH 8.5	16h, 4°C	0.1M-CH_3COONa, pH 5.5 for 1 h at a flow rate of 2.5ml/min at 20°C
Invertase and Glucose Oxidase (Grade 1, Boehringer)	8 4	4.8	Hydrolytic 3.65M-HCl at 45°C for 40 min	3	0.2M-KH_2PO_4, pH 7.8	16h, 4°C	0.1M-CH_3COONa, pH 5.0 for 1h at a flow rate of 2.5ml/min at 20°C
Pyruvate Kinase and Rabbit Muscle Lactate Dehydrogenase	2 2	1.6	Non-hydrolytic DMAP at 70°C for 16h	3	0.1M-KH_2PO_4, 1.0mM-EDTA, 0.1mM-DTT, pH7.8	16h, 4°C	0.1M-KH_2PO_4, 0.06M-KCl, 4mM-$MgCl_2$, 0.5mM-DTT, pH7.4 for 1h at a flow rate of 2.5ml/min at 20°C

2.3. Measurement of Enzymic Activity

2.3.1. Glucose Oxidase

Glucose oxidase activity was determined according to the method described in the Seravac Catalogue (Seravac Laboratories (Pty.) Ltd., Holyport, Maidenhead, Berks, U.K.). Assays were performed at 25°C in the presence of 0.1M-potassium phosphate buffer, pH5.9, by measurement of the oxygen uptake, which results from the oxidation of β-D-glucose, using a Yellow Springs Model 53 oxygen monitor (Yellow Springs Instrument Company, Yellow Springs, Ohio, U.S.A.).

2.3.2. Catalase

The catalase activity of glucose oxidase preparations was determined by the spectrophotometric method of Beers & Sizer (1952).

2.3.3. Penicillinase

Penicillinase activity was determined titrimetrically by measuring the initial rate of hydrolysis of benzylpenicillin using a TTT 1c titrator assembly (Radiometer). All assays were performed at pH6.7 in a total reaction volume of 100ml and in each case the reaction was started by the addition of enzyme.

2.3.4. Lactate Dehydrogenase

Lactate dehydrogenase activity was determined by measuring the rate of decrease of extinction at 340nm, when NADH is oxidized by excess pyruvic acid in the presence of the enzyme.

All assays were carried out in a 3ml reaction volume contained in 1cm light path cuvette at 30°C in the presence of 0.1M-KH_2PO_4, 0.5mM-DTT, 0.06M-KCl, 4mM-$MgCl_2$, 0.2mM-pyruvic acid, 0.15mM-NADH, pH7.4. The reaction was always started by the addition of enzyme.

For studies of the effect of pH on the rate of enzymic activity a mixed buffer system of the following composition was used, 25mM-phosphate, 25mM-acetate and 25mM-borate, titrated with either strong acid or alkali to the appropriate pH.

2.3.5. Pyruvate Kinase

Pyruvate kinase activity was determined by either the method of Cooper & Kornberg (1969) or by the procedure described by Tietz & Ochoa (1958).

By the former method pyruvate kinase activity is determined by the colorimetric measurement of the pyruvic acid which is formed in the enzymic reaction, as its hydrazone with 2,4-DNPH.

A 10ml reaction volume, which contained 0.1M-KH_2PO_4, 0.2mM-phosphoenolpyruvate, 0.06M-KCl, 4mM-$MgCl_2$, 0.5mM-DTT, 0.1mM-ADP, pH7.4 was equilibrated at 30°C and the reaction started by the addition of enzyme. After 5min a 1ml aliquot was withdrawn, added to 1ml of 0.03% 2,4-DNPH in 2M-HCl, incubated for a further 10min and then 1ml of 4M-NaOH added. The extinction at 445nm was then measured after a further 10min, against a reagent blank. The pyruvic acid concentration was determined by reference to a standard curve compiled by subjecting known amounts of

pyruvic acid to the assay procedure. The effect of pH on enzymic activity was determined by using a mixed buffer system of 25mM-acetate, 25mM-borate and 25mM-phosphate, titrated with strong acid or alkali to the required pH.

In the latter method the pyruvic acid, formed in the course of the reaction, was measured spectrophotometrically by using lactate dehydrogenase in the presence of excess NADH.

All assays were carried out in a 3ml reaction volume contained in a 1cm light path cuvette at 30°C in the presence of 0.1M-potassium phosphate, 0.15mM-NADH, 0.5mM-DTT, 0.06M-KCl, 4mM-$MgCl_2$, 0.2mM-ADP, 0.2mM-phosphoenolpyruvate and 0.7-7 units lactate dehydrogenase, pH7.4. In each case the reaction was started by the addition of the pyruvate kinase.

For studies of the effect of pH on the rate of enzymic activity a mixed buffer system of 25mM-potassium phosphate, 25mM-acetate and 25mM-borate was used, titrated to the required pH with either strong acid or base.

2.4. Assay of Immobilized Enzyme Derivatives

2.4.1. Tube-Supported Derivatives

Nylon tube-co-supported pyruvate kinase and lactate dehydrogenase was assayed by perfusion through the tube with solutions of the substrates at predetermined flow rates and analysis of the effluent for reaction products. The enzyme tube was employed in the form of a coil 4cm in diameter, and maintained at 30°C±0.01 by immersion

in a thermostatically controlled water bath. Flow rates were determined by collecting known volumes of effluent and noting the time taken for their emergence. The system was always allowed to attain a steady state, wherein the concentration of the reaction products in the effluent was constant, before determination of the amount of NADH oxidation was undertaken. This was done by measuring the difference in extinction at 340nm between the substrate solution and the effluent.

Solutions of ADP in the concentration range 0.2-1.0mM were assayed in the presence of 0.1M-KH_2PO_4,0.06M-KCl,4mM-$MgCl_2$,0.05mM-DTT, 1.0mM-phosphoenolpyruvate,0.15mM-NADH,pH7.4. For studies of the effect of pH on the coupled enzymic reaction, the substrates were prepared in a mixed buffer system of the following composition: 25mM-acetate, 25mM-borate and 25mM-phosphate, titrated with either strong acid or alkali to the required pH.

The pyruvate kinase activity of the co-supported pyruvate kinase and lactate dehydrogenase nylon tube was measured separately by colorimetric measurement of the pyruvic acid formed in the enzymic reaction, as its hydrazone with 2,4-DNPH.

A 1ml aliquot of the effluent was collected, added to 1ml of 0.03%(v/v) 2,4-DNPH in 2M-HCl and the solution incubated for 10 min. 1ml of 4M-NaOH was then added, the solution incubated for a further 10min and the extinction at 445nm determined with reference to a reagent blank. The pyruvic acid concentration was then interpolated by reference to a standard curve compiled by subjecting known amounts of pyruvic acid to the prescribed assay procedure.

Solutions of ADP in the concentration range 0.2-0.1mM were assayed in the presence of 0.1M-KH_2PO_4,0.06M-KCl,4mM-$MgCl_2$, 0.5mM-DTT,1.0mM-phosphoenolpyruvate,pH7.4. The effect of pH on the pyruvate kinase activity was determined by using a mixed buffer system of 25mM-acetate, 25mM-borate and 25mM-phosphate, titrated to the required pH with either strong acid or alkali.

2.5. Automated Analysis

2.5.1. Equipment

Automated analytical techniques employing immobilised enzyme derivatives were based on the "Technicon" continuous flow through method. The sampler(S),pump(P),heating bath(HB) and dialyser module(D) were all standard "Technicon" equipment. Other symbols used in the figures describing the flow systems are as follows: MC,mixing coil; DB,debubbler; W,waste.

Extinction was recorded with a Beckman DB spectrophotometer (Beckman Instruments, Glenrothes, Fife, U.K.), fitted with a matched pair of 1cm light path flow cells of 0.12ml internal volume.

2.6. Analytical Methods

2.6.1. Nylon Powder-Supported Enzymes

General Procedures

Nylon powder-supported enzymes were used in the form of small packed beds (4.0cmx0.2cm bore), maintained at 37°C by their immersion in a thermostatically controlled water bath.

Samples were first air-segmented and then mixed with the appropriate buffer. However, since it is not possible to perfuse a packed bed with an air-segmented stream, it was necessary to de-gas the sample stream before perfusing it through the packed bed of nylon powder-supported enzyme. The sample stream was air-segmented again immediately after leaving the packed bed and then mixed with the appropriate colour reagents for the determination of reaction products.

2.6.1.1 Determination of Glucose using Nylon Powder-Supported Glucose Oxidase

Glucose was determined spectrophotometrically by measuring the extinction at 349nm of I_2 formed by the reaction of H_2O_2, produced in the enzymic reaction, with acid/KI. The KI reagent was always used within 12h of its preparation.

$$\beta\text{-D-glucose} + O_2 \longrightarrow \text{D-gluconono-}\delta\text{-lactone} + H_2O_2$$

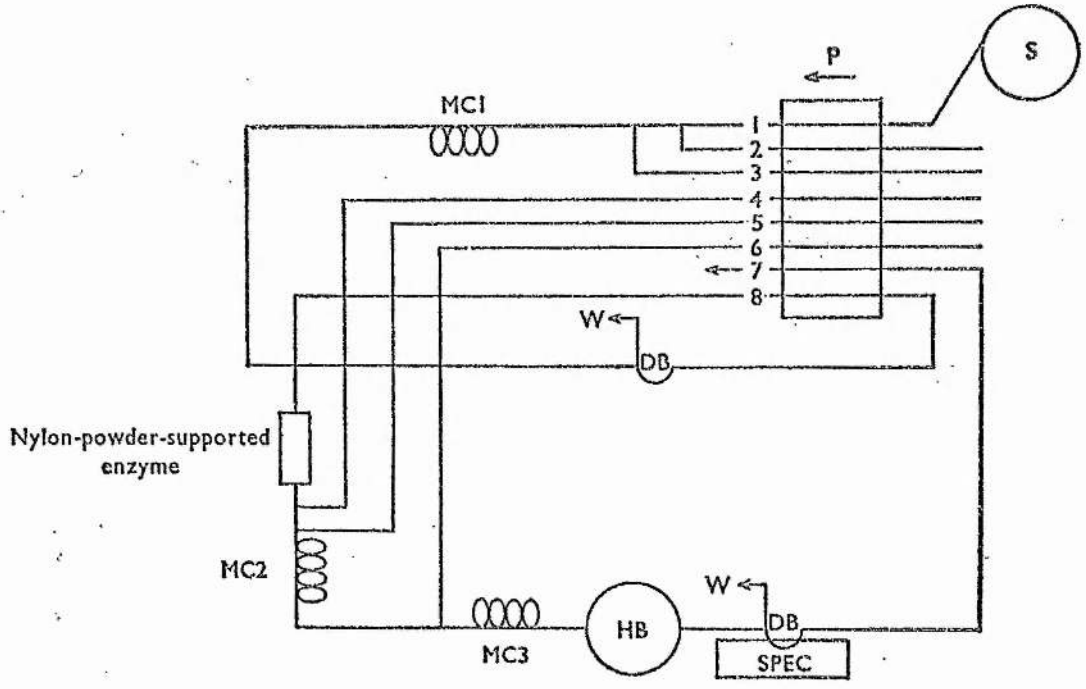
$$\text{D-gluconono-}\delta\text{-lactone} + H_2O \longrightarrow \text{gluconic acid}$$

$$H_2O_2 + 2KI + 2HCl \longrightarrow I_2 + 2KCl + 2H_2O$$

Fig.2 shows the flow system used for the measurement of glucose using nylon powder-supported glucose oxidase. The sample stream containing glucose was first air-segmented and then mixed with 0.2M-sodium acetate buffer, pH5.0. The stream was then degassed and perfused through the packed bed of nylon powder-supported glucose oxidase. After leaving the packed bed the stream was immediately air-segmented, acidified with 1.25M-HCl, mixed with a solution of 0.25M-KI and then passed through a standard mixing coil. The liquid stream was then degassed and its extinction at 349nm recorded.

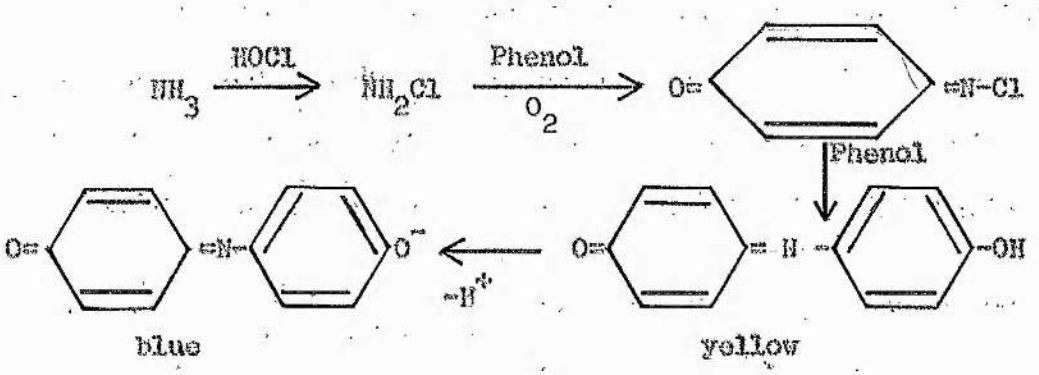
Fig. 2. Flow system for the use of nylon powder-supported glucose oxidase and nylon powder-supported urease in the form of small packed beds in automated analysis.

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(v/v) wash/sample ratio was used. The packed bed of nylon powder-supported enzyme was maintained at 37°C. The mixing coil (MC2) and the heating bath (HB) were not included in the flow system. Substrate, air, 0.5M-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(v/v) 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-0.5M-KH_2PO_4 buffer, pH7.0, air, reagent A and reagent B were pumped through the pump lines 1,2,3,4,5 and 6 respectively. For the meaning of the symbols see section 2.5.1.


S
P
MC1
1
2
3
4
5
6
7
8
W
DB
Nylon-powder-supported
enzyme
MC2
MC3
HB
W
DB
SPEC

2.6.1.2 Determination of Urea using Nylon Powder-Supported Urease

Urea was determined by measurement of the ammonia formed in the enzymic reaction by the method of Chaney & Marbach (1962).



The flow system used for the determination of urea using nylon powder-supported urease is shown in Fig.2. Before entering the packed bed of nylon powder-supported urease, the sample stream containing urea was segmented with air, mixed with 0.5M-KH_2PO_4 buffer, 5.0mM-EDTA, pH7.0 and then degassed. Immediately after leaving the packed bed the sample stream was air-segmented and mixed with Reagent A(0.006%(w/v) sodium nitroprusside in aq.4.7%(w/v) phenol) and then with Reagent B (NaOCl in 0.5M-NaOH, conteining 0.10-0.15% available chlorine). The sample stream was then passed through a standard delay coil at 37°C, after which it was degassed and its extinction at 630nm recorded.

2.6.2. Nylon Membrane-Supported Enzymes

General Procedures

Nylon membrane-supported enzymes were incorporated into automated flow systems by mounting the membrane between the plates of a standard dialyser module. To ensure equilibration of substrate across the membrane-supported enzyme the sample or donor stream was always pumped

into the dialyser module at a flow rate greater than it was pumped out to the exit stream. This ensured a continuous flow of sample from the donor stream to the recipient stream.

2.6.2.1. Determination of Glucose using Nylon Membrane-Supported Glucose Oxidase

Glucose was determined spectrophotometrically by the acid/KI method (see section 2.6.1.1.).

The flow system used for the determination of glucose with nylon membrane-supported glucose oxidase is shown in Fig.3. The sample stream was air-segmented, mixed with 0.2M-sodium acetate buffer, pH5.0 before being pumped into the dialyser module as the donor stream, and the recipient stream consisted of air-segmented 0.2M-sodium acetate buffer, pH5.0. Upon leaving the dialyser module the donor stream was pumped to waste and the recipient stream was acidified with 1.25M-HCl followed by 0.25M-KI. After passage through a standard mixing coil the stream was degassed and its extinction at 349 nm recorded.

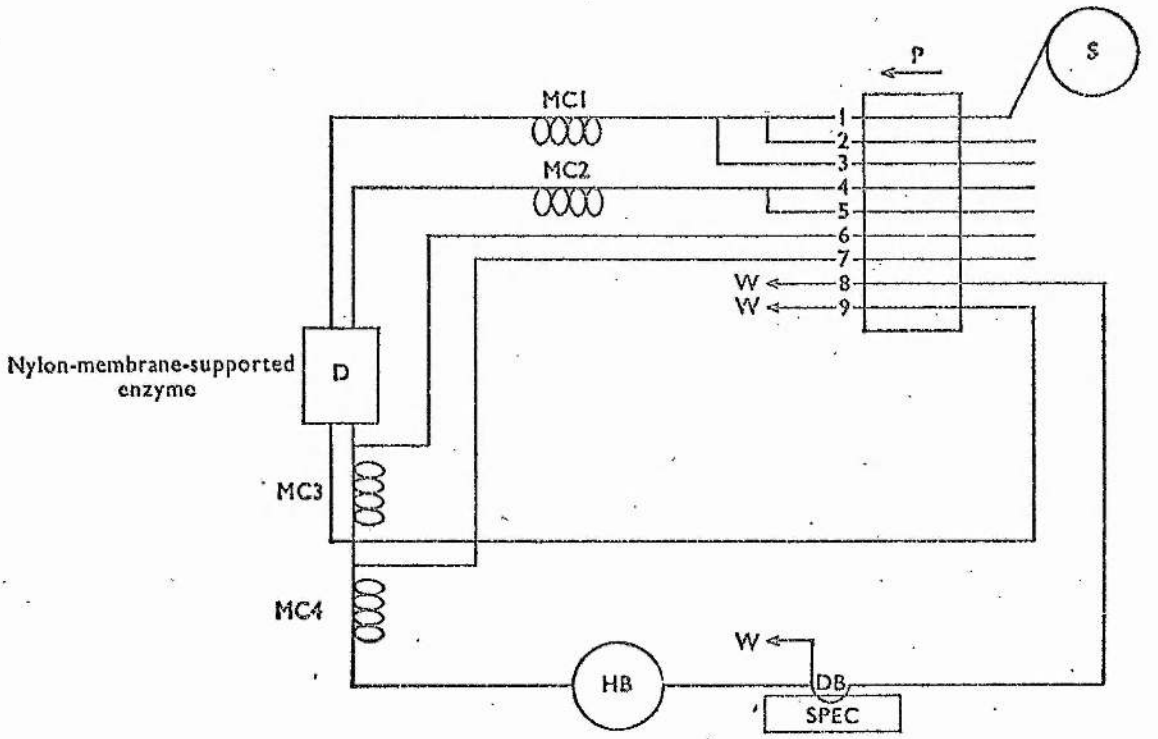
2.6.2.2. Determination of Urea using Nylon Membrane-Supported Urease

Urea was determined by measurement of the ammonia formed in the enzymic reaction by the method of Chaney & Marbach (1962) (see section 2.6.1.2).

The flow system used for the determination of urea using nylon membrane-supported urease is shown in Fig.3. The sample stream containing urea, was first air-segmented, mixed with 0.1M-KH_2PO_4 buffer,

Fig.3. Flow system for the use of nylon membrane-supported glucose oxidase and nylon membrane-supported urease 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(v/v) wash/sample ratio was used. The nylon membrane-supported enzyme was maintained at 37°C. The mixing coil (MC3) 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(v/v) 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 meaning of the symbols see section 2.5.1.


P
S
MC1
MC2
1
2
3
4
5
6
7
W
W
8
9
Nylon-membrane-supported enzyme
D
MC3
MC4
W
HB
DB
SPEC

5mM-EDTA, pH7.0 and then pumped into the upper channel of the dialyser module, containing the nylon membrane-supported urease. An air-segmented stream of 0.1M-KH_2PO_4 buffer, 5mM-EDTA, pH7.0 was perfused through the lower channel and upon leaving the dialyser module was mixed first with Reagent A(0.006%(w/v) sodium nitroprussido in aq.4.7%(v/v) phenol) followed by Reagent B(NaOCl in 0.5M-NaOH containing 0.10-0.15% available chlorine). The liquid stream was then passed through a standard delay coil at 37°C, after which it was degassed and its extinction at 630nm recorded.

2.6.3. Nylon Tube-Supported Enzymes

General Procedures

Nylon tube-supported enzymes were incorporated into automated flow systems in the form of coils, 4cm in diameter and maintained at constant temperature by their immersion in a thermostatically controlled water bath.

Analytical Methods involving Nylon Tube-Supported Oxidoreductases

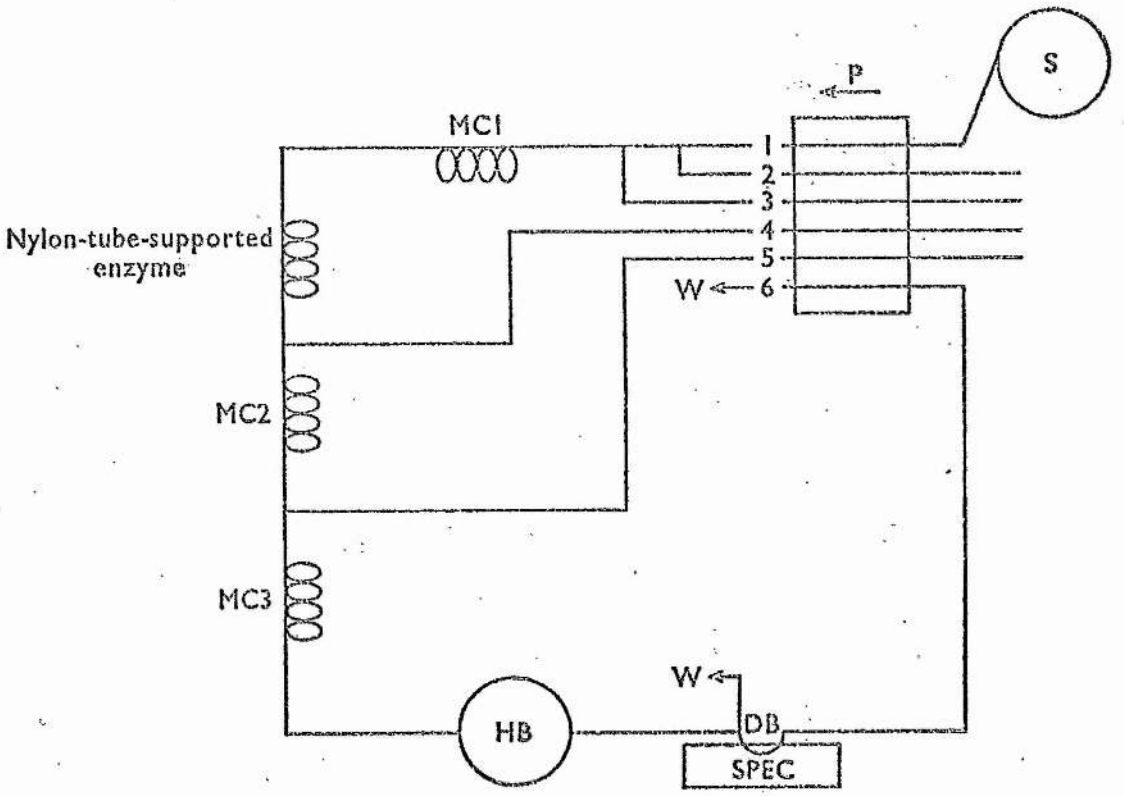
2.6.3.1. Determination of Glucose using Nylon Tube-Supported Glucose Oxidase

Glucose was determined spectrophotometrically by the acid/KI method (see section 2.6.1.1.).

The flow system used for the determination of glucose using a 3m length of nylon tube-supported glucose oxidase is shown in Fig.4. After beingair-segmented and mixed with 0.5M-sodium acetate buffer, pH5.0 the sample stream was perfused through the nylon tube-supported

Fig. 4. Flow system for the use of nylon tube-supported glucose oxidase and nylon tube-supported urease 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(v/v) wash/sample ratio was used. The nylon tube-supported enzyme was maintained at 37°C. The mixing coil (MC2) and the heating bath (HB) were not included in the flow system. Substrate, air, 0.5M-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-0.5M-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 section 2.5.1.


S
P
MC1
1
2
3
4
5
W
6
Nylon-tube-supported
enzyme
MC2
MC3
W
HB
DB
SPEC

glucose oxidase, which was maintained at 37°C. The effluent, containing H_2O_2 as one of the products of the enzymic reaction, was first mixed with 1.25M-HCl and then 0.25M-KI. After being passed through a standard mixing coil, it was degassed and its extinction at 349nm recorded.

2.6.3.2 Determination of Glucose using Nylon Tube-Co-Supported Glucose Oxidase and Peroxidase

Glucose was determined by linking the glucose oxidase reaction to peroxidase and measuring the extinction at 620nm due to the reaction between the hydrogen donor, perid, and the H_2O_2 in the presence of peroxidase.

$$\text{Perid} + H_2O_2 \longrightarrow \text{oxidised perid} + 2H_2O$$

Fig.5 shows the flow system used for the determination of glucose by this method. The sample stream containing glucose, was air-segmented, mixed with 0.5M-sodium acetate buffer, pH5.5 and then with 5mM-perid, before being perfused through a 3m length of nylon tube-co-supported glucose oxidase and peroxidase maintained at 25°C. The effluent from the tube was then degassed and its extinction at 620nm recorded.

2.6.3.3 General Procedure for Methods involving Nylon-Tube-Supported Dehydrogenases

Fig. 6 shows the flow system used for the automated determination of pyruvate, oxaloacetate, ethanol and ADP using nylon tube-supported lactate, malate, alcohol dehydrogenases and nylon tube-co-supported pyruvate kinase and lactate dehydrogenase respectively.

Fig. 5. Flow system for the use of nylon tube-co-supported glucose oxidase and peroxidase in automated analysis.

For the determination of glucose with nylon tube-co-supported glucose oxidase and peroxidase, the pump tubing lines 1,2,3,4 and 5 gave flow rates of 1.40,0.32,0.60,0.42 and 2.00ml/min respectively. The nylon tube-co-supported enzymes were maintained at 25°C and a 2:1(v/v) wash/sample ratio was used. Substrate, air, 0.5M-sodium acetate buffer, pH5.5 and 5mM-perid were pumped through lines 1,2,3 and 4 respectively. For the meaning of the symbols see section 2.5.1.

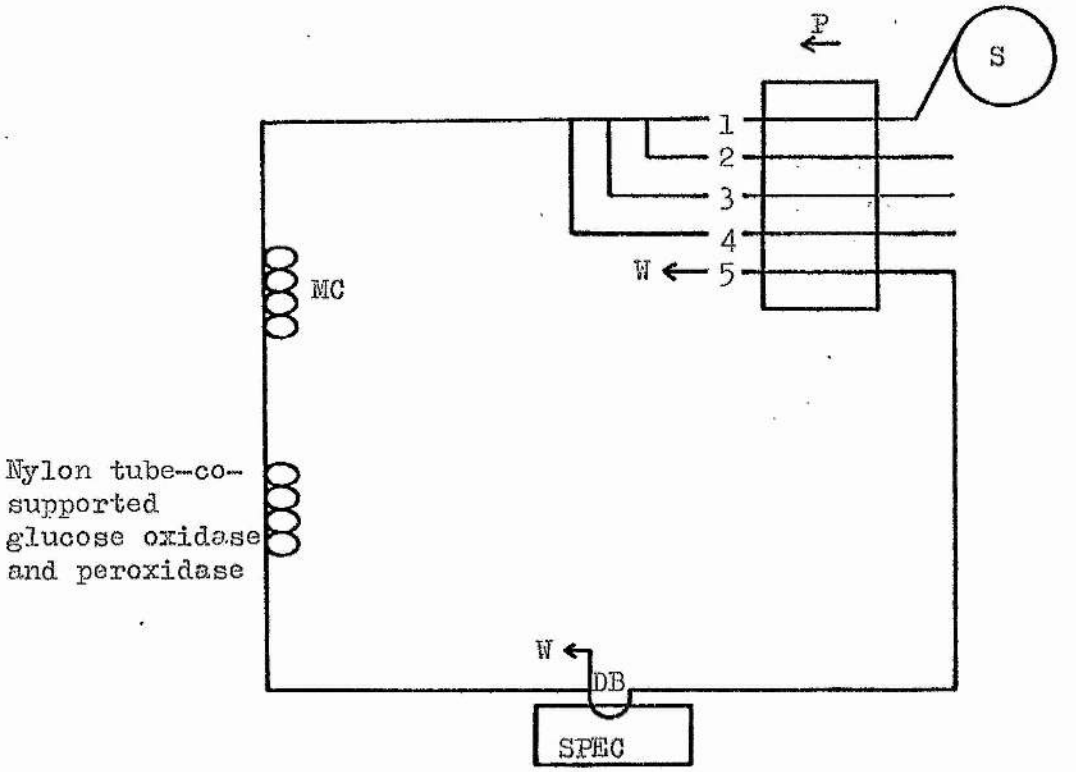
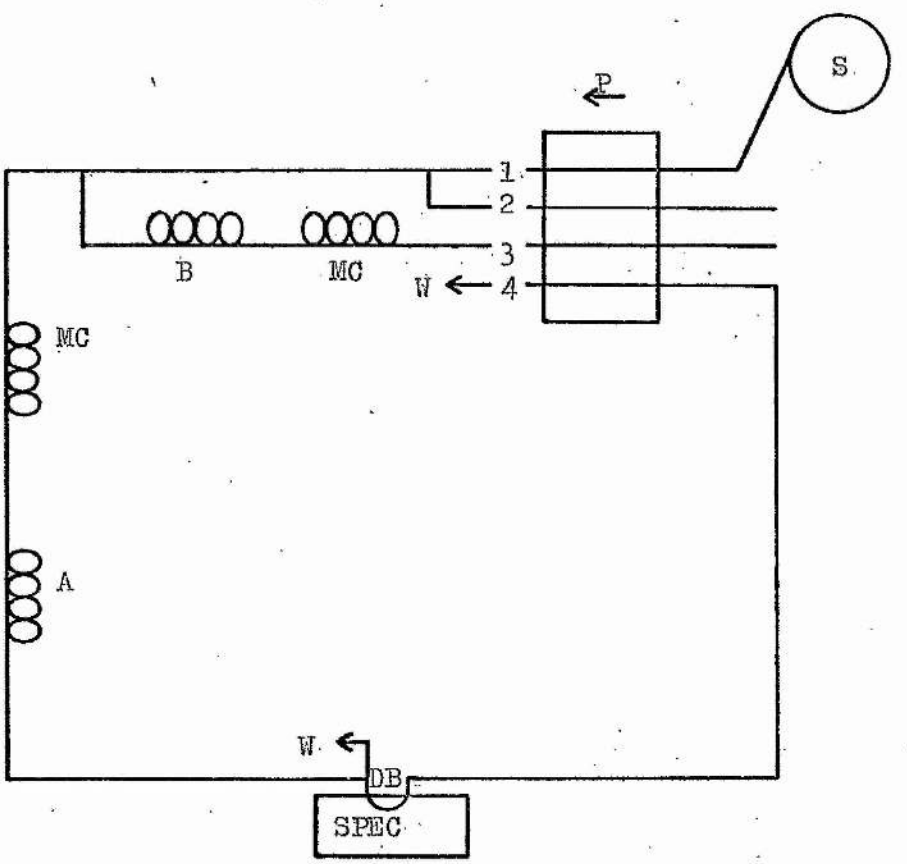

P
S
1
2
3
4
W
5
MC
Nylon tube--co-
supported
glucose oxidase
and peroxidase
W
DB
SPEC

Fig. 6. Flow system for the use of nylon tube-supported dehydrogenases in automated analysis.

For the determination of pyruvate, oxalacetate and ethanol using nylon tube-supported lactate, malate and alcohol dehydrogenases respectively, the pump tubing lines 1,2,3 and 4 gave flow rates of 2.00,0.42,0.42 and 2.00ml/min respectively. Substrate, air and buffer were pumped through lines 1, 2 and 3 respectively. The nylon tube-supported enzymes were maintained at 25°C, a 2:1(v/v) wash/sample ratio was used and analyses were performed at the rate of 30/h. The tube B was not included in the flow system. For the determination of pyruvate and oxalacetate using nylon tube-supported lactate and malate dehydrogenases respectively, and using the nylon tube-supported alcohol dehydrogenase to continuously generate the NADH, the pump tubing lines 1,2,3 and 4 gave flow rates of 2.00,0.42,0.42 and 2.00ml/min respectively. The nylon tube-supported alcohol dehydrogenase was inserted at position B in the flow system. Substrate, air and buffer were pumped through lines 1, 2 and 3 respectively. The nylon tube-supported enzymes were maintained at 25°C, a 2:1(v/v) wash/sample ratio was used and analyses were performed at the rate of 30/h.

For the determination of ADP using nylon tube-co-supported pyruvate kinase and lactate dehydrogenase, the pump tubing lines 1,2,3 and 4 gave flow rates of 2.00,0.42,0.42 and 2.00ml/min respectively. Substrate, air and buffer were pumped through lines 1, 2 and 3 respectively. The nylon tube-co-supported enzymes were maintained at 25°C, a 2:1(v/v) wash/sample ratio was used and analyses were performed at the rate of 30/h. For the meaning of the symbols see section 2.5.1.


S
P
1
2
3
4
B
MC
W
MC
A
W
DB
SPEC

In each case the pyruvate, oxalacetate or ethanol samples were assayed by recording either the decrease or increase of the absorbance at 340nm due to the utilisation or production of NADH.

$$\text{Pyruvate} + \text{NADH} \longrightarrow \text{lactate} + \text{NAD}^+$$

$$\text{Oxalacetate} + \text{NADH} \longrightarrow \text{malate} + \text{NAD}^+$$

$$\text{Ethanol} + \text{NAD}^+ \longrightarrow \text{acetaldehyde} + \text{NADH}$$

ADP was assayed by linking the pyruvate kinase reaction to lactate dehydrogenase and determining the pyruvate produced in the pyruvate kinase reaction with excess NADH.

$$\text{ADP} + \text{PEP} \longrightarrow \text{pyruvate} + \text{ATP}$$

$$\text{Pyruvate} + \text{NADH} \longrightarrow \text{lactate} + \text{NAD}^+$$

$$\text{ADP} + \text{PEP} + \text{NADH} \longrightarrow \text{ATP} + \text{lactate} + \text{NAD}^+$$

2.6.3.4. Determination of Pyruvate using Nylon Tube-Supported Lactate Dehydrogenase

Pyruvate was determined by inserting a 3m length of nylon tube-supported lactate dehydrogenase at position A in the flow system shown in Fig. 6 and buffer of the following composition 0.1M-KH_2PO_4, 1mM-EDTA, 0.1mM-DTT, 1.0mM-NADH, pH7.5 pumped through line 3.

2.6.3.5. Determination of Oxalacetate using Nylon Tube-Supported Malate Dehydrogenase

Oxalacetate was determined by inserting a 3m length of nylon tube-supported malate dehydrogenase at position A in the flow system shown in Fig.6 and buffer of the following composition 0.1M-KH_2PO_4, 1mM-EDTA, 0.1mM-DTT,1.0mM-NADH,pH7.5 pumped through line 3.

2.6.3.6. Determination of Ethanol using Nylon Tube-Supported Alcohol Dehydrogenase

For the determination of ethanol, a 3m length of alcohol dehydrogenase was inserted at position A in the flow system shown in Fig. 6 and 0.1M-KH_2PO_4 buffer, 1.0mM-EDTA, 0.1mM-DTT, 1.0mM-NAD^+, pH7.5 pumped through line 3.

2.6.3.7. Determination of ADP using Nylon Tube-Co-Supported Pyruvate Kinase and Lactate Dehydrogenase

ADP was determined by inserting a 3m length of nylon tube-co-supported pyruvate kinase and lactate dehydrogenase at position A in the flow system shown in Fig.6 and buffer of the following composition 0.4M-KH_2PO_4, 2.5mM-PEP, 1.0mM-DTT, 1.0m-NADH, 0.3M-KCl, 20mM-$MgCl_2$, pH7.4 pumped through line 3.

2.6.3.8. General Procedure for the Continuous Production of NADH from NAD^+ for the Assay of Pyruvate and Oxalacetate

Nylon tube-supported alcohol dehydrogenase was inserted at position B in Fig. 6 and continuously perfused with 0.1M-KH_2PO_4 buffer, 1.0mM-EDTA, 0.1mM-DTT, 2mM-NAD^+, 400mM-ethanol, pH7.5. The effluent from the alcohol dehydrogenase tube was then mixed with the air-segmented sample stream and perfused through the second enzyme coil inserted at position A.

$$\text{Ethanol} + NAD^+ \longrightarrow \text{Acetaldehyde} + NADH$$

$$\text{Pyruvate or Oxalacetate} + NADH \longrightarrow \text{Lactate or Malate} + NAD^+$$

2.6.3.9. Determination of Pyruvate using Nylon Tube-Supported Lactate Dehydrogenase and Nylon Tube-Supported Alcohol Dehydrogenase

Nylon tube-supported lactate dehydrogenase and nylon tube-supported alcohol dehydrogenase were inserted at positions A&B respectively in Fig.6 and 0.1M-KH_2PO_4 buffer, 1mM-EDTA, 0.1mM-DTT, 2mM-NAD^+, 400mM-ethanol, pH7.5 pumped through line 3.

2.6.3.10. Determination of Oxalacetate using Nylon Tube-Supported Malate Dehydrogenase and Nylon Tube-Supported Alcohol Dehydrogenase

Nylon tube-supported malate dehydrogenase and nylon tube-supported alcohol dehydrogenase were inserted at positions A&B respectively in Fig.6 and buffer of the composition 0.1M-KH_2PO_4, 1mM-EDTA, 0.1mM-DTT, 2mM-NAD^+, 400mM-ethanol, pH7.5 pumped through line 3.

Analytical Methods used for the Determination of Disaccharides

2.6.3.11. Determination of Sucrose using Nylon Tube-Supported Invertase

Sucrose was determined by measurement of the glucose formed in the enzymic reaction by the DNSA method of Bruner (1964).

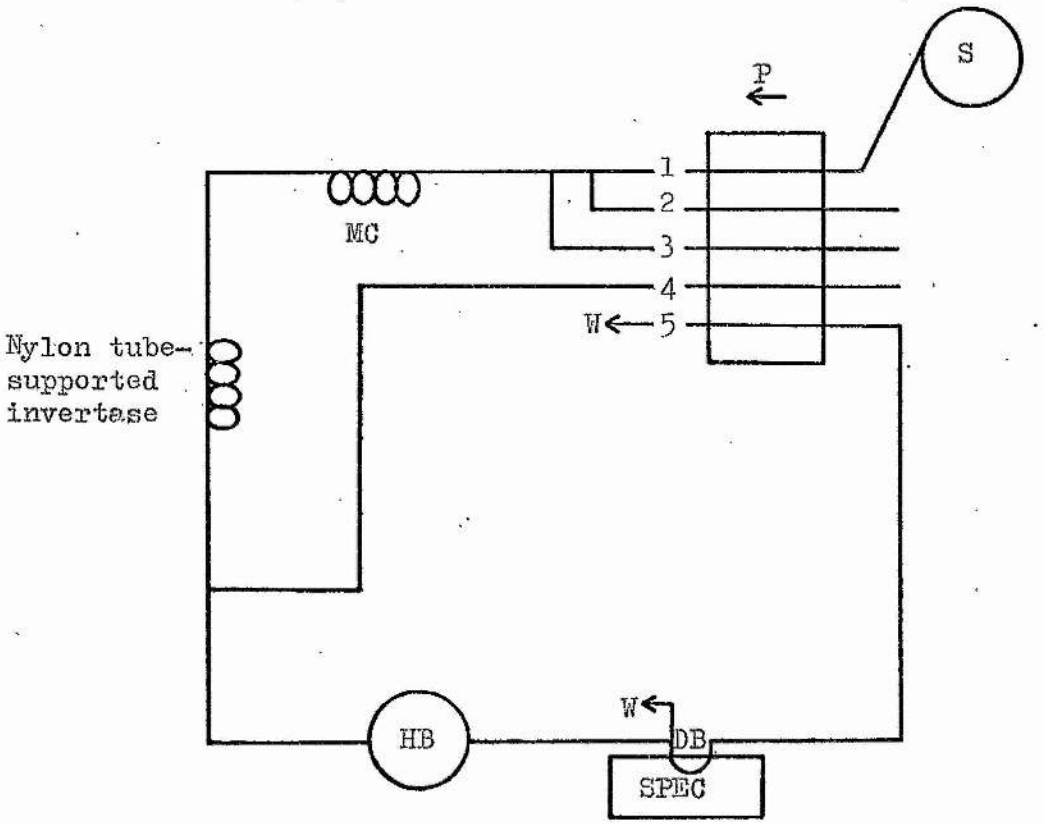
$$\text{Sucrose} \longrightarrow \alpha\text{-D-glucose} + \text{D-fructose}$$

$$\text{3,5-Dinitrosalicylic acid} \xrightarrow[\text{KOH}]{\text{reducing sugar}} \text{3-amino-5-nitro salicylic acid}$$

The flow system used for the determination of sucrose using a 3m length of nylon tube-supported invertase is shown in Fig. 7. After being air-segmented, the sample stream containing sucrose was mixed with 0.5M-sodium acetate buffer, pH5.5 prior to being perfused through the nylon tube-supported invertase which was maintained at 37°C. The effluent from the tube, containing glucose as one of the reaction

Fig. 7. Flow system for the use of nylon tube-supported invertase in automated analysis.

For the determination of sucrose with nylon tube-supported invertase, 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 nylon tube-supported invertase was maintained at 37°C and the heating bath (HB) 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) DNSA in 1M-KOH were pumped through pump tubing lines 1,2,3 and 4 respectively. For the meaning of the symbols see section 2.5.1.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Nylon tube-
supported
invertase
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HB
DB
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products, was then mixed with 0.6% (v/v) DMSA in 1M-KOH and passed through a standard delay coil at 65°C. It was subsequently degassed and its extinction at 540nm recorded.

2.6.3.12. General Procedure for the Determination of Disaccharides using Linked Enzyme Systems

2 methods were used for the determination of disaccharides using linked enzyme systems. 1) The appropriate disaccharidase and glucose oxidase were immobilised onto the same 3m length of nylon tube and inserted at position A in the flow system shown in Fig.8 and 2) the appropriate disaccharidase and glucose oxidase were immobilized onto separate lengths of nylon tube and inserted into the flow system shown in Fig. 8 at positions A&B respectively. In each case the glucose liberated was determined spectrophotometrically by the acid/KI method (see section 2.6.1.1.).

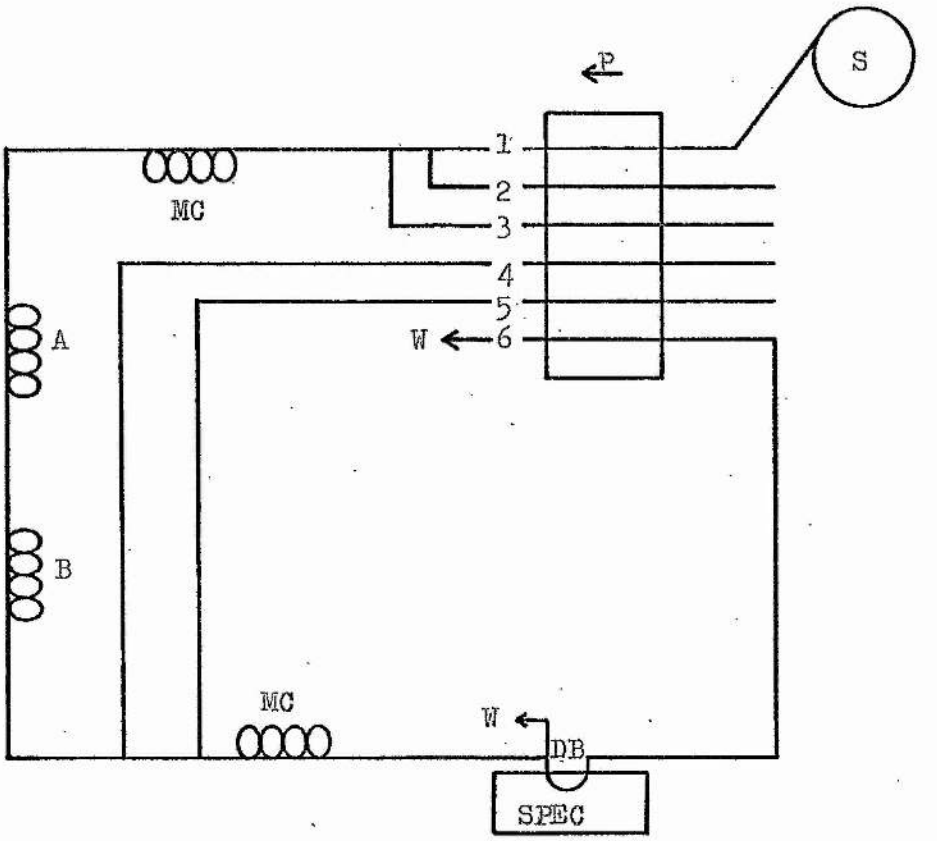
The sample stream containing the disaccharide, was air-segmented and mixed with buffer before being perfused through the linked enzyme coil or coils. The sample stream was then acidified with 1.25M-HCl followed by 0.25M-KI and passed through a standard mixing coil. Finally, the solution was degassed and its extinction at 349nm recorded.

2.6.3.13. Determination of Sucrose

Fig. 8 shows the flow system used for the determination of sucrose using invertase and glucose oxidase immobilized as described in methods 1 and 2 (see section 2.6.3.12). Buffer of the following

Fig. 8. Flow system for the use of nylon tube-supported linked enzyme systems in automated analysis.

For the determination of sucrose, maltose and lactose using nylon tube-supported invertase, amyloglucosidase and β-galactosidase respectively, placed in series with nylon tube-supported 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. The nylon tube-supported enzymes were maintained at 37°C and a 2:1(v/v) wash/sample ratio was used. Substrate, air, buffer, 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 sucrose and maltose using nylon tube-co-supported invertase and glucose oxidase and nylon tube-co-supported 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 nylon tube-co-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 section 2.5.1.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composition 0.5M-sodium acetate, pH5.0 was pumped through line 3.

$$\text{Sucrose} \longrightarrow \alpha\text{-D-glucose} + \text{D-fructose}$$

$$\alpha\text{-D-Glucose} \rightleftharpoons \beta\text{-D-glucose}$$

$$\beta\text{-D-Glucose} + O_2 \longrightarrow \text{D-glucono-}\delta\text{-lactone} + H_2O_2$$

$$\text{D-Glucono-}\delta\text{-lactone} + H_2O \longrightarrow \text{gluconic acid}$$

$$H_2O_2 + 2KI + 2HCl \longrightarrow I_2 + 2KCl + 2H_2O$$

2.6.3.14. Determination of Lactose

The flow system used for the determination of lactose using β-galactosidase and glucose oxidase, immobilized as described in method 2 (see section 2.6.3.12) is shown in Fig. 8. 0.5M-KH_2PO_4 buffer, 5mM-$MgCl_2$, 0.25mM-EDTA, pH6.5 was pumped through line 3.

$$\text{Lactose} \longrightarrow \text{D-galactose} + \beta\text{-D-glucose}$$

$$\beta\text{-D-Glucose} \rightleftharpoons \alpha\text{-D-glucose}$$

$$\beta\text{-D-Glucose} + O_2 \longrightarrow \text{D-glucono-}\delta\text{-lactone} + H_2O_2$$

$$\text{D-Glucono-}\delta\text{-lactone} + H_2O \longrightarrow \text{gluconic acid}$$

$$H_2O_2 + 2KI + 2HCl \longrightarrow I_2 + 2KCl + 2H_2O$$

2.6.3.15 Determination of Maltose

Fig. 8 shows the flow system for the determination of maltose using amyloglucosidase and glucose oxidase immobilized as described in methods 1 and 2 (see section 2.6.3.12). Buffer of the following composition 0.5M-sodium acetate, pH5.5 was pumped through line 3.

$$\text{Maltose} \longrightarrow \alpha\text{-D-glucose} + \beta\text{-D-glucose}$$

$$\beta\text{-D-Glucose} + O_2 \longrightarrow \text{D-glucono-}\delta\text{-lactone} + H_2O_2$$

$$\text{D-Glucono-}\delta\text{-lactone} + H_2O \longrightarrow \text{gluconic acid}$$

$$H_2O_2 + 2KI + 2HCl \longrightarrow I_2 + 2KCl + 2H_2O$$

2.6.3.16. Determination of Penicillin G using Nylon Tube-Supported Penicillinase

Penicillin G was determined colorimetrically by measuring the decrease in extinction at 578nm of starch/iodine solution due to the reaction with penicilloic acid liberated during the enzymic reaction.

$$\text{Penicillin G} + H_2O \longrightarrow \text{Penicilloic acid}$$

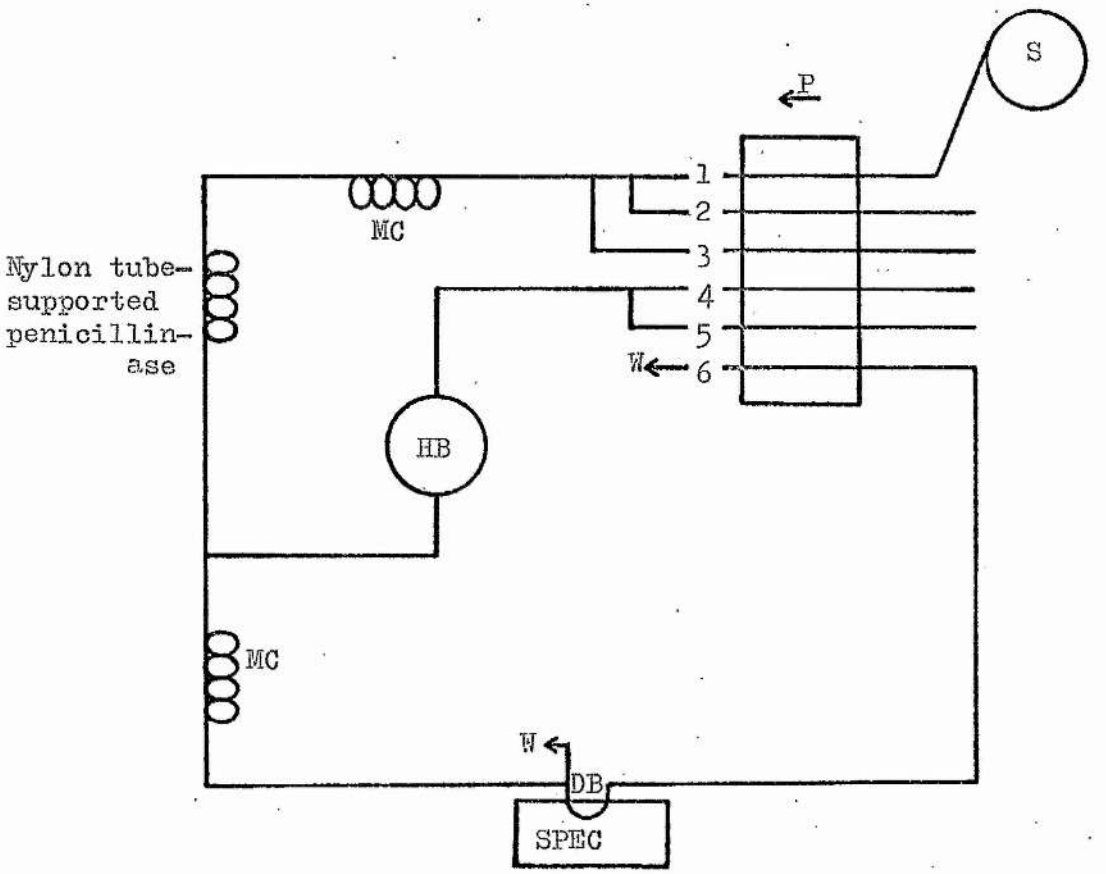
Fig. 9 shows the flow system used for the determination of penicillin G using nylon tube-supported penicillinase. The sample stream containing penicillin G, was air-segmented, mixed with 0.2M-KH_2PO_4 buffer, pH6.5 and then passed through the enzyme tube maintained at 25°C. Upon leaving the tube the stream was mixed with a solution of 0.12%(w/v) soluble starch, 40mM-KH_2PO_4, 0.08mM-iodine, pH6.5 and passed through a standard mixing coil. Finally, the sample stream was degassed and its extinction at 578nm recorded.

2.6.3.17. Determination of Urea using Nylon Tube-Supported Urease

Urea was determined by measurement of the ammonia formed in the reaction by the method of Chaney & Marbach (1962) (see section 2.6.1.2).

Fig. 9. Flow system for the use of nylon tube-supported penicillinase in automated analysis.

For the determination of penicillin G with nylon tube-supported penicillinase the pump tubing lines 1,2,3,4,5 and 6 gave flow rates of 0.60,0.60,0.42,2.50,0.42 and 2.9ml/min respectively. The nylon tube-supported penicillinase and the heating bath (HB) were maintained at 25°C. Substrate, air, 0.2M-KH_2PO_4 buffer, pH6.5, 0.15% soluble starch in 0.05M-KH_2PO_4 buffer, pH6.5 and 0.5mM-iodine were pumped through the pump tubing lines 1,2,3,4 and 5 respectively. A 2:1(v/v) wash/sample ratio was used. For the meaning of the symbols see section 2.5.1.


S
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MC
Nylon tube-supported penicillin-ase
HB
MC
W
DB
SPEC

The flow system used for the determination of urea using nylon tube-supported urease is shown in Fig. 4., The sample stream containing urea, was first air-segmented and then mixed with 0.5M-KH_2PO_4 buffer,5mM-EDTA,pH7.0 prior to being perfused through the nylon tube-supported urease, which was maintained at 37°C. After leaving the tube, the effluent containing ammonia, an one of the products of the reaction, was first mixed with Reagent A(0.006%(w/v) sodium nitroprusside in aq.4.7%(v/v) phenol) followed by Reagent B (NaOCl in 0.5MNaOH containing 0.10-0.15% available chlorine). The sample stream was then passed through a standard delay coil at 37°C, after which it was degassed and its extinction at 630nm recorded.

Analysis of Fermentation Products using Nylon Tube-Supported Enzymes

2.6.3.18. Determination of Penicillin G in Fermentation Broths

Cultures of *Penicillium Chrysogenum* were grown in the following manner: a Roper-Steep slope culture of *P.Chrysogenum* (strain NRRL-1951-B25, a gift from Dr. E.G. Jeffreys, Imperial Chemical Industries Ltd., Alderley Park, Cheshire) was sub-cultured into 2 litres of Hockenhulls defined medium (Hockenhull 1969) which also contained 0.05%(w/v) phenylacetic acid,0.025%(w/v) $MgSO_4$,0.3% (v/v) $NaNO_3$,0.004%(w/v) $ZnSO_4$,0.05%(v/v) KH_2PO_4,0.002%(v/v) $MnSO_4$, pH6.5. The culture was rapidly stirred with aeration at 25°C without pH control.

Samples were removed from the fermenter at regular intervals and the pH measured, after which the penicillin G level was

determined using the method described in section 2.6.3.16. The penicillin G content was interpolated from a standard curve compiled by subjecting known amounts of penicillin G to the assay procedure.

2.6.3.19 Determination of Ethanol and Glucose in Yeast Cultures

Glucose was fermented in the following manner: 2g of dried bakers yeast (Distillers Company Ltd.) were suspended in 1 litre of medium containing 24g Malt extract (Boots Pure Drug Co.Ltd.), 36g glucose and 5g citric acid, pH5.5. The medium was prepared 24h before use to allow complete muterotation of the glucose. The mixture was stirred at 37°C and samples removed at regular intervals in order that the glucose and ethanol levels could be determined. For this, glucose was determined using the method described in section 2.6.3.1. and ethanol was determined using the method described in section 2.6.3.6.

2.7. Muterotation of Glucose

2.7.1. Muterotation of Glucose using Nylon Tube-Supported Invertase and Nylon Tube-Supported Glucose Oxidase

Fig. 8 shows the flow system used to follow the muterotation of glucose using nylon tube-supported invertase inserted at position A and nylon tube-supported glucose oxidase at position B. A series of delays were introduced into the flow system between positions A & B by inserting a variety of mixing coils and delay coils. In each case the nylon tube-supported enzymes were assayed by the procedure described in section 2.6.3.13.

2.7.2. Measurement of the Rate Constants for the Mutarotation of Glucose

The rate constants for the mutarotation of glucose were determined at 37°C in acetate buffers, pH5.0 using a Hilger & Watts Polarimeter (Hilger & Watts (Ltd.), London).

0.9g of 'Analar' glucose were dissolved in the appropriate acetate buffer at 37°C, placed in a 5cm light path cell and mounted into the polarimeter. Readings were taken of the angle of rotation every 2mins and the results plotted in the form of a first order log plot. The log of the change in rotation was plotted against time giving a line of negative slope. The rate constant of mutarotation was then calculated by measurement of the slope of the line.

$$\alpha\text{-D-Glucose} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \beta\text{-D-glucose}$$

The system behaves as a first order one with effective rate constant equal to the sum of k_1 and k_2.

Let α_0 = angle of rotation at t=0 mins

" α_t = " t=t "

" α_e = " equilibrium

Since the system obeys first order kinetics, it can be shown that

$$\log_e(\alpha_t - \alpha_e) - \log_e(\alpha_0 - \alpha_e) = -(k_1 + k_2)\, t$$

or $$\log_{10}(\alpha_t - \alpha_e) = \log_{10}(\alpha_0 - \alpha_e) - \frac{(k_1 + k_2)\, t}{2.303}$$

Thus a plot of $\log_{10}(\alpha_t - \alpha_e)$ against t should yield a straight line of slope $-(k_1+k_2)/2.303$.

2.8. Determination of Protein Concentration

Protein in solution was always determined by the microbiuret method of Itakahi & Gill (1964). In each case reference was made to a standard curve compiled by subjecting known amounts of egg albumen to the assay procedure.

Results and Discussion

3.1. Supports and Enzyme Immobilization

3.1.1. Enzyme Immobilization

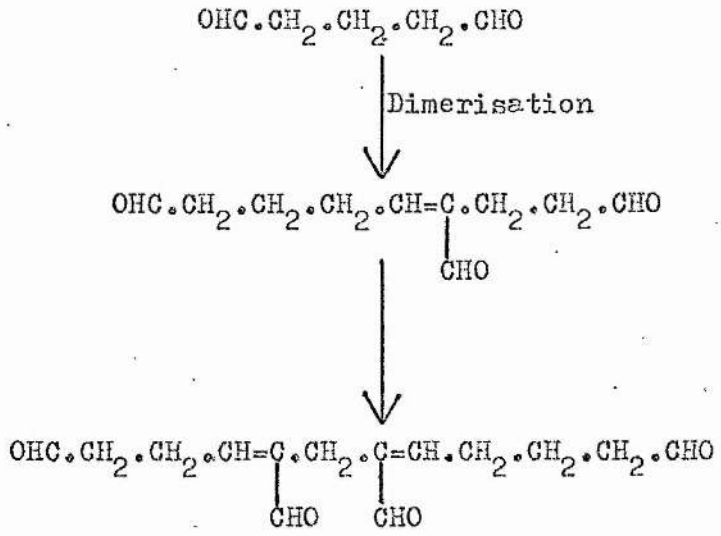
Glutaraldehyde has some properties, which make it an attractive compound for use in enzyme immobilization. For example, it is relatively non-toxic, easy to handle and readily available at low cost. Furthermore, it affords a facile method for the preparation of immobilized enzyme derivatives.

Enzymes have been immobilized using glutaraldehyde by three different methods. Quiocho & Richards (1966) cross-linked carboxypeptidase by incubating the enzyme and glutaraldehyde together at room temperature and in 1967 Habeeb immobilized trypsin on AE-cellulose by cross-linking the enzyme both to and around the support with glutaraldehyde, by incubating all three materials together at room temperature. In 1970 Sundaram & Hornby immobilized urease onto hydrolytically cleaved nylon tube by first perfusing the tube with glutaraldehyde, washing free of physically adsorbed glutaraldehyde and then perfusing with urease. Since glutaraldehyde is a small molecule (Mol.wt.≈ 100), the possibility exists in the first two methods that the glutaraldehyde may react extensively with the enzyme thus producing an over-substituted derivative whose catalytic activity may be impaired. However, this possibility is more remote in the Sundaram & Hornby procedure, since the glutaraldehyde is immobilized onto the nylon matrix before the enzyme is added. As a consequence, the reactivity of the glutaraldehyde towards the enzyme may be greatly

restricted and so it is probable that the groups on the protein molecule reacting with the glutaraldehyde are limited to those situated on the periphery of the molecule.

Ogata et al. (1968) presented evidence that glutaraldehyde combines with the free ε-amino groups of lysine residues, whilst Habeeb & Hiramoto (1968) implicated the amino acids histidine, tyrosine, cysteine as well as lysine. Furthermore, the nature of the linkage between the glutaraldehyde and the protein is uncertain and two possibilities have been discussed by Richards & Knowles (1968). The first proposal considers that the glutaraldehyde exists in solution in an oligomeric form, as a result of aldol condensation reactions, which result in unsaturated molecules. It is then possible for protein molecules containing unprotonated amino groups to react via a Michael-type addition across these double bonds (Fig.10). The second proposal considers that the glutaraldehyde reacts with the free amino groups on the enzyme with the formation of a Schiff base-type linkage. The conditions necessary for the two reactions are similar, for example, the free amino groups would need to be unprotonated for both reactions to occur and consequently pH values favouring dissociation of the amino groups would favour either method. However, the functional capacity of glutaraldehyde to react as a bifunctional reagent is essentially the same regardless of the mechanism of its reaction with amino groups, since the end result in each case is an effective immobilization of the enzyme.

(a) Aldol Condensations



(Structure 1)

(b) Cross-linking Reactions

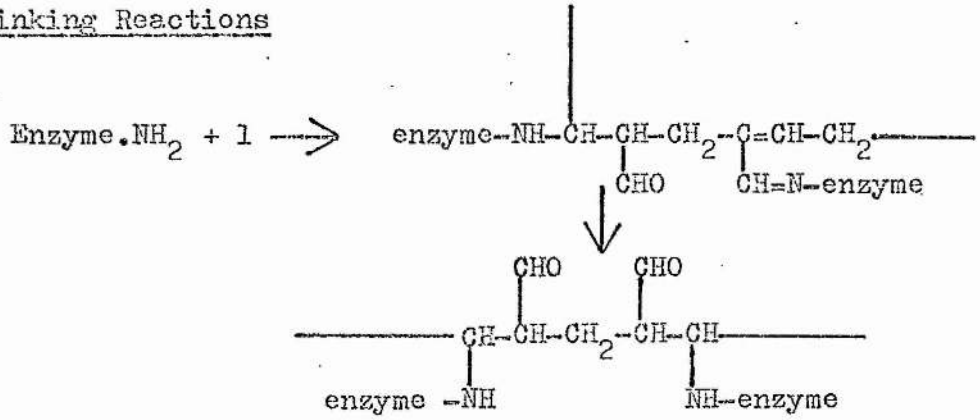


Fig.10. The proposed polymerisation reactions of glutaraldehyde and the subsequent reactions with unprotonated amino groups.

3.1.2. Support Structures

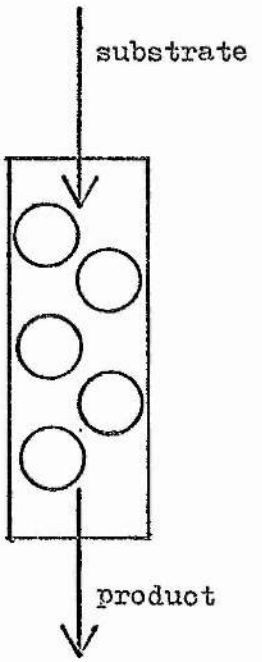
Three different types of support structure can be considered for the preparation of immobilized enzymes for application in automated analytical flow systems. These structures are: a tube used in the form of a coil with the enzyme immobilized onto the inside surface, a powder used in the form of a small packed bed and a membrane with the enzyme immobilized around and within the matrix of the membrane and used in a continuous dialyser module. Fig. 11 shows these three different structures and the ways in which they may be used in continuous flow-through processes.

Of the three different types of support structure described, only the nylon powder has readily available free amino groups (see section 2.1.2.). However, after cleavage of the inside surface of nylon tube, free amino groups may be detected by perfusion of the tube with 2,4,6-trinitrobenzene sulphonic acid in saturated sodium tetraborate and observation of the characteristic yellow colour. Therefore, the immobilization of protein to these two structures, using glutaraldehyde, may be envisaged as proceeding via a reaction between the free amino groups on the support and one of the aldehyde functions of the glutaraldehyde and between the other aldehyde function of the glutaraldehyde and free amino groups on the enzyme protein.

On the other hand, nylon membranes have to be made from high molecular weight nylon, since low molecular weight nylon is too brittle and lacks the mechanical properties required for membrane formation. Therefore, the nylon membrane has correspondingly few free

(a) A Tube

(b) A Packed Bed



(c) A Membrane

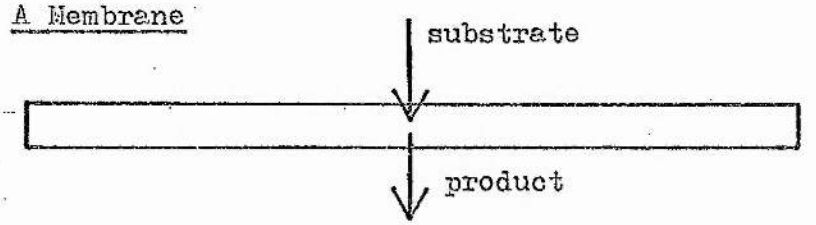


Fig.11. The use of immobilized enzyme structures in continuous flow-through processes.

groups and by virtue of the requirement for mechanical strength it cannot be hydrolysed to liberate more free end groups. Consequently, the immobilisation of an enzyme on a nylon membrane requires a method which is not dependent upon the presence of free amino groups. Thus the enzyme and gluteraldehyde may be added to the membrane together, resulting in a general cross-linking of the protein around and within the pores of the nylon membrane matrix.

3.1.3. Preparation of Nylon Tube-Supported Enzymes

Table 3 shows the effect of pretreatment of the nylon tubes with 18.6%(v/v) $CaCl_2$,18.6%(v/v) water in methenol on the activity of nylon tube-supported enzyme derivatives. Both the nylon tube-supported triázole-treated glucose oxidase derivatives were prepared under identical conditions, except for the pretreatment of the pitted tube, and their activity was determined by assaying samples containing 5mM-glucose using the method described in section 2.6.3.1. Similarly, both nylon tube-supported urease derivatives were prepared under identical conditions, except for the pretreatment of the pitted tube and their activity was determined by assaying samples containing 0.1mM-urea using the method described in section 2.6.3.17.

These results show that this process increases the activity of the nylon tube-supported urease ninefold, and that of the nylon tube-supported glucose oxidase eightfold, in both cases relative to the comparable derivatives prepared from non-pitted nylon tube. The pitting process removes the regions of amorphous nylon from the nylon structure leaving the regions of crystalline nylon intact (Du Pont de Nemours & Co., 1970). As a result of this process, the wettability

Table 3

The effect of pretreatment of the nylon tube with 18.6%(v/v) $CaCl_2$, 18.6%(v/v) water in methanol on the activity of nylon tube-supported enzyme derivatives.

Derivative	Activity of derivative prepared with non-pitted nylon tube	Activity of derivative prepared with pitted nylon tube
Nylon tube-supported triazole-treated glucose oxidase	0.021[a]	0.170[a]
Nylon tube-supported urease	0.028[b]	0.250[b]

a) expressed as the increase in extinction at 349nm/m length of nylon tube-supported triazole-treated glucose oxidase when a 5mM-glucose sample was assayed as described in section 2.6.3.1.

b) expressed as the increase in extinction at 630nm/m length of nylon tube-supported urease when a 0.1mM-urea sample was assayed as described in section 2.6.3.17.

of the nylon surface is increased, thereby making it more hydrophilic and correspondingly more favourable as an environment on which to immobilize an enzyme. At the same time, the removal of the amorphous nylon decreases the weight of the nylon structure (Du Pont de Nemours & Co., 1970) without destroying its structural integrity and so this process must also increase the surface area of the structure. As a consequence of this, there is a greater surface area available per unit length of tube for the immobilization of enzyme.

These two effects can account for the enhanced activity of the nylon tube-supported enzyme derivatives, which were prepared with pitted nylon tube; i.e. the greater surface area results in more enzyme being immobilized and the increased hydrophilicity results in a more favourable environment for enzymic activity.

3.1.4. Preparation of Nylon Membrane-Supported Enzymes

Table 4 shows the effect of added enzymatically inert protein on the preparation of membrane-supported glucose oxidase. For this, a nylon membrane derivative was prepared using the triazole-treated glucose oxidase and its activity compared to that of a similar membrane prepared using the triazole-treated glucose oxidase, to which had been added 10mg/ml of haemoglobin. In each case the membrane-supported derivatives were assayed using the method described in section 2.6.2.1. The results show that the derivative prepared in the presence of haemoglobin was approximately twenty times as active as the derivative prepared in the absence of haemoglobin.

Table 4

The effect of catalytically inert protein on the activity of nylon membrane-supported triazole-treated glucose oxidase.

Enzyme coupling solution	Activity
3.5mg/ml triazole-treated glucose oxidase	0.04[a]
3.5mg/ml triazole-treated glucose oxidase containing 10mg/ml haemoglobin	0.275[b]

a) expressed as the increase in extinction at 349nm when a 20mM-glucose sample was assayed as described in section 2.6.2.1.

b) expressed as the increase in extinction at 349nm when a 5mM-glucose sample was assayed as described in section 2.6.2.1.

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

3.1.5. Preparation of Nylon Powder-Supported Enzymes

Enzymes can be immobilized onto nylon powder by the methods described in section 2.2. A prerequisite of this method is the availability of free amino groups on the surface of the nylon powder for reaction with the bifunctional reagent. These may be generated by one of two processes. Either the polymerisation of the nylon may be aborted at an early stage, thus producing low molecular weight nylon with a corresponding high equivalent of end groups, or the end groups may be liberated from commercial high molecular weight nylon powder by processes such as those described in section 2.1. The latter process has the disadvantage that the treatment, whereby the amino groups are liberated, may produce fines which would impair the flow characteristics of a packed bed prepared from this material. Consequently, in all subsequent experiments low molecular weight nylon powder was used for the preparation of immobilized enzyme derivatives.

3.2. Automated Analysis

3.2.1. Determination of Glucose using Nylon-Supported Triazole-Treated Glucose Oxidase

Fig. 12 shows the effect of sampling rate on the calibration curves obtained when standard solutions of glucose were assayed using the three nylon-supported triazole-treated glucose oxidase derivatives; nylon tube (Fig. 12a), nylon powder (Fig.12b) and nylon membrane (Fig.12c). The assay procedures used with each of these derivatives are described in sections 2.6.1.1, 2.6.2.1 and 2.6.3.1 for the nylon powder, nylon membrane and nylon tube derivatives respectively. In all cases increasing the sampling rate decreased the sensitivity of the assay procedure.

By reference to standard curves, which were concurrently compiled by subjecting known concentrations of H_2O_2 to each of the three assay procedures, the efficiencies of the nylon-supported triazole-treated glucose oxidase derivatives 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 0.5 were obtained for tube, membrane and powder derivatives respectively.

3.2.2. Determination of Urea using Nylon-Supported Urease

Fig.13 shows the effect of sampling rate on the calibration curves obtained when standard solutions of urea were assayed using the three nylon-supported urease derivatives, nylon tube (Fig.13a),

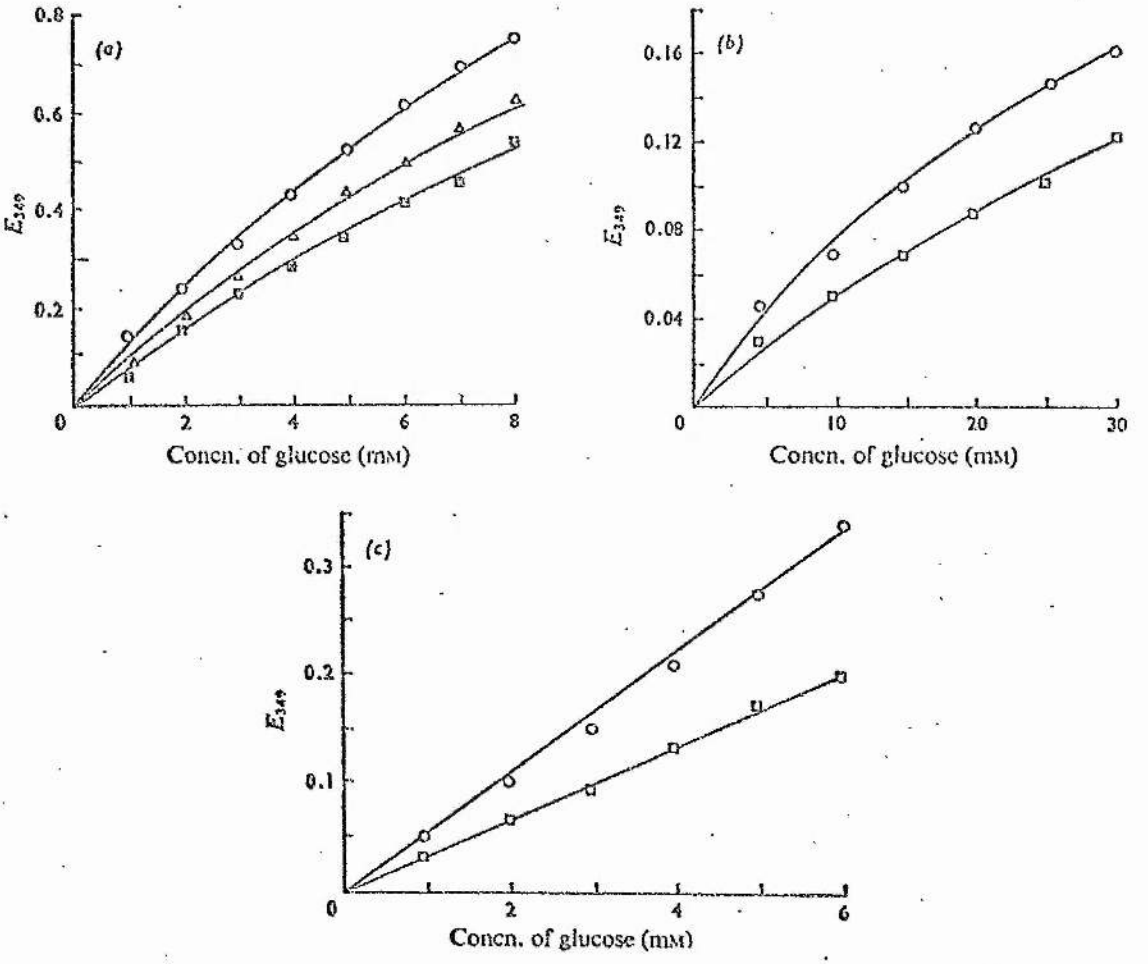


Fig.12. Standard curves for the automated determination of glucose with a 3m length of nylon tube-supported triazole-treated glucose oxidase(a),250mg of nylon powder-supported triazole-treated glucose oxidase in the form of a small packed bed(b) and nylon membrane-supported triazole-treated glucose oxidase(c).

○,20samples/h;□,30samples/h;△,40samples/h;▣,60samples/h.

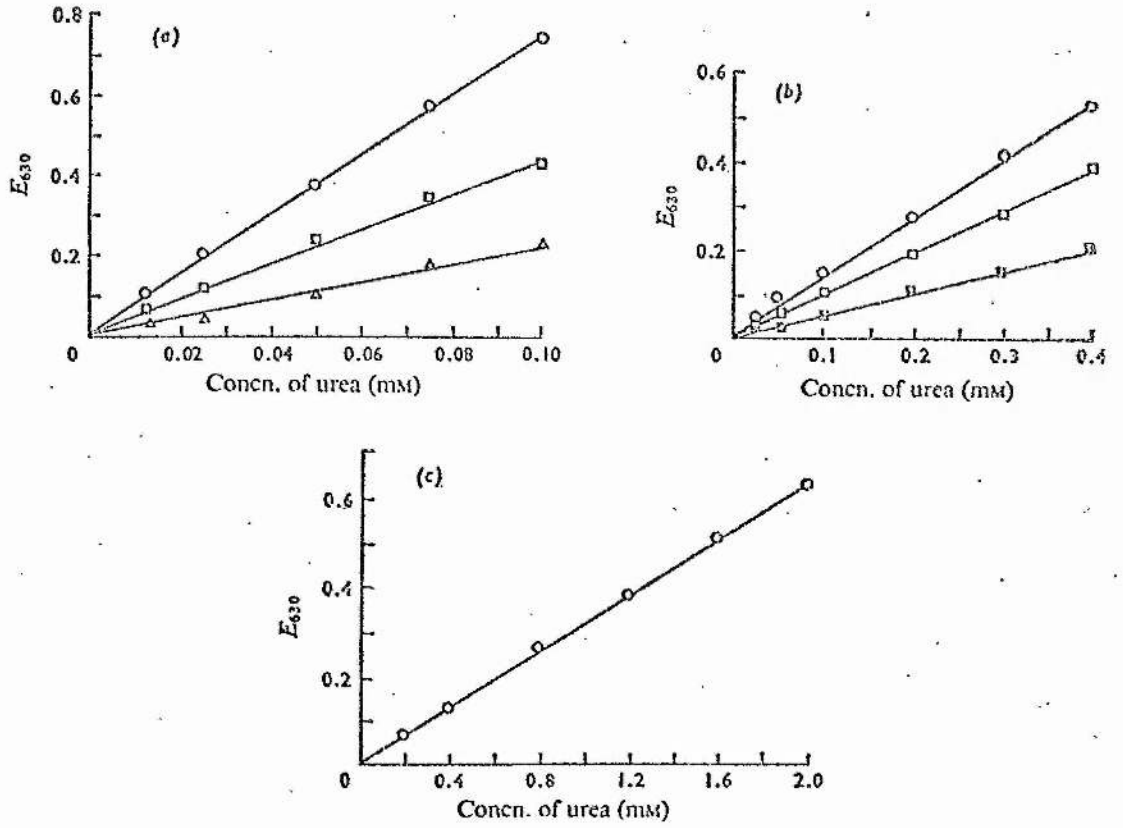


Fig.13. Standard curves for the automated determination of urea with a 3m length of nylon tube-supported urease(a),250mg of nylon powder-supported urease in the form of a small packed bed(b) and nylon membrane-supported urease(c). ○,20samples/h;□,30samples/h;▩40samples/h;△,60samples/h.

nylon powder (Fig.13b) and nylon membrane (Fig.13c). The assay procedures for each of these derivatives are described in sections 2.6.1.2, 2.6.2.2 and 2.6.3.17 for the nylon powder, nylon membrane and nylon tube derivatives respectively. For both the nylon tube- and nylon powder-supported derivatives an increase in the sampling rate caused a decrease in the sensitivity of the assay.

All three flow systems were 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.

3.2.3. General Considerations on the use of Nylon-Supported Enzymes in Automated Analysis

Table 5 summarises the results obtained for the three triazole-treated glucose oxidase and the three urease derivatives. It may be seen that in each case the nylon tube-supported derivative yielded the highest percentage conversion resulting in the highest product concentration and consequently the greatest sensitivity. However, the nylon powder-supported derivatives had the highest activities, in terms of the percentage conversion /s residence time in the immobilised enzyme structure. This is because the powder has a much greater surface area for the attachment of enzyme than either the tube or the membrane and therefore the concentration of immobilized enzyme in the packed bed will be correspondingly higher than the

Table 5

The activity of nylon-supported enzyme derivatives

Derivative	Product Concentration (mM)	% Conversion	Activity[a]
Nylon tube-supported triazole-treated glucose oxidase	0.185	3.7	0.06
Nylon powder-supported triazole-treated glucose oxidase	0.025	0.5	0.33
Nylon membrane-supported triazole-treated glucose oxidase	0.147	2.9	0.02
Nylon tube-supported urease	0.162	81.3	1.2
Nylon powder-supported urease	0.032	16.0	10.7
Nylon membrane-supported urease	0.015	7.5	0.05

a) expressed as the percentage conversion /s residence time in the immobilized enzyme structure.

Glucose assays were performed with 5mM-glucose and at 20 samples/h.

Urea assays were performed with 0.1mM-urea and at 20 samples/h.

concentration of immobilized enzyme in either the tube or the membrane structure.

However, the greater percentage conversion of substrate to product in the tube-supported derivatives compared to that of the powder-supported derivatives can be accounted for by the greater residence time of the substrate in the former structure. For instance, the void volume of a packed bed (4.0x0.2cm) is approximately 0.05ml and perfusion through the packed bed at a flow rate of 1.20ml/min results in a residence time of 2.5 s. Whilst for a 3m length of nylon tube of internal diameter 0.1cm, the internal volume is 2.35ml and when the nylon tube is perfused at a flow rate of 2.23ml/min the residence time in the tube is 63s. Therefore the sample has approximately a 25 times greater residence time in the nylon tube-supported derivative than in the nylon powder-supported derivative.

Figs. 12 and 13 show that increasing the sampling rate decreased the sensitivity of the assays with the nylon tube-supported enzyme structures. This is a common observation with all automated analytical techniques, which are based on the Technicon continuous flow-through principle. The cause of this has been accounted for in terms of an increase in cross- contamination due to the shorter wash times between samples.

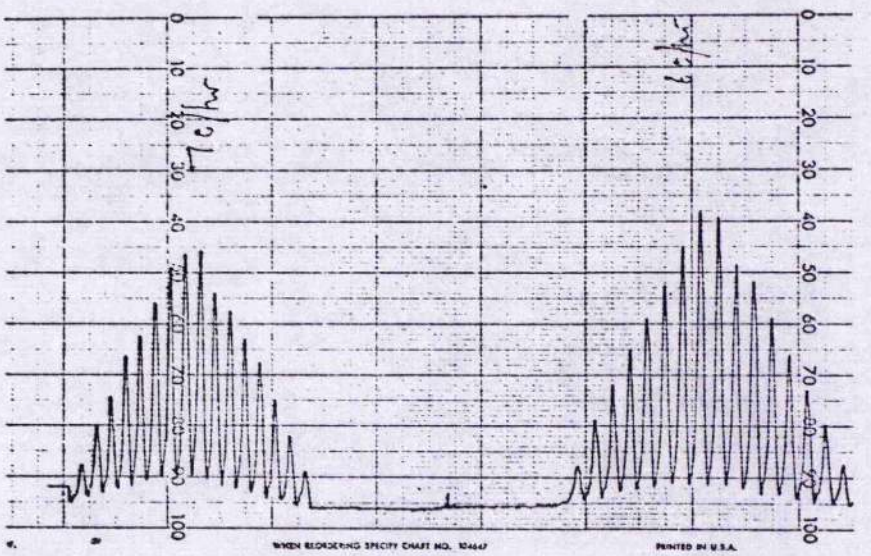
It was possible to use higher sampling rates with the tube-supported derivatives than with either the corresponding powder- or membrane-supported derivatives. At sampling rates in excess of 40/h

for the powder-supported derivatives and in excess of 30/h for the membrane-supported derivatives the shape of the sample peaks from the recorder were considerably distorted and significant base line drift was observed. However, as shown in Fig.14 the recorder traces obtained with the tube-supported derivatives were not significantly distorted by increasing the sampling rate from 20/h to 60/h. The distortion in the recorder traces observed with the powder-supported derivatives and membrane-supported derivatives are presumably due to the mixing of the sample stream when the air-segmentation is removed and to lateral diffusion in the dialyser respectively.

Furthermore, of the three immobilized enzyme structures studied, the nylon tube-supported derivatives were most easily incorporated into conventional Technicon Autoanalyser flow systems. The use of the nylon powder-supported derivatives was complicated by the necessity for 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.

Therefore, in terms of ease of application, sensitivity of assay and the number of samples assayed per unit time, the nylon tube-supported enzyme preparations are the preferred structures for use in automated analysis. However, since one of the main advantages in using an immobilized enzyme is economy of enzyme protein, it was essential to establish whether or not savings in terms of enzyme were

(a)



(b)

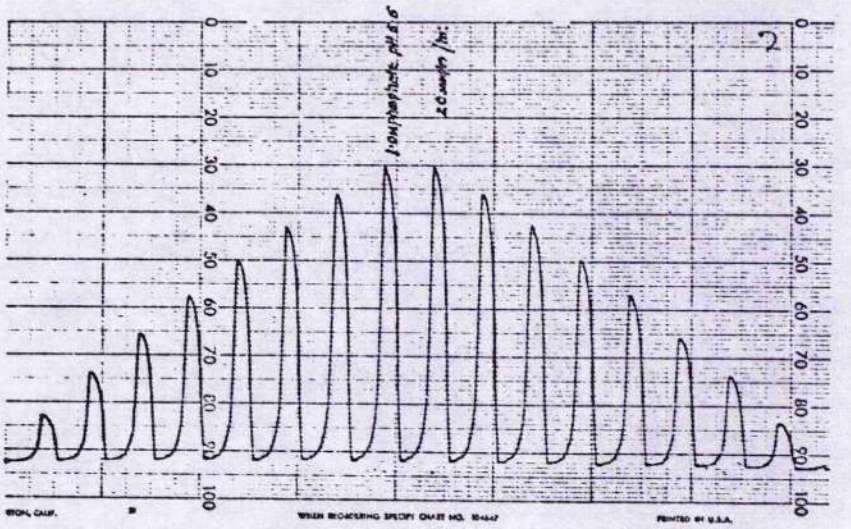


Fig.14. The effect of sampling rate on the recorder traces obtained when standard solutions of glucose were determined using nylon tube-supported triazole-treated glucose oxidase. The sampling rates were 70/h(left hand trace,Fig.14a),60/h (right hand trace,Fig.14a) and 20/h(Fig.14b)

being made as relatively large quantities of enzyme protein were used for the preparation of the tube-supported derivatives. 35mg of triazole-treated glucose oxidase and 10mg of urease were used for the preparation of nylon tube-supported glucose oxidase and nylon tube-supported urease derivatives respectively and these preparations were used over a 30 day period for the estimation of 3500 glucose samples and 5000 urea samples respectively. These quantities of enzyme, which were used for the preparation of these derivatives when used free in solution were sufficient for not more than 1500 glucose samples and 1100 urea samples respectively. Thus a significant economy in enzyme utilisation is achieved using tube-supported enzyme derivatives. However, greater savings could be realised by improving the chemistry of the coupling step and by using enzyme preparations of greater activity and purity. This latter point is verified in the following section.

3.2.4. An Improved Method for the Determination of Glucose using Nylon Tube-Supported Glucose Oxidase

Fig. 15 shows the results obtained when standard solutions of glucose were assayed using a 3m length of nylon tube-supported glucose oxidase prepared from Boehringer glucose oxidase (specific activity = 140 units/mg). By reference to a calibration curve, which was concurrently compiled by assaying standard solutions of H_2O_2, the results are also presented in terms of the H_2O_2 produced.

For samples containing 0.1-0.6mM-glucose, the conversion to product is approximately 65%, in terms of the H_2O_2 produced. However,

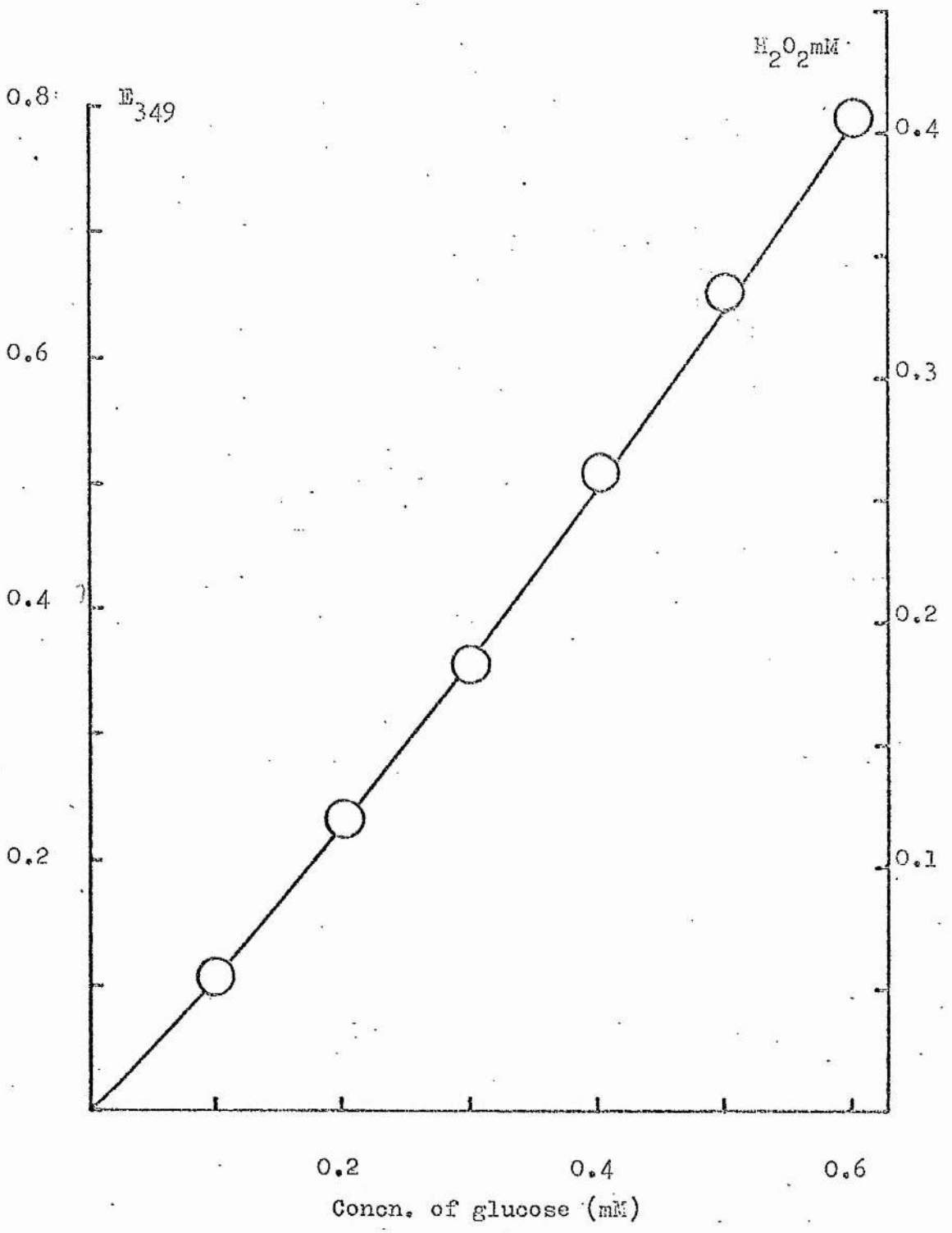


Fig.15. Standard curve for the automated determination of glucose with a 3m length of nylon tube-supported Boehringer glucose oxidase. Samples were assayed at the rate of 20/h.

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 (Swoboda & Massey 1964) then the percentage conversion of available substrate to product is greater than 90%. 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. It may be seen by comparison of Figs.12a and 15 that using an enzyme preparation of greater specific activity and purity results in an order of magnitude increase in the sensitivity of the assay procedure for the determination of glucose.

3.2.5. Determination of Glucose using Nylon Tube-Co-Supported Glucose Oxidase and Peroxidase

Fig. 16 shows the results obtained when standard solutions of glucose were assayed with a 3m length of nylon tube-co-supported glucose oxidase and peroxidase using the method described in section 2.6.3.2. The results show that this procedure may be used for the determination of glucose in the concentration range 1-10mM.

However, it was observed over an 8h period that the hydrogen donor perid, became deposited on the wall of the nylon tube-co-supported derivative causing a gradual darkening in its appearance. Although there was no detectable loss of catalytic activity during this period, nevertheless this continual deposition could prove to be a serious drawback to the use of this particular co-supported derivative. Two other hydrogen donors; o-dianisidine and o-tolidine were used instead of perid, but a similar deposition was observed in each case.

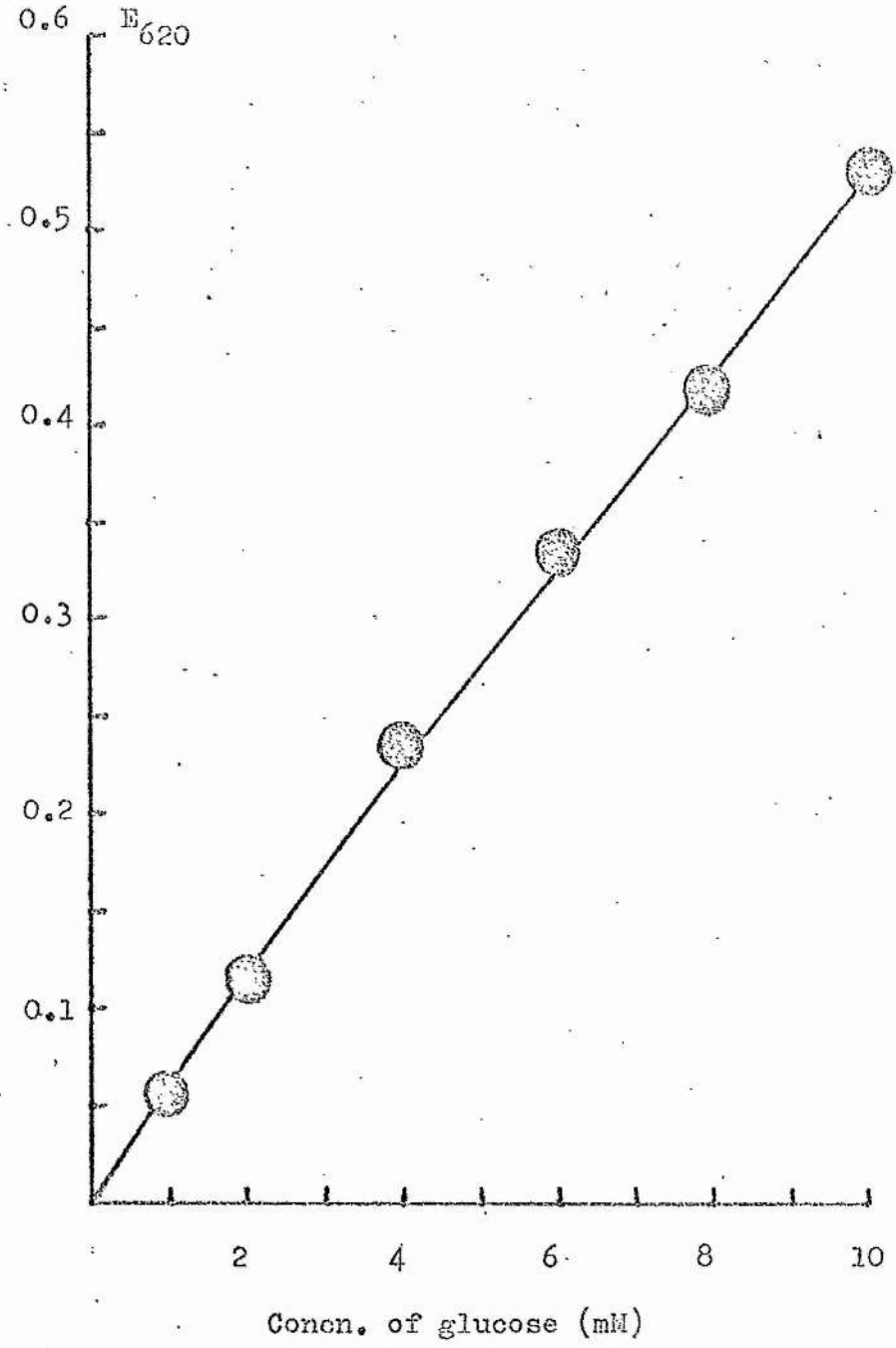


Fig.16. Standard curve for the automated determination of glucose with a 3m length of nylon tube-co-supported glucose oxidase and peroxidase. Samples were assayed at the rate of 20/h.

The glucose oxidase and peroxidase linked enzyme system is commonly used in clinical laboratories for the determination of glucose in solution. This procedure is expensive in terms of enzymes and hydrogen donor, if a non-carcinogenic hydrogen donor such as perid is used. Alternatively, a relatively cheaper hydrogen donor such as o-dianisidine or o-tolidine may be used, but their general usefulness is impaired due to their carcinogenic character.

Therefore, of the two immobilised enzyme systems described for the determination of glucose in solution, that using the nylon tube-supported glucose oxidase and acid/KI is the more attractive than the co-supported glucose oxidase and peroxidase structure, since it is an order of magnitude more sensitive, it does not require peroxidase and precludes the use of a hydrogen donor. The only advantage that the coupled system could have compared with the single enzyme system is that the effect of catalase impurity in the sample would be minimised. However, such catalase contamination may be removed by dialysis of the sample stream, prior to being perfused through the nylon tube-supported glucose oxidase, in which case this consideration is not important.

3.2.6. Determination of Pyruvate, Oxalacetate and Ethanol using Nylon Tube-Supported Dehydrogenases

Pyruvate, oxalacetate and ethanol were determined using 3m lengths of nylon tube-supported lactate dehydrogenase, malate dehydrogenase and alcohol dehydrogenase as described in sections 2.6.3.4, 2.6.3.5 and 2.6.3.6 respectively. Pyruvate in the concentration

range 0.04-0.20mM (Fig. 17a), oxalacetate in the concentration range 0.002-0.16mM (Fig. 17b) and ethanol in the concentration range 10-100mM (Fig. 17c) may be determined by these methods. The three nylon tube-supported dehydrogenases were used over a 20 day period without incurring any loss in catalytic activity and during this time each derivative was used for at least 1000 separate analyses. However, solutions of the three enzymes, stored under comparable conditions lost at least 90% of their activity over the same period.

This clearly demonstrates a considerable enhancement in stability of the immobilized enzyme derivative compared with that of the soluble enzyme. Since many enzyme based assays are not feasible due to the instability in solution of the enzyme and the high cost of the enzyme a marked increase in stability on immobilization would overcome both of these problems and make the assay a more attractive proposition.

3.2.7. The Continuous Production of NADH for the Automated Determination of Pyruvate and Oxalacetate

Pyruvate and oxalacetate were determined using 3m lengths of nylon tube-supported lactate dehydrogenase and malate dehydrogenase as described in sections 2.6.3.9 and 2.6.3.10 respectively. In both cases the NADH required by these enzymes was continuously produced in situ, by perfusing ethanol and NAD^+ through a 3m length of nylon tube-supported alcohol dehydrogenase. Pyruvate in the concentration range 0.01-0.16mM (Fig. 18a) and oxalacetate in the concentration range 0.05-0.20mM (Fig. 18b) were determined by these methods.

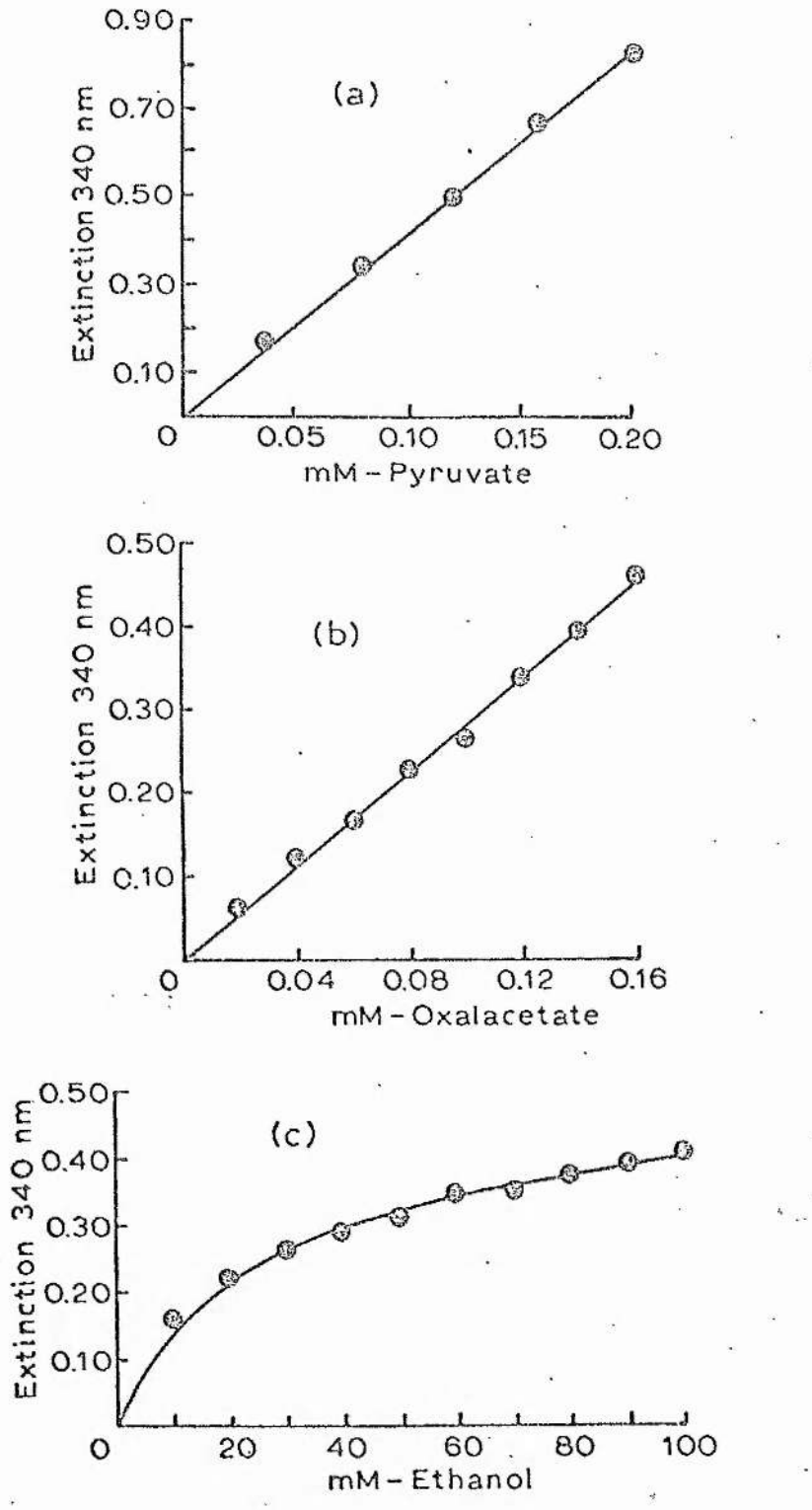


Fig.17. Standard curves for the automated determination of pyruvate with a 3m length of nylon tube-supported lactate dehydrogenase(a), oxalacetate with a 3m length of nylon tube-supported malate dehydrogenase(b) and ethanol with a 3m length of alcohol dehydrogenase(c). Samples were assayed at the rate of 30/h.

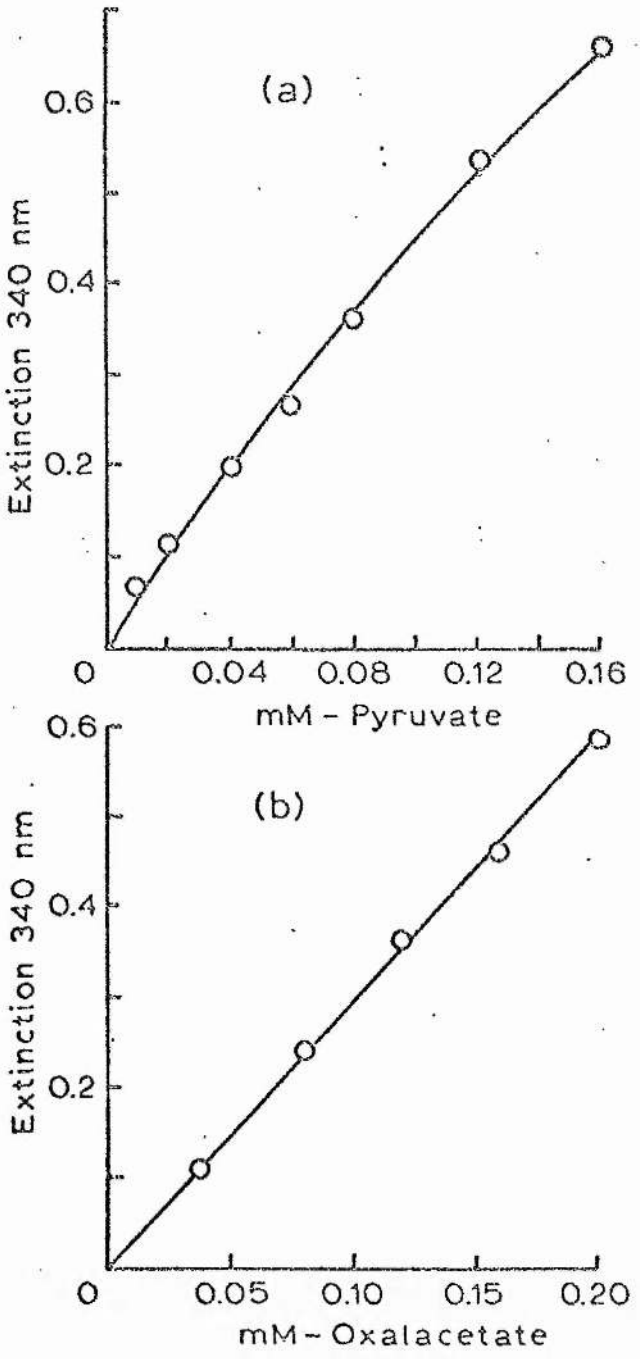


Fig.18. Standard curves for the automated determination of pyruvate with a 3m length of nylon tube-supported lactate dehydrogenase(a) and oxalacetate with a 3m length of nylon tube-supported malate dehydrogenase(b). The NADH was generated continuously from NAD^+ with a 3m length of nylon tube-supported alcohol dehydrogenase. Samples were assayed at the rate of 30/h.

3.2.8. General Considerations on the use of Dehydrogenases

Tube-supported enzymes have been used in automated analysis exclusively for the determination of their substrates. The use of the tube-supported enzyme for the production of an expensive analytical material such as NADH from a cheaper material such as NAD^+, represents a new way of exploiting their potential. One distinct advantage of this system is that the amount of NADH in the system may be calculated from the change in extinction when the alcohol dehydrogenase tube is inserted into the flow system. Continuous generation of NADH from NAD^+ results in at least a two-fold saving in the cost of pyridine nucleotide for analytical systems requiring this coenzyme.

It may be seen from inspection of Figs. 17a and 18a and between Figs.17b and 18b that there is little difference between the two assay systems for the determination of pyruvate and oxalacetate respectively. In conclusion therefore, the two systems described for the determination of pyruvate and oxalacetate using the NADH generating system are preferable to the comparable systems using commercial NADH.

In an earlier section (3.1.1.) the nature of the linkage between glutaraldehyde and free amino groups was discussed and the possibility of the linkage being of a Schiff base-type was considered. Schiff bases may be converted into stable secondary amines by reduction with sodium borohydride. Since the dehydrogenases are unstable in solution, the nylon tube-supported enzyme derivatives were perfused with sodium borohydride, so that if any Schiff base linkages existed they would be reduced and this might possibly increase the stability of the immobilized enzyme derivatives.

3.2.9. Determination of ADP using Nylon Tube-Co-Supported Pyruvate Kinase and Lactate Dehydrogenase

Fig. 19 shows the results obtained when ADP solutions of known concentration were assayed by the method described in section 2.6.3.7 using a 3m length of nylon tube-co-supported pyruvate kinase and lactate dehydrogenase. ADP in the concentration range 0.1-0.5mM may be assayed by this method. Since ADP in free solution is determined by the linked enzyme system of pyruvate kinase and lactate dehydrogenase the linked immobilized enzyme system could prove to be quite important in analysis, due to the high cost of each of these enzymes. Further characterisation of this derivative is described in section 3.3.3.

Determination of Disaccharides

3.2.10. Determination of Sucrose using Nylon Tube-Supported Invertase

Sucrose was determined using a 3m length of nylon tube-supported invertase as described in section 2.6.3.11 and the results are presented in Fig.20. By reference to a standard curve, concurrently compiled by assaying solutions of known glucose concentration, the results are also presented in terms of the glucose produced. These results show that the method may be used for determining sucrose in the concentration range 0.5-5.0mM.

3.2.11. Determination of Sucrose using the Linked Enzyme System of Invertase and Glucose Oxidase

Fig.21 shows the calibration curve obtained when standard solutions of sucrose were assayed as described in method 1 (see

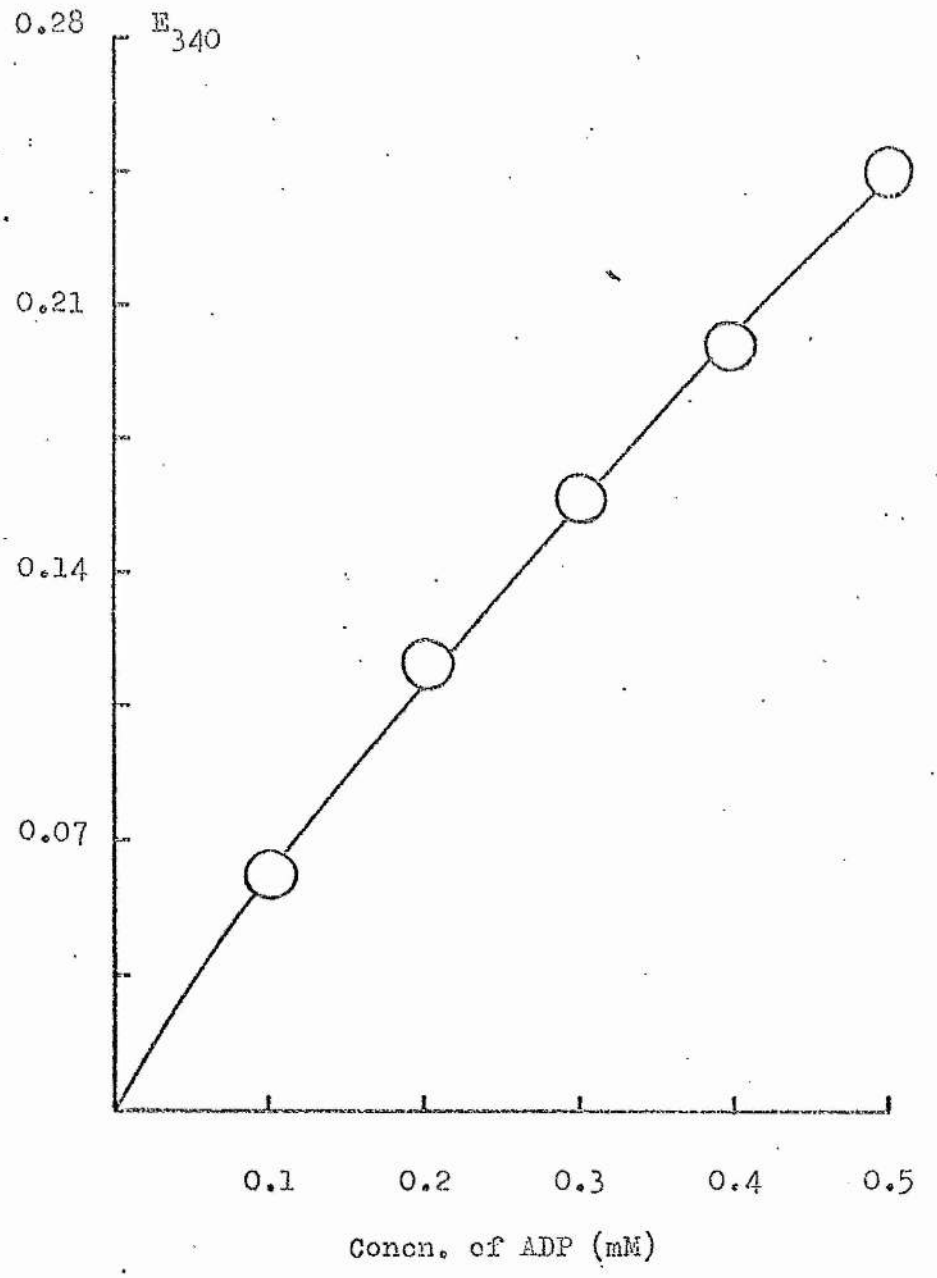


Fig.19. Standard curve for the automated determination of ADP with a 3m length of nylon tube-co-supported pyruvate kinase and lactate dehydrogenase. Samples were assayed at the rate of 30/h.

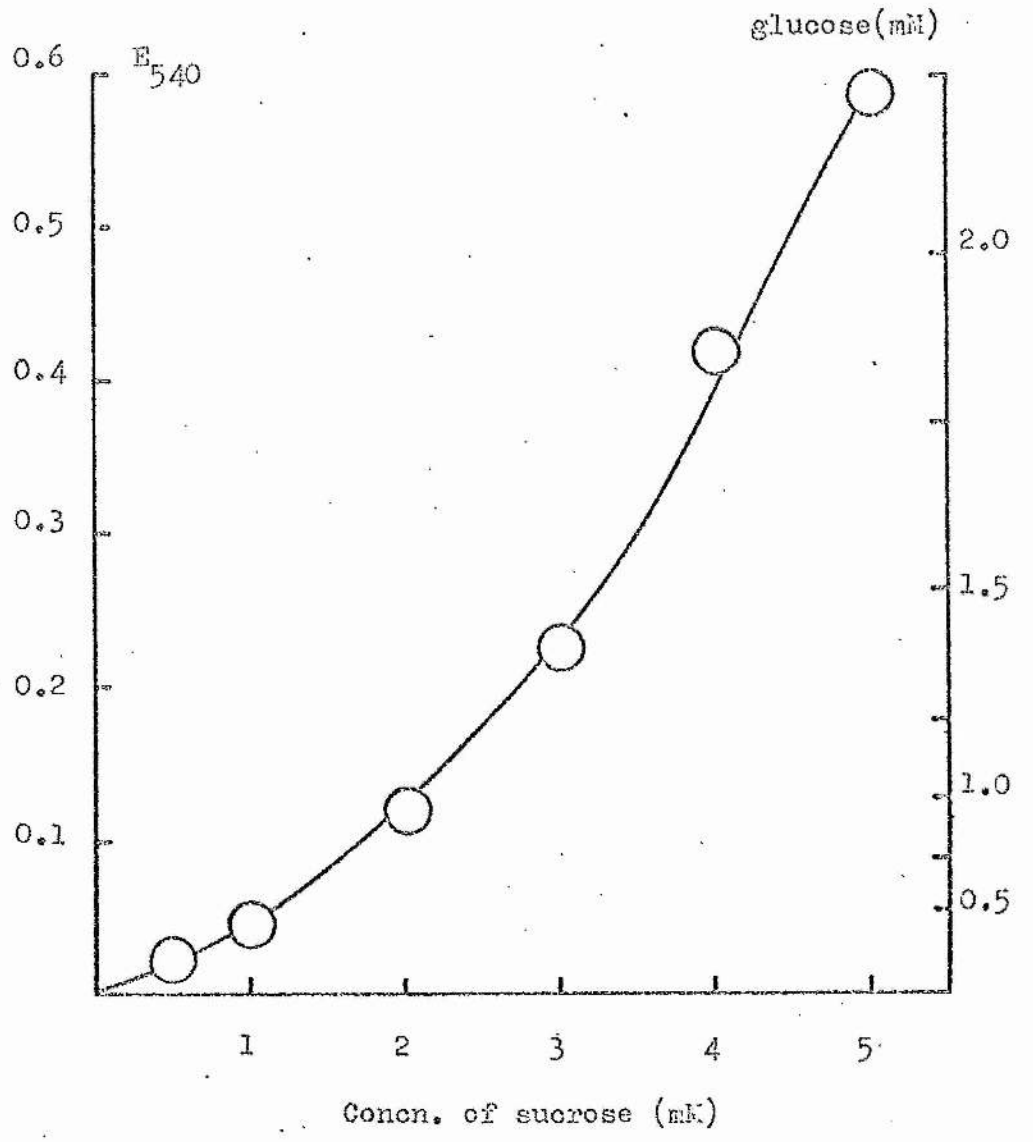


Fig.20. Standard curve for the automated determination of sucrose with a 3m length of nylon tube-supported invertase. Samples were assayed at the rate of 20/h.

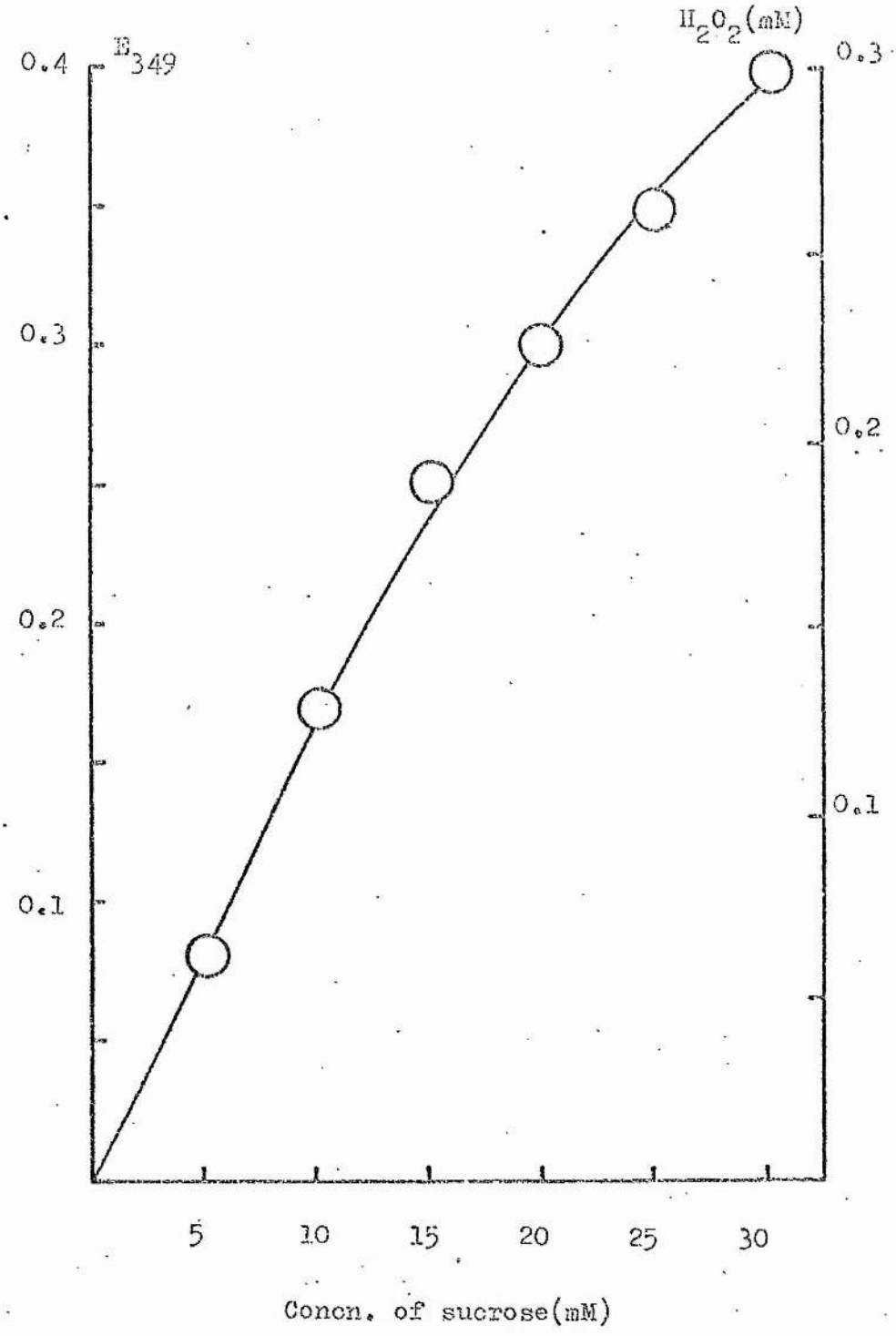


Fig.21. Standard curve for the automated determination of sucrose with a 3m length of nylon tube-co-supported invertase and glucose oxidase. Samples were assayed at the rate of 20/h.

section 2.6.3.13) using a 3m length of nylon tube-co-supported invertase and glucose oxidase, prepared from a coupling mixture containing a 2:1 ratio of invertase to glucose oxidase. As the results show, this procedure may be used for the determination of sucrose in the concentration range 5-30mM. By reference to a standard curve, concurrently compiled by assaying solutions of known H_2O_2 concentration, the results are also presented in terms of the H_2O_2 produced. The effect of sampling rate on the calibration curves obtained when sucrose was determined by this method are shown in Fig. 22.

Sucrose was also determined according to method 2 (see section 2.6.3.13) using a 3m length of nylon tube-supported invertase placed in series with a 3m length of nylon tube-supported glucose oxidase and the results of this experiment are shown in Fig. 23. H_2O_2 solutions of known concentration were subjected to the assay procedure and the results are also presented in terms of the H_2O_2 produced. It can be seen that this procedure may be used for determining sucrose in the concentration range 5-30mM.

Two different nylon tube-co-supported invertase and glucose oxidase derivatives were prepared, one using an enzyme solution containing equal amounts of invertase and glucose oxidase and the second from an enzyme solution containing a 2:1 ratio of invertase to glucose oxidase. In each casethree separate activities were defined for each derivative; the conversion of sucrose to H_2O_2 (as described in section 2.6.3.13); the conversion of glucose to H_2O_2

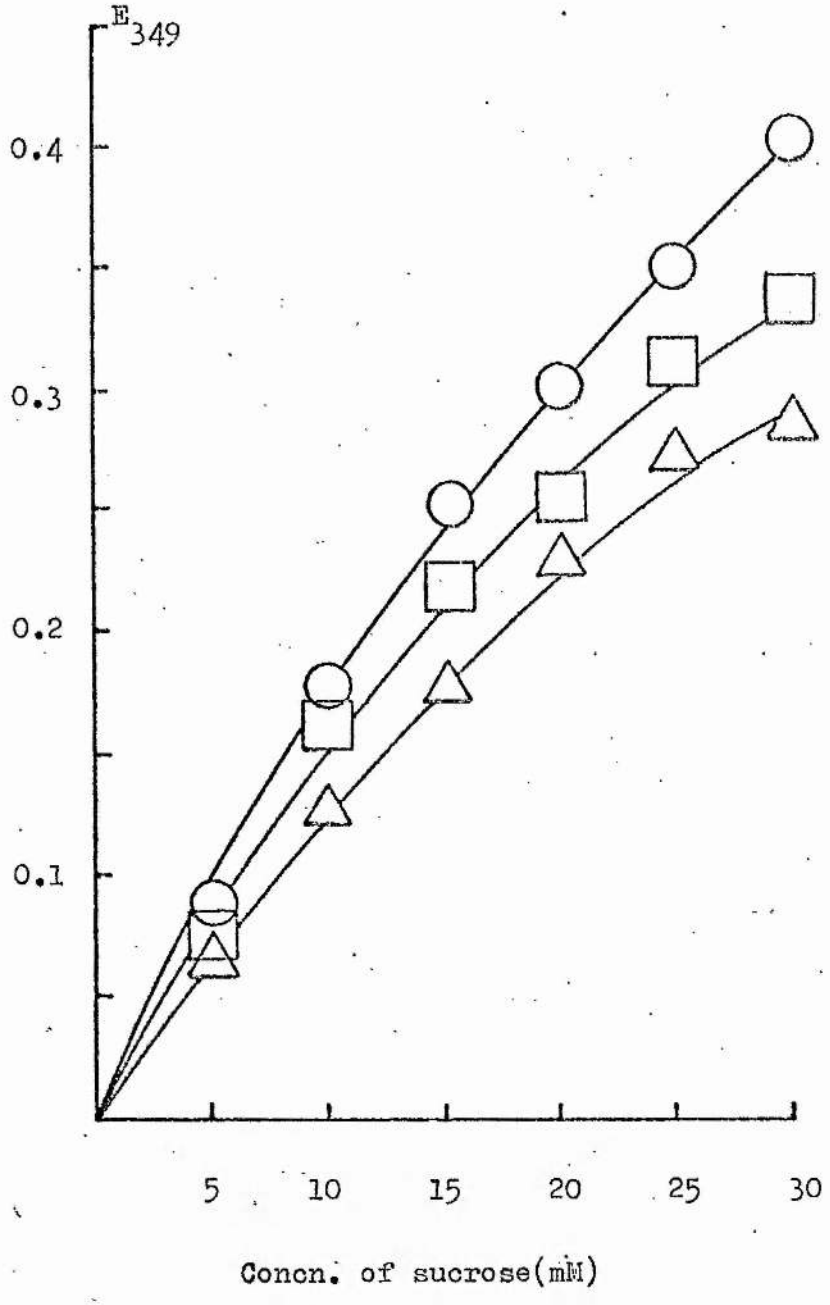


Fig.22. The effect of sampling rate on the standard curves for the automated determination of sucrose with a 3m length of nylon tube-co-supported invertase and glucose oxidase. ○, 20samples/h; □, 30samples/h; △, 40samples/h.

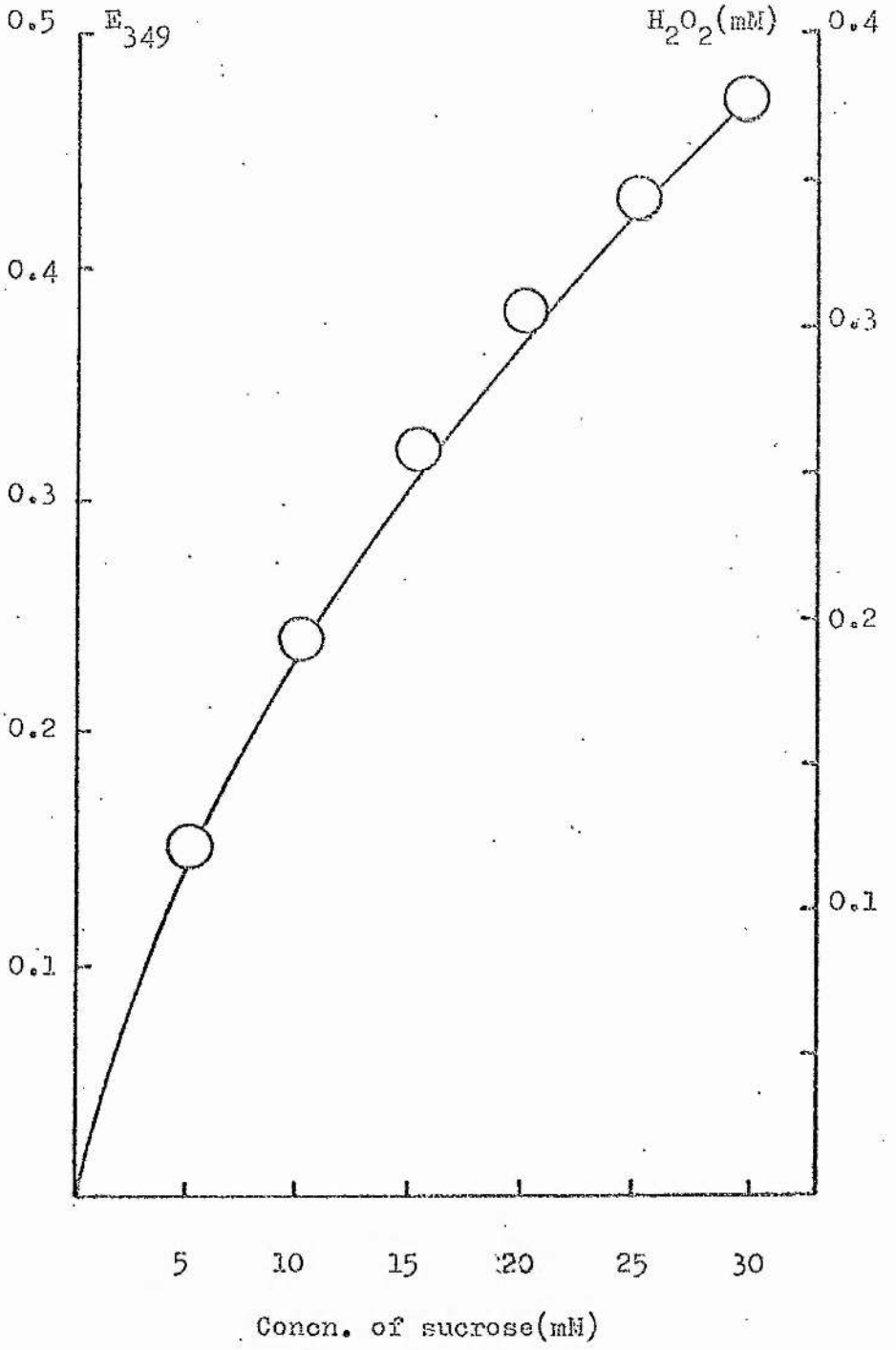


Fig.23. Standard curve for the automated determination of sucrose with a 3m length of nylon tube-supported invertase placed in series with a 3m length of nylon tube-supported glucose oxidase. Samples were assayed at the rate of 20/h.

(as described in section 2.6.3.1), and finally the conversion of sucrose to glucose (as described in section 2.6.3.11). The latter value was corrected to compensate for the glucose converted to D-glucono-δ-lactone.

The separate activities of the 3m length of nylon tube-supported invertase, for the conversion of sucrose to glucose (see section 2.6.3.11) and for the 3m length of nylon tube-supported glucose oxidase, for the conversion of glucose to H_2O_2 (see section 2.6.3.1) were also determined. Finally, the overall conversion of sucrose to H_2O_2 (see section 2.6.3.13) for this system was determined. These results are presented in Table 6.

3.2.12. Determination of Maltose using the Linked Enzyme System of Amyloglucosidase and Glucose Oxidase

Fig. 24 shows the results obtained when maltose was determined using a 3m length of nylon tube-co-supported amyloglucosidase and glucose oxidase as described in method 1 (see section 2.6.3.15). By reference to a standard curve, concurrently compiled by assaying solutions of known H_2O_2 concentration, the results are also presented in terms of the H_2O_2 produced. These results show that maltose solutions in the concentration range 1-6mM may be determined by this procedure.

Maltose was also determined according to the procedure described in method 2 (see section 2.6.3.15) using a 3m length of nylon tube-supported amyloglucosidase placed in series with a 3m length of nylon tube-supported glucose oxidase. The results of this experiment

Table 6

The separate enzymic activities of nylon tube-co-supported invertase and glucose, nylon tube-supported invertase and nylon tube-supported glucose oxidase.

Derivative	% Conversion Sucrose → H_2O_2	% Conversion Sucrose → Glucose	% Conversion Glucose → H_2O_2
Nylon tube-supported invertase and nylon tube-supported glucose oxidase (system 1)	1.3	22.5	65.0
System 1 with a 1.51 min delay between coils	3.0	22.5	65.0
System 1 with a 26.5 min delay between coils	11.8	22.5	65.0
Nylon tube-co-supported invertase and glucose oxidase (system 2)	0.6	10.1	66.5
Nylon tube-co-supported invertase and glucose oxidase (system 3)	1.06	17.6	66.0

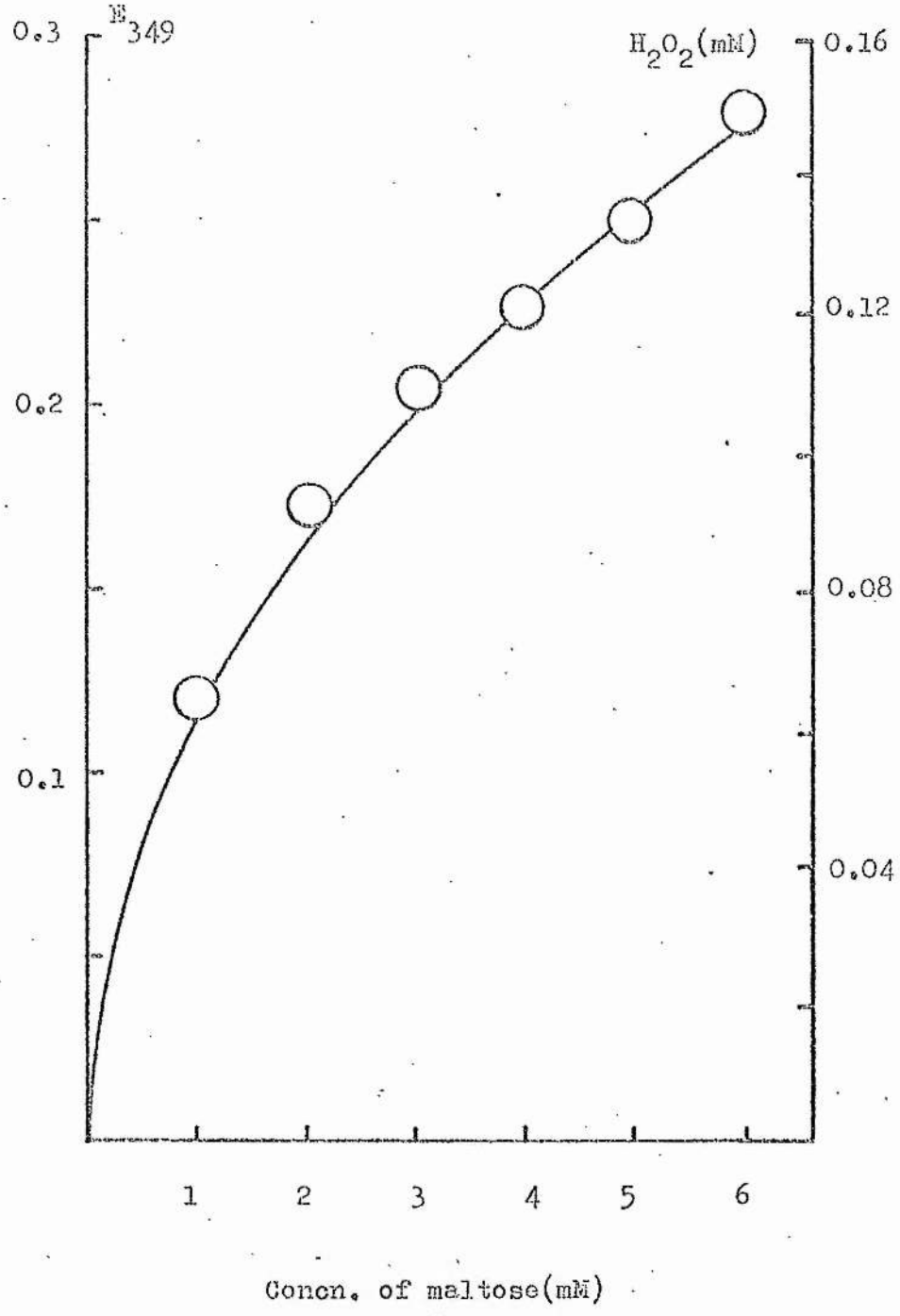


Fig.24. Standard curve for the automated determination of maltose with a 3m length of nylon tube-co-supported amyloglucosidase and glucose oxidase. Samples were assayed at the rate of 20/h.

are also presented in terms of the H_2O_2 produced (Fig. 25) and show that maltose in the concentration range 5-30mM may be determined by this method.

3.2.13. Determination of Lactose using the Linked Enzyme System of β-Galactosidase and Glucose Oxidase

Lactose was determined using a 3m length of nylon tube-supported β-galactosidase and a 3m length of nylon tube-supported glucose oxidase as described in method 2 (see section 2.6.3.14). The results of this experiment are shown in Fig. 26. H_2O_2 solutions of known concentration were subjected to the assay procedure and the results are also presented in terms of the H_2O_2 produced. These results show that the method may be used for the determination of lactose in the concentration range 0.1-0.6mM. Standard solutions of lactose were assayed using this procedure at a number of different sampling rates and the results of this experiment are shown in Fig.27.

3.2.14. Measurement of the Rate Constant for the Mutarotation of Glucose

Fig. 28 shows the effect of time on the mutarotation of glucose in the presence of 0.1M-sodium acetate buffer, pH5.0 in the form of a plot of $\log_{10}(\alpha_t - \alpha_{\infty})$ against t(Smith 1937) using the method described in section 2.7.2. The slope of this line is 0.05min^{-1} which corresponds to an effective first order rate constant of 0.106min^{-1} for the mutarotation of glucose.

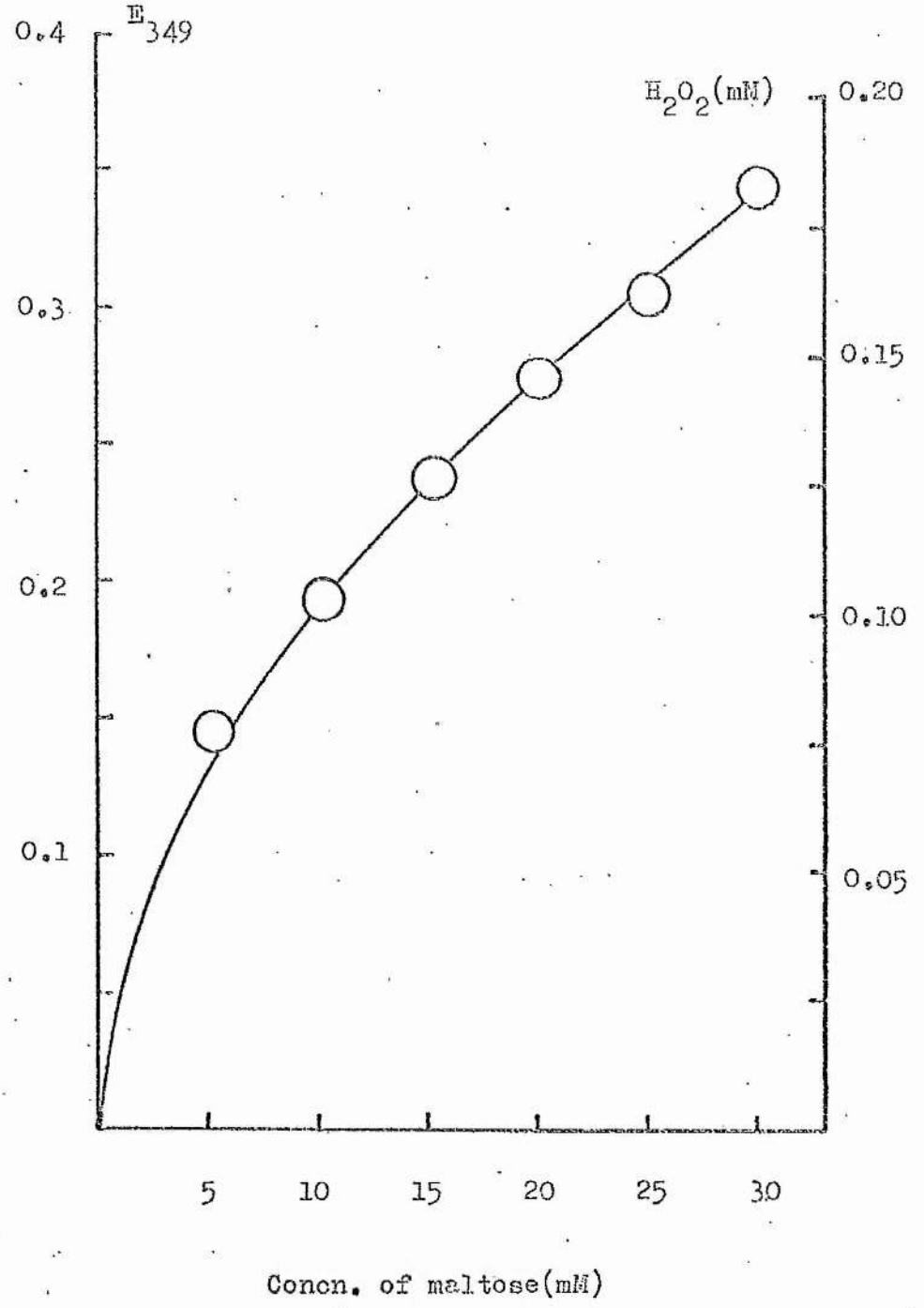


Fig.25. Standard curve for the automated determination of maltose with a 3m length of nylon tube-supported amyloglucosidase placed in series with a 3m length of nylon tube-supported glucose oxidase. Samples were assayed at the rate of 20/h.

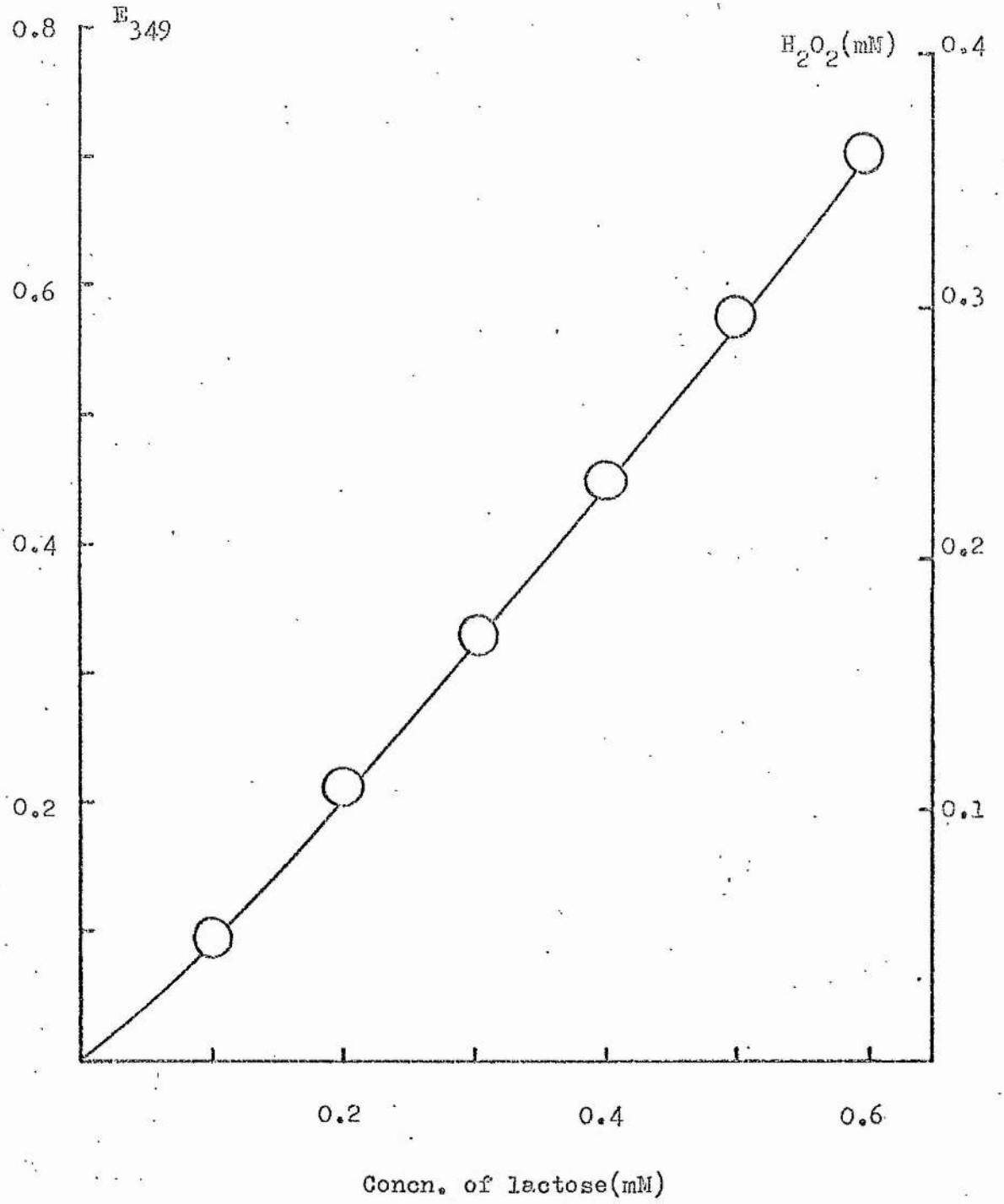


Fig.26. Standard curve for the automated determination of lactose with a 3m length of nylon tube-supported β-galactosidase placed in series with a 3m length of nylon tube-supported glucose oxidase. Samples were assayed at the rate of 20/h.

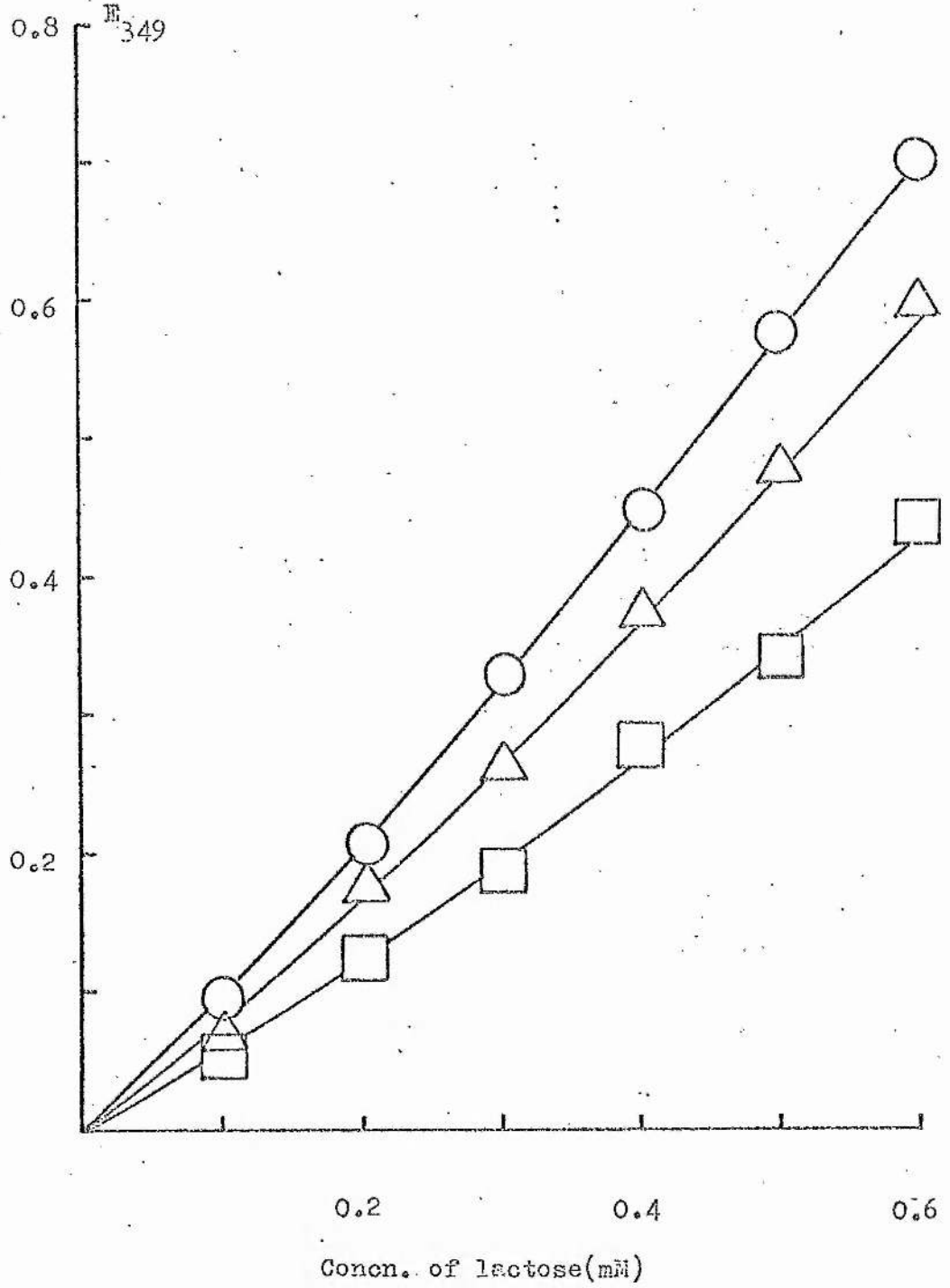


Fig.27. The effect of sampling rate on the standard curves for the automated determination of lactose with a 3m length of nylon tube-supported β-galactosidase placed in series with a 3m length of nylon tube-supported glucose oxidase.
○, 20samples/h; △, 30samples/h; □, 40samples/h.

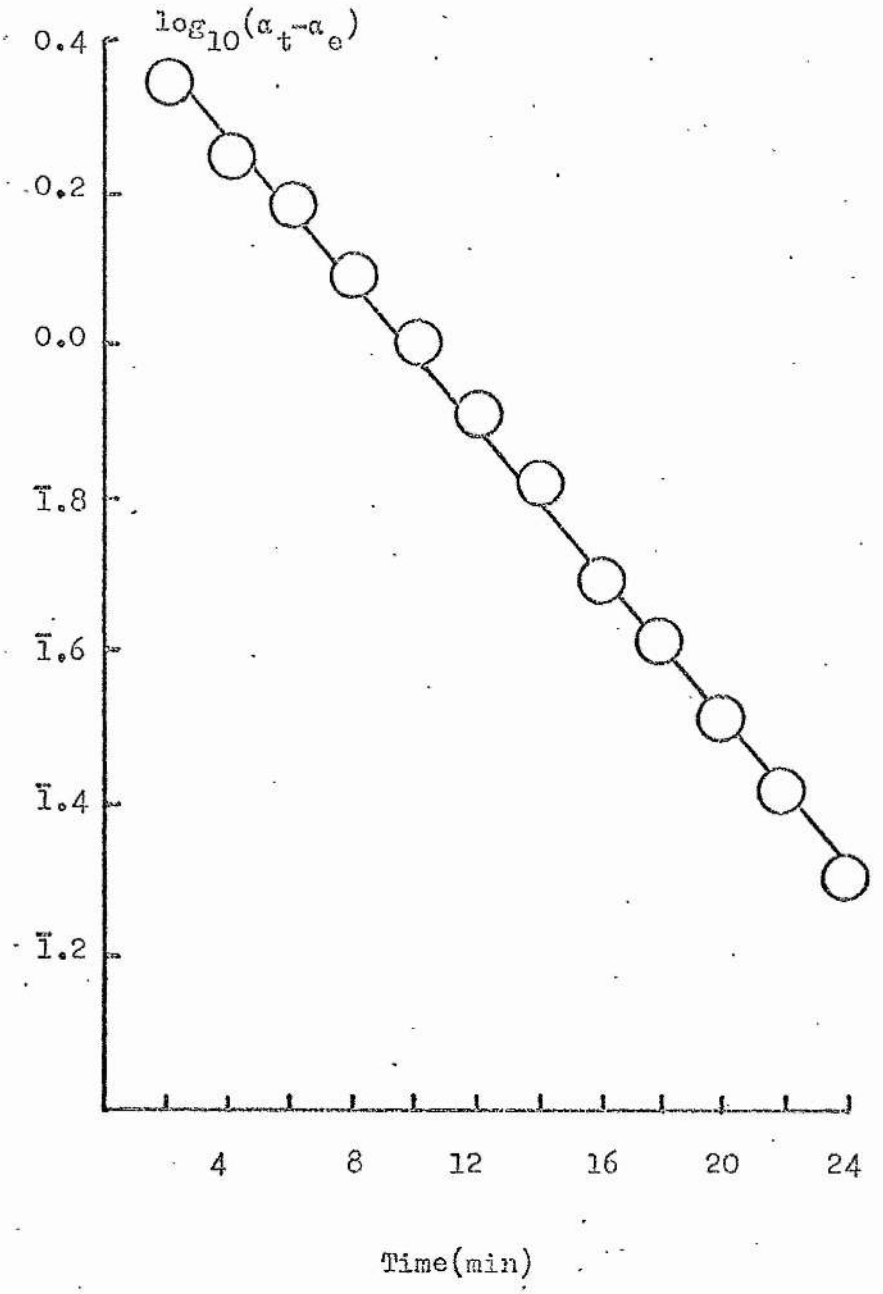


Fig.28. The effect of time on the mutarotation of glucose in the presence of 0.1M-sodium acetate buffer, pH5.0 at 37°C.

3.2.15. Mutarotation of Glucose using the Linked Enzyme System of Invertase and Glucose Oxidase

Fig. 29 shows the effect of delay time on the calibration curves obtained when standard solutions of sucrose were assayed as described in section 2.7.1 using a 3m length of nylon tube-supported invertase placed in series with a 3m length of nylon tube-supported glucose oxidase. These results show that the method can be used for the determination of sucrose in the concentration range 0.5-2.5mM and that there is a four-fold increase in sensitivity by increasing the delay time between the two coils from 1.51 min to 26.5 min.

The H_2O_2 product concentrations obtained, when samples containing 2.5mM sucrose were assayed at each delay time, were plotted as a function of the percentage concentration of the β-anomer in the mixture, which was determined using the data presented in section 3.2.14. When the results are plotted in this manner (Fig.30), the H_2O_2 concentration is seen to be proportional to the concentration of the β-anomer. As the % of the β-anomer in the mixture approaches the equilibrium value of 64% the curvature of the line increases showing that longer delay times between the enzyme coils would have little effect on increasing the overall sensitivity of the assay system. Therefore the increase in sensitivity of the linked enzyme system caused by an increase in delay time correlates with the formation of the β-anomer.

3.2.16. General Considerations on the Determination of Disaccharides using Linked Enzyme Systems

Two different linked immobilized enzyme systems were used for the determination of maltose, one using a 3m length of nylon tube-

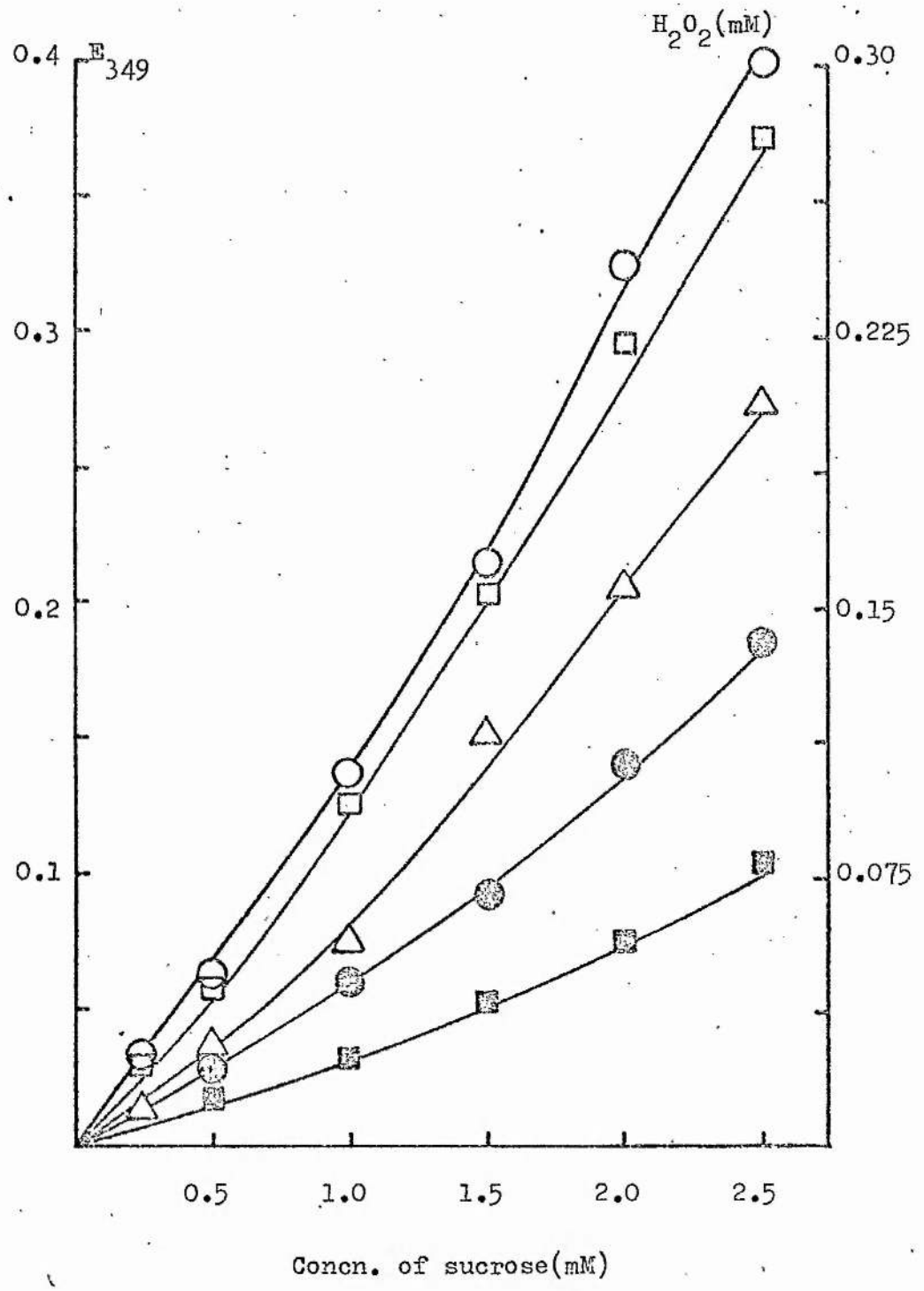


Fig.29. The effect of delay time between a 3m length of nylon tube-supported invertase placed in series with a 3m length of nylon tube-supported glucose oxidase for the automated determination of sucrose. Samples were assayed at the rate of 20/h.

○,26.5min delay;□,13.25 min delay;△,6.9min delay;●,3.45 min delay;■,1.51min delay.

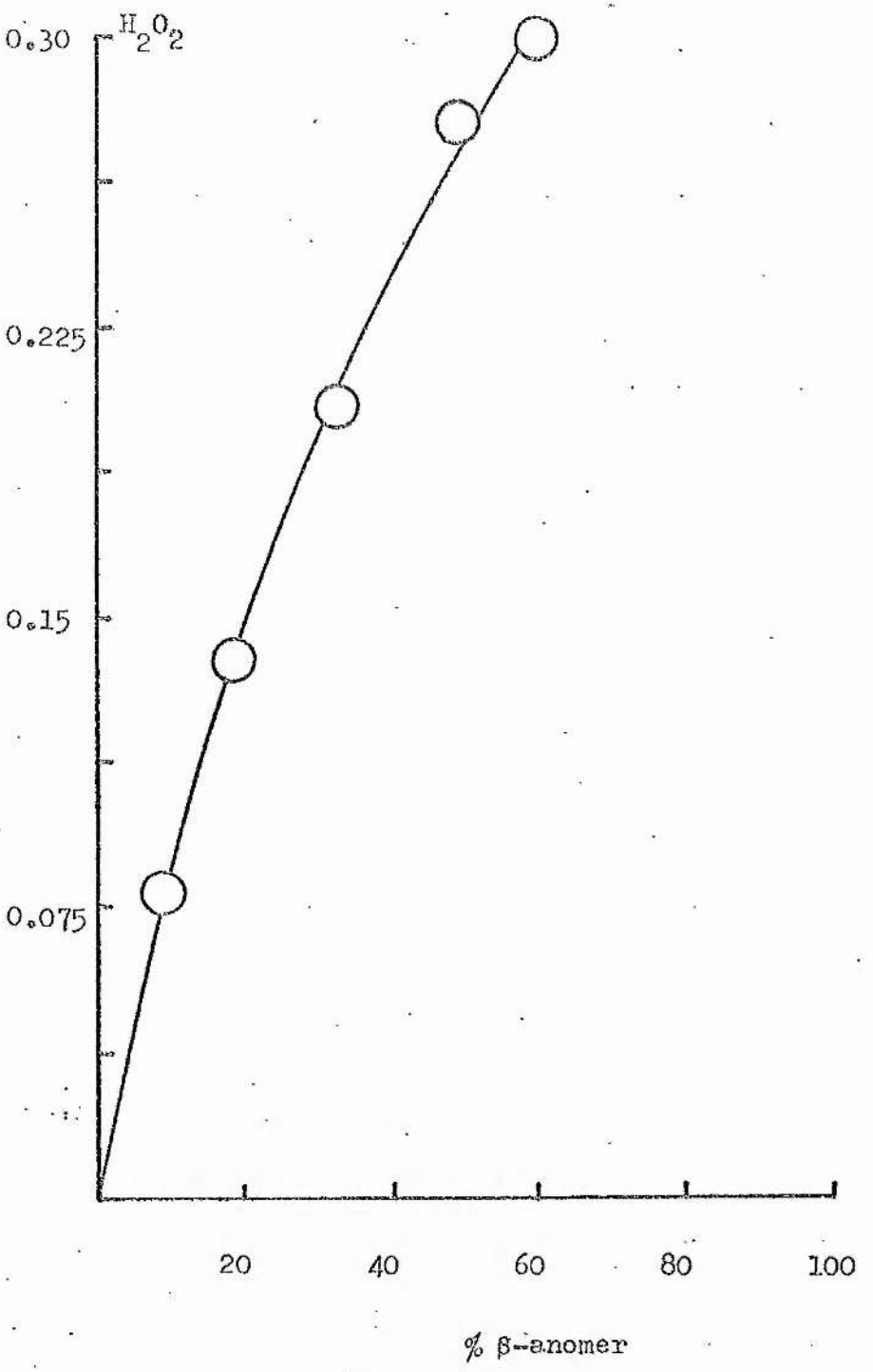


Fig.30. Relationship between the H_2O_2 concentration produced, at a substrate concentration of 2.5mM-sucrose, for each delay time (see Fig.29) and the concentration of the β-anomer of glucose in the sample stream.

co-supported amyloglucosidase and the other using a 3m length of nylon tube-supported amyloglucosidase and a 3m length of nylon tube-supported glucose oxidase. Although the former system was twice as sensitive as the latter system no great significance is attached to this finding, since the enzyme solutions used for the preparation of each derivative differed significantly in concentration. However, the principal objective of the experiments was achieved in that two different linked enzyme systems for the determination of maltose were devised.

In this work three different linked enzyme structures were investigated as analytical reagents for the determination of sucrose, these were, invertase and glucose oxidase immobilised onto separate lengths of nylon tube and used sequentially in series (system 1). Two immobilised enzyme structures, both with the two enzymes co-immobilised onto 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 6 showed the separate enzymic activities of the enzymes in each structure together with the overall activity of the respective structures for the complete linked enzyme reaction. In each case the glucose oxidase activity in terms of the overall % oxidation of glucose in each system was in excess of 64%, and as discussed previously this may represent an even greater overall conversion of substrate in view of the fact that the glucose contained initially only 64% of the β-anomer. On the other hand the invertase activity of the structures, in terms of the overall % hydrolysis of sucrose to glucose and fructose was 10.1, 17.6 and 22.5

for the systems 2, 3 and 1 respectively. The results show that increasing the ratio of invertase to glucose oxidase from 1:1 to 2.1, in the coupling mixture, had no effect on the glucose oxidase activity of the linked enzyme structures, 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, as previously explained 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

$$\text{sucrose} \longrightarrow \text{D-fructose} + \alpha\text{-D-glucose}$$

$$\alpha\text{-D-glucose} \rightleftharpoons \beta\text{-D-glucose}$$

$$\beta\text{-D-glucose} + O_2 \longrightarrow \text{D-glucono-}\delta\text{-lactone} + H_2O_2$$

$$\text{D-glucono-}\delta\text{-lactone} + H_2O \longrightarrow \text{gluconic acid}$$

may be controlled to a greater extent by neither of the enzyme catalysed steps, but by the spontaneous mutarotation. This proposal is strengthened from 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 to D-glucono-δ-lactone four-fold.

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 onto Sephadex G-50. The same authors (1970) also reported an enhancement in the overall conversion of glucose-6-phosphate to gluconc-lactone-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 slow intermediate non-enzyme-catalysed mutarotation of the α-D-glucose.

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 operating conditions for the individual immobilized enzyme systems. For instance, it is easier to define the optimum coupling conditions for a single immobilized enzyme system than for a linked enzyme system because the conditions which 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 using non-hydrolytically cleaved nylon tube, whereas the glucose oxidase preferred the hydrolytically cleaved nylon tube. Therefore a co-supported linked

system of these two enzymes was not feasible since each preferred a different support. A support with no residual charge might have proved successful as support for the co-supported system, but again this would have represented a compromise because each enzyme preferred its own differently charged support.

The optimum storage conditions of immobilized enzyme preparations will differ between enzymes with respect to pH, ionic strength, temperature and solvent composition. Therefore, immobilizing enzymes onto 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 co-supported linked enzyme system 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 onto separate coils because the solvent composition of the sample stream can be readily altered after leaving the first coil and prior to being perfused through the second coil.

Therefore, the major advantage derived from the immobilization of two enzymes, each onto a separate tube, compared with their immobilization onto 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.8 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 latter value from the former value then, is a measure of the disaccharide concentration in the sample and by reference to the appropriate standard curve the disaccharide level can be determined.

3.2.17. Determination of Penicillin G using Nylon Tube-Supported Penicillinase

Fig. 31 shows the effect of sampling rate on the calibration curves obtained when standard solutions of penicillin G were assayed as described in section 2.6.3.16 using a 1m length of nylon tube-supported penicillinase. This method may be used for the determination of penicillin G in the concentration range 20-100µm.

The stability of a 1m length of nylon tube-supported penicillinase was studied by continuously subjecting standard solutions of penicillin G to the assay procedure. Fig.32 shows that over a period of 14 days, during which time 12,000 separate analyses were performed, the nylon tube-supported penicillase did not incur any loss in catalytic activity. By reference to a standard curve, concurrently compiled by subjecting known concentrations of penicilloic acid to the assay procedure, the results are also presented in terms of the penicilloic acid produced.

The contents of one sealed vial of penicillinase, containing 900 enzymes units, were used to prepare a 3m length of nylon tube-supported penicillinase and for the stability study a 1m length was used for the determination of 12,000 separate samples. Therefore the 3m length could be used for a minimum of 36,000 separate assays. 900 enzyme units

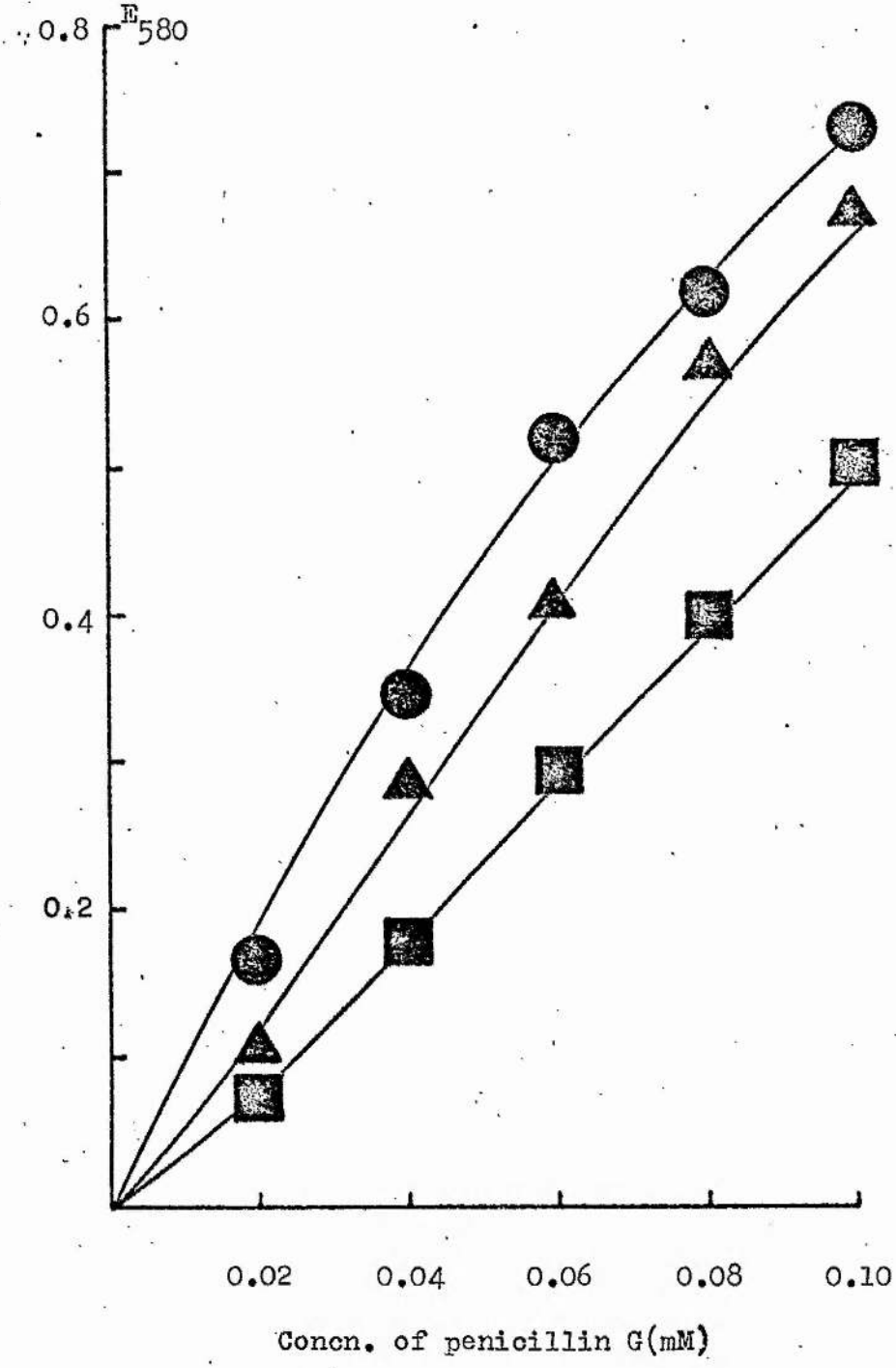


Fig.31. The effect of sampling rate on the standard curves for the automated determination of penicillin G with a 1m length of nylon tube-supported penicillinase.
●, 20samples/h; ▲, 40samples/h; ■, 60samples/h.

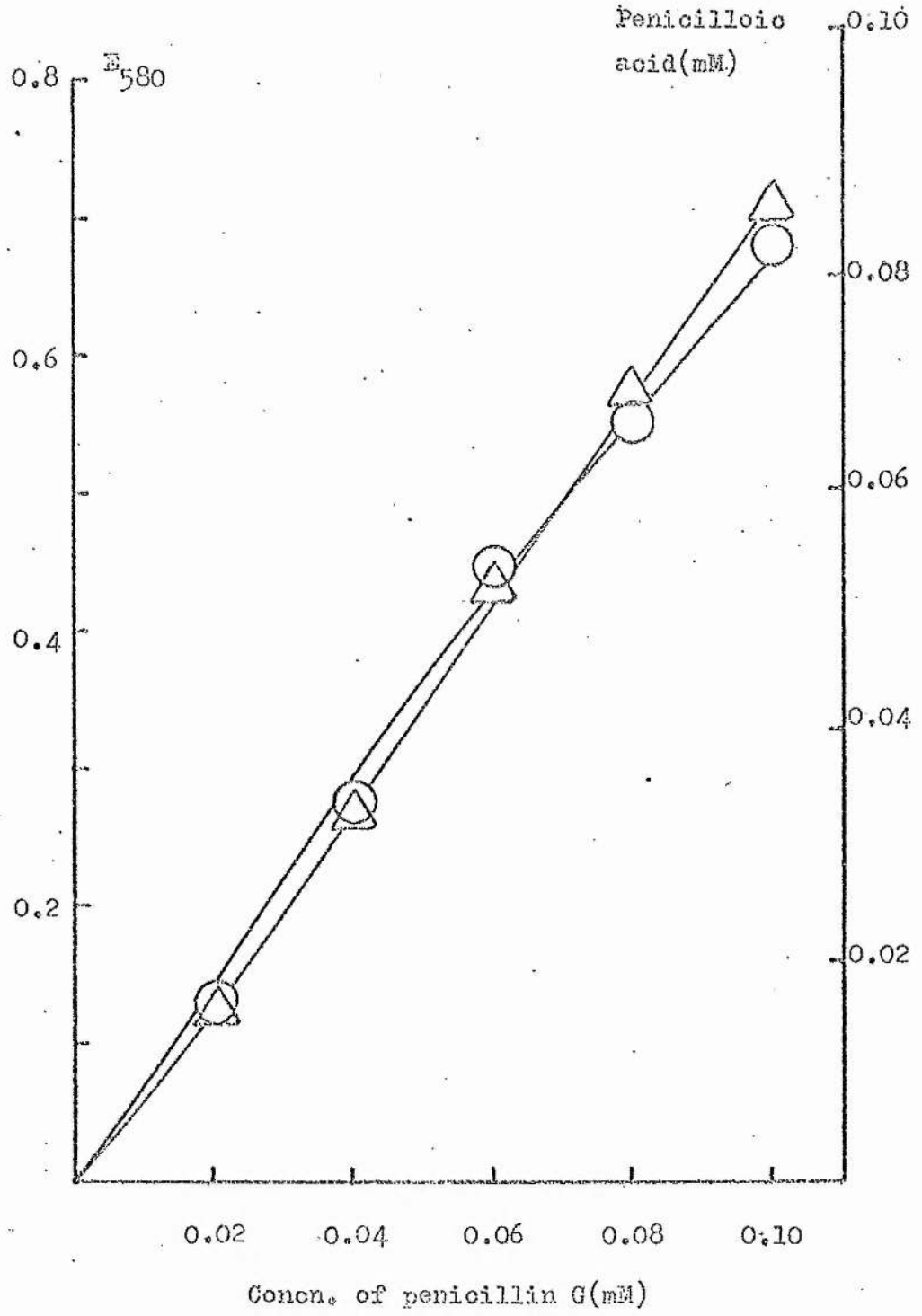


Fig.32. Standard curves for the automated determination of penicillin G with a 1m length of nylon tube-supported penicillinase. ◯, 1st standard curve and △, 1,200th standard curve.

of penicillinase when used free in solution are sufficient for not more than 4,000 separate penicillin determinations. This again illustrates the considerable savings in terms of enzyme protein which can be realised by using an enzyme in an immobilized form, when compared with the same amount of enzyme used in free solution.

It has been observed that penicillinase is strongly adsorbed onto glass surfaces (Pollock 1960) even from very dilute solutions. Thus an automated flow system for the determination of penicillin using soluble penicillinase would be influenced by this effect and consequently standardisation of the assay procedure would be more arduous.

3.2.18. Determination of Penicillin G in Fermentation Broths

Fig. 33 shows the results obtained when the penicillin G level in growing cultures of P.chrysogenum was determined using a 1m length of nylon tube-supported penicillinase as described in section 2.6.3.18. A number of different parameters may be used as an index of the stage of growth of P.chrysogenum culture (Hockenhull 1969). For instance, dry cell weight, penicillin level, pH changes and estimations of specific medium constituents such as lactose have all been used. The penicillin G concentration was interpolated by reference to a standard curve compiled by subjecting known amounts of penicillin G to the assay procedure. It was essential to establish whether or not the fermenter medium was interfering with the enzymic reaction or with the subsequent colour reaction. In order to establish if either of these two effects were important, two standard curves were compiled. The first was compiled by assaying known concentrations of penicillin G in the presence of fermenter medium, and the second curve was compiled by assaying known concentrations of penicillin G in the presence of

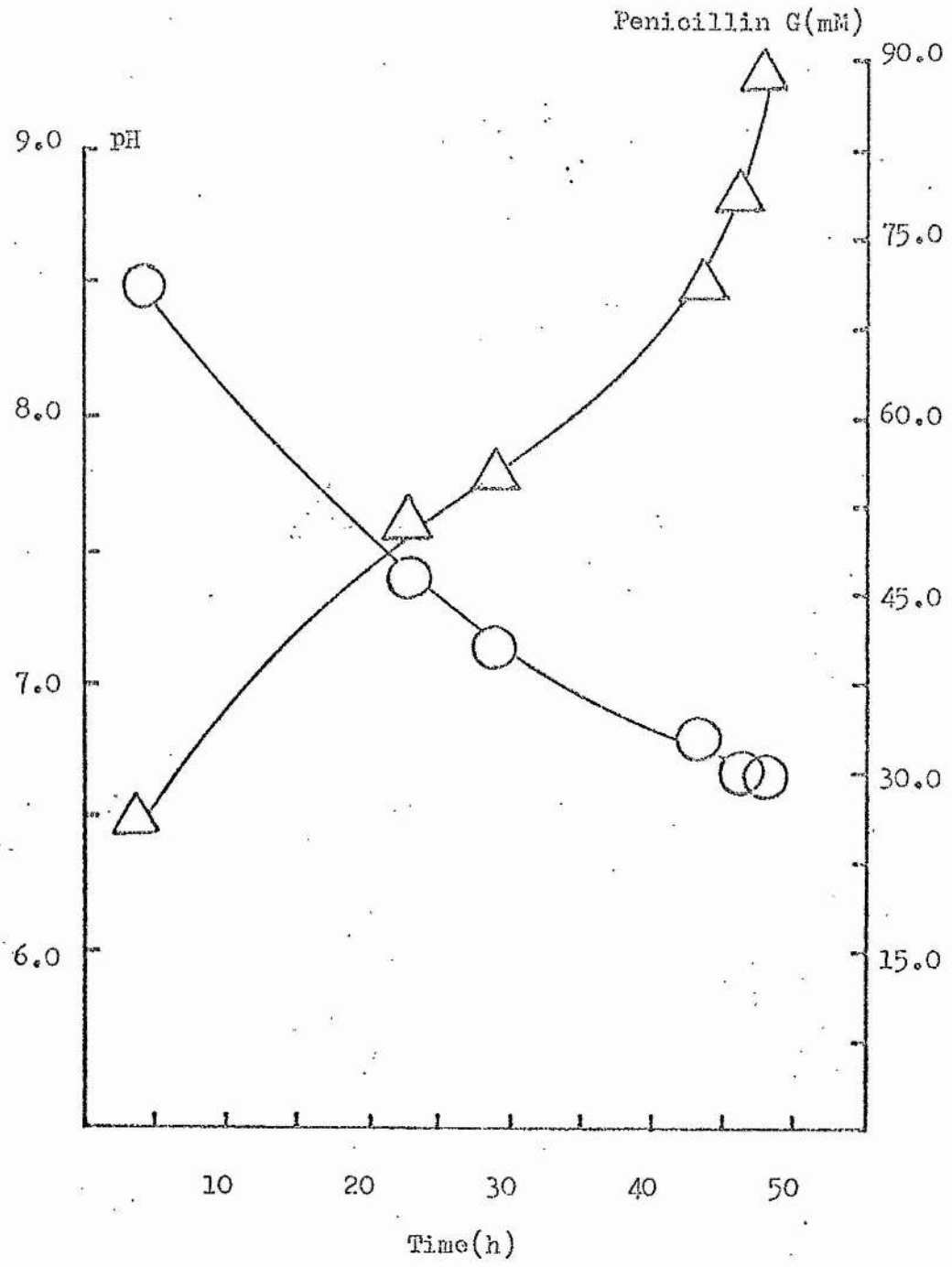


Fig.33. Changes in the penicillin G concentration and the pH of the medium constituents during submerged culture of Penicillium chrysogenum.

distilled water. The results of these experiments (Fig. 34) show that there was no detectable interference in the assay procedure due to the presence of the fermenter medium.

3.2.19. Determination of Ethanol and Glucose in Yeast Cultures

Fig. 35 shows the results obtained when the glucose and ethanol levels in cultures of yeast fermenting glucose were determined using 3m lengths of nylon tube-supported glucose oxidase and nylon tube-supported alcohol dehydrogenase, by the methods described in sections 2.6.3.1. and 2.6.3.6. respectively. In each case the glucose and ethanol levels were interpolated by reference to standard curves compiled by subjecting known amounts of glucose and ethanol to the respective assay procedures. As may be seen from these results, the conversion of glucose to ethanol is approximately 80%. It might be expected that the conversion of glucose to ethanol would have been 100%, but this disparity of 20% is not considered significant since the glucose is probably metabolised by other pathways besides the fermentation to ethanol.

3.2.20. General Considerations on the Analysis of Fermentation Broths using Nylon Tube-Supported Enzymes

The analysis of fermentation broths is obviously very important as a means of monitoring cell growth, end-product production or medium utilisation. Continuous monitoring of a fermentation broth using an enzyme-based assay procedure would require a large amount of enzyme in view of the protracted length of such fermentations. Since it has already been established that the immobilization of an enzyme leads

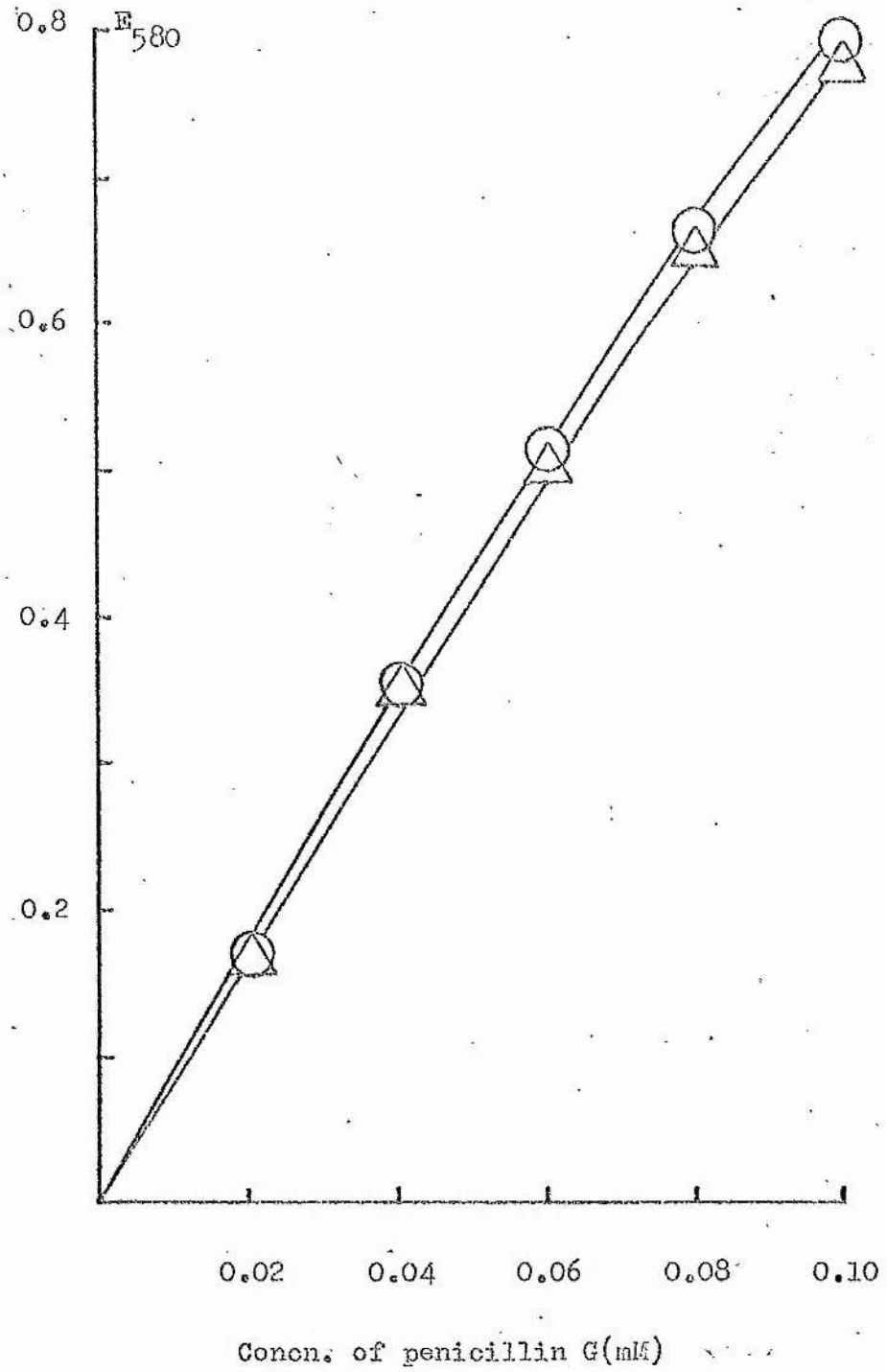


Fig.34. Standard curves for the automated determination of penicillin G, in the presence(△) and in the absence(○) of fermenter medium.

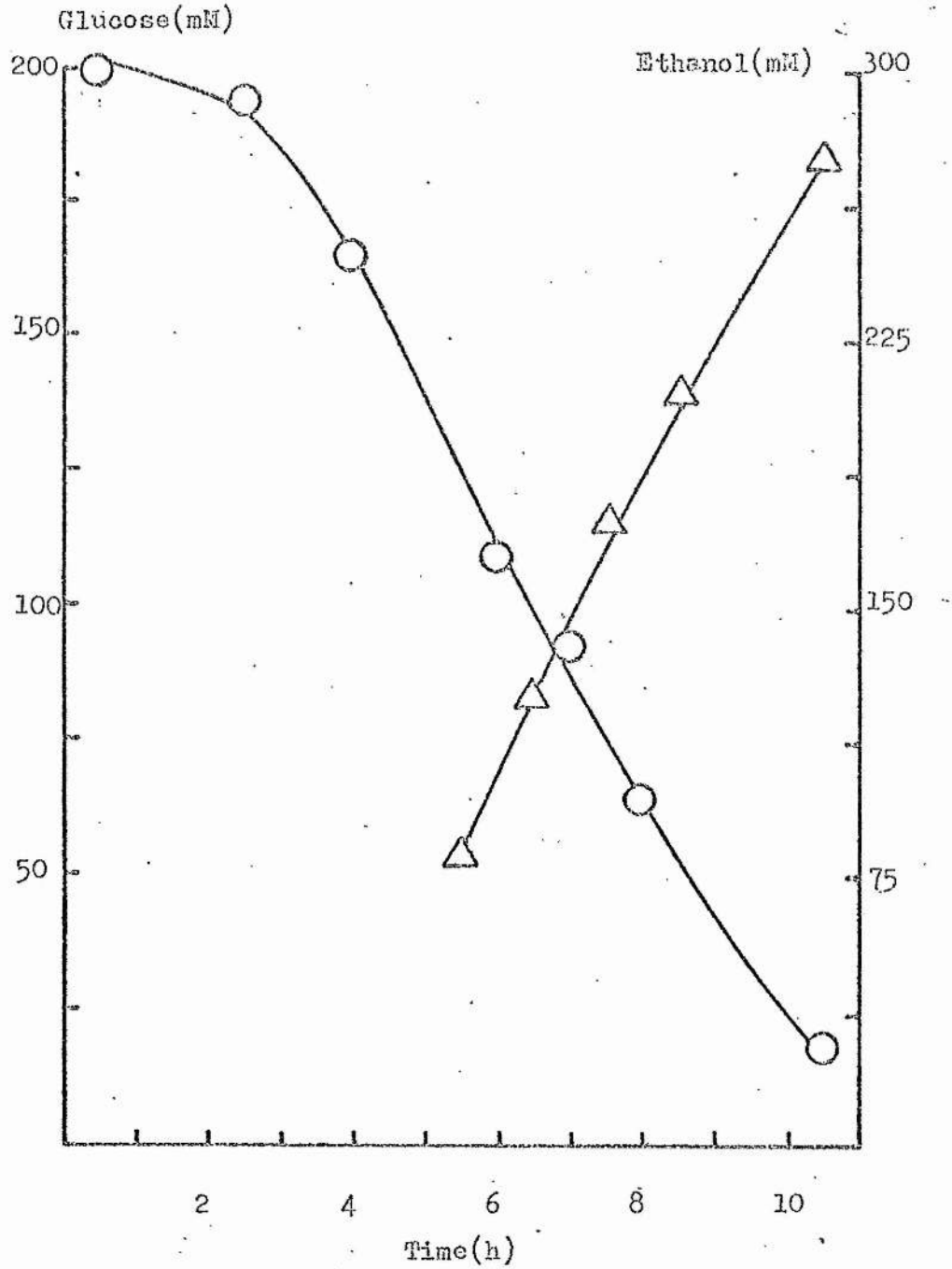


Fig.35. Changes in the glucose and ethanol concentrations in the medium of a yeast culture fermenting glucose. ○, glucose; △, ethanol.

to substantial savings in terms of enzyme protein the use of nylon tube-supported enzymes should find wide application in the fermentation industry. Before a nylon tube-supported enzyme can be used in this manner, it is essential to ensure that the fermenter medium is not interfering with the enzymic reaction or subsequent colour reaction. As was shown with the nylon tube-supported enzymes used in this section for monitoring fermentations, the fermentation media neither interfered with the activity of the immobilized enzyme nor with the subsequent colour reactions employed for the detection of reaction products.

3.3. Properties of Immobilized Enzyme Derivatives

Immobilized enzymes have been characterised in a variety of ways. For example, the stability of some preparations has been described, their Michaelis parameters have been measured, their activity dependence on pH identified and the enzyme protein of some preparations has been evaluated (Melrose, 1971).

The stability of an immobilized enzyme derivative, which is going to be used in automated analysis, is very important, because if more assays can be obtained from the enzyme in free solution than when it is immobilized, there is no virtue in immobilising the enzyme. Secondly, if the enzyme loses activity at a slow rate, then this will complicate the standardisation of the assay procedure. It is essential to know the Michaelis constant for an immobilized enzyme derivative to be used in automated analysis so that the enzyme may be assayed at substrate concentrations less than 1.5 times the K_m value. As the

substrate concentration of an enzyme catalysed reaction is increased up to the K_m value the plot of velocity against substrate concentration becomes more curved. Therefore, the sensitivity of the assay procedure decreases, because the velocity change per unit increase in substrate concentration becomes smaller.

The maximum sensitivity of an immobilized enzyme based assay can only be realised when the derivative is assayed at its pH optimum. Therefore, it is essential that the activity dependence on pH for the immobilized enzyme derivative is determined. The enzyme protein content of an immobilized enzyme derivative for use in automated analysis is not of comparable importance. This is because the protein content is not an indication of the activity of the derivative, and it is the activity of the derivative which is of paramount importance.

3.3.1. Stability of Immobilized Enzyme Derivatives

Table 7 shows the results of stability studies for seven of the immobilized enzyme preparations used in this work. The results are presented in terms of the number of assays for which the derivative was used and the time period during which these assays were performed. None of these derivatives showed any detectable loss in catalytic activity during these stability studies. (Horvath & Solomon (1972) have observed similar high stabilities for enzymes immobilized onto nylon tubes.

It may be possible to immobilize a large number of enzymes onto nylon structures using glutaraldehyde and for the products to show

Table 7

Stability Studies for nylon-supported enzyme derivatives

Derivative	Duration of use without any detectable loss in catalytic activity (days).	Number of assays performed without detectable loss in catalytic activity.
Nylon membrane-supported triazole-treated glucose oxidase	14	2,000
Nylon tube-supported triazole-treated glucose oxidase	30	3,500
Nylon tube-supported urease	30	5,000
Nylon tube-supported alcohol dehydrogenase	20	1,000
Nylon tube-supported lactate dehydrogenase	20	1,000
Nylon tube-supported malate dehydrogenase	20	1,000
Nylon tube-supported penicillinase	14	12,000

enzymic activity. Some of these enzyme preparations will rapidly become inactivated either on storage or in the course of operation. Enzymes catalysing the same reaction, but isolated from different sources will also show variations in stability between one another. For example, β-galactosidase from Saccharomyces fragilis was used for the preparation of nylon tube-supported β-galactosidase, but after being usedover a 2h period for the determination of 40 lactose samples, the preparation had lostmore than 90% of its catalytic activity. Whereas, the corresponding derivative prepared from E.coli β-galactosidase showed no detectable loss in activity when similarly used for 200 separate lactose determinations over a 7 day period. Since all the other derivatives were used over a minimum of 48h for the determination of at least 100 separate analyses without any detectable loss in activity, the rate of inactivation is probably quite small. This idea is strengthened by the observations of O'Neill et al. (1971) and by Sharp (1968) that the rate of inactivation of immobilised enzyme preparations can be approximately described by first order kinetics, that is, if a preparation loses 10% activity in 24h it will lose less than 10% activity in the next 24h. If this is the case, then the major factors responsible for any subsequent inactivation will probably be poisoning of the enzyme due to impurities in the sample stream or to accidental misuse.

3.3.2. Michaelis Parameters

The Michaelis parameters of an enzyme are measured conventionally by observing the dependence of the initial reaction velocity of the enzyme-catalysed reaction on the substrate concentration.

The data thus obtained may be analysed in a variety of ways for the evaluation of these constants, for example, by the method of Lineweaver & Burk (1934). In the special case of tube-supported enzyme structures the measurement of an initial reaction velocity is very arduous, since the structure only readily permits the determination of the extent of the reaction after a fixed residence time of the substrate in the tube. This parameter is clearly defined by the geometry of the tube and the rate of flow of substrate through the tube. However, provided that the percentage conversion of substrate to product during passage through the tube is not in excess of about 10 % then the substrate concentration is not significantly depleted in the course of the operation. In which case, the amount of product formed in the course of the residence time of the substrate in the tube may be used as an approximate measure of the velocity. In this way Sundaram & Hornby (1970) determined the apparent K_m value for nylon tube-supported urease and Hornby et al. (1970) used the same procedure for determining the apparent K_m value for polystyrene tube-supported glucose oxidase. This approach was used for the determination of the apparent K_m of some of the nylon tube-supported enzymes used in this work. Fig. 36 shows, for example, the dependence of velocity on substrate concentration for nylon tube-supported triazole-treated glucose oxidase in the form of a Lineweaver & Burk plot.

Table 8 shows the apparent K_m values for some of the nylon tube-supported derivatives used in this work when calculated by the method of Lineweaver & Burk (1934). The apparent K_m of glucose for the triazole-treated glucose oxidase derivatives was measured in the presence

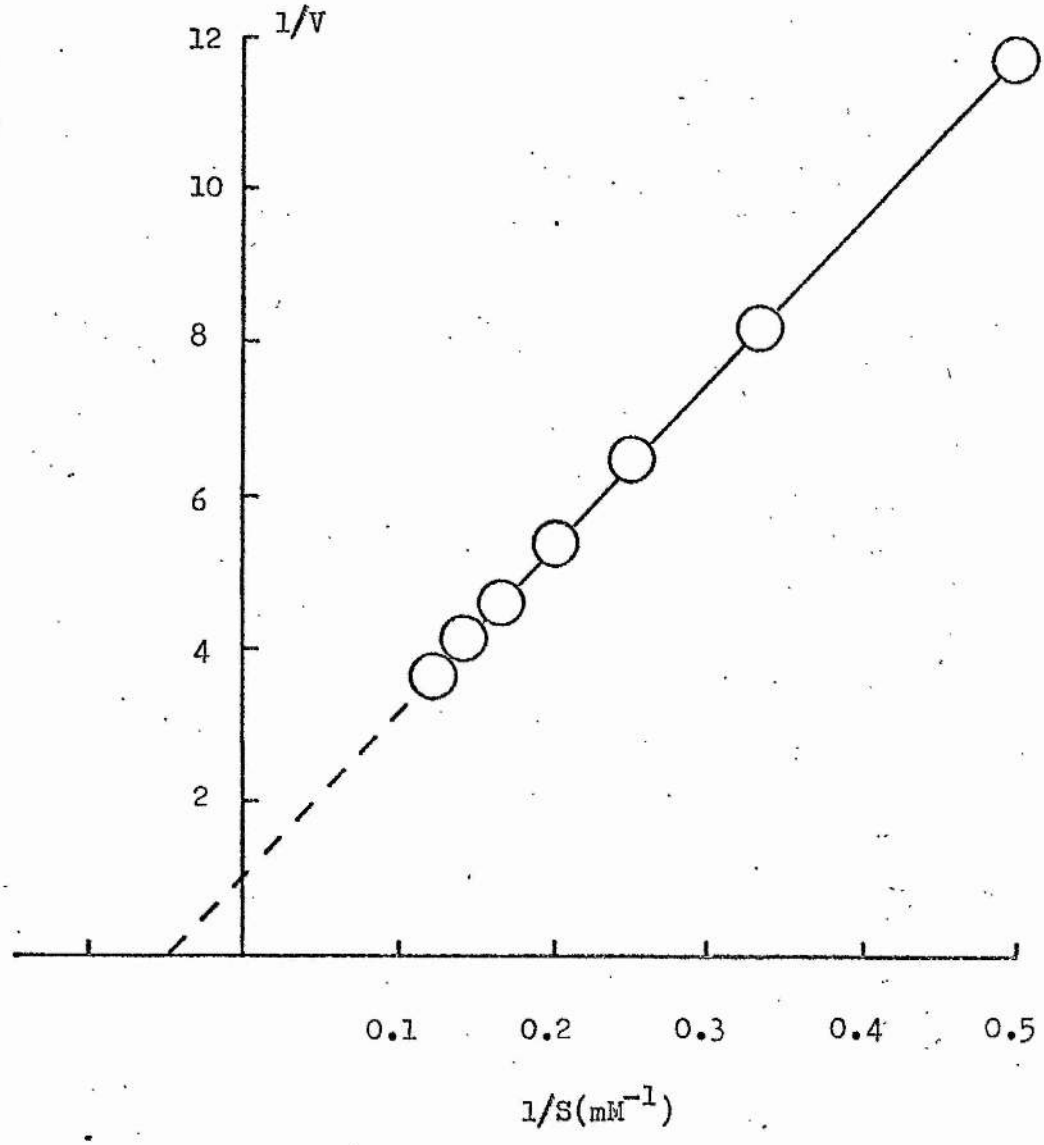


Fig.36. The effect of substrate concentration on the formation of H_2O_2 by a 3m length of nylon tube-supported triazole-treated glucose oxidase at 37°C in 0.1M-sodium acetate buffer, pH5.0. V is expressed as the concentration of H_2O_2 in the effluent, (mM).

Table 8

Apparent K_m values for nylon tube-supported enzyme derivatives

Derivative	Charge on tube	Apparent K_m of nylon tube-supported enzyme (mM)	K_m of enzyme in free solution (mM)
Penicillinase	(-)	0.4	0.25[a]
Triazole-treated glucose oxidase	(-)	25.0	33.0 [b]
Amyloglucosidase	(-)	19.0	18.5 [b]
Invertase	(-)	18.2	9.1 [b]
Urease	(-)	20.0	10.0 [b]

a) determined titrimetrically according to the method described in section 2.3.3.

b) obtained from the values quoted by Barman (1969).

of air-saturated water. No attempt was made to calculate the apparent K_m value for other two substrate enzyme derivatives, since none of these enzymes were under saturating conditions with respect to one substrate and so an apparent K_m value would have been of dubious significance. The K_m value for the enzymes in free solution is shown in the right hand column of Table 8. As the results show there are no significant differences between the apparent K_m value of the immobilized enzyme derivative and the K_m value for the same enzyme in free solution.

Two proposals have been suggested to explain the distortions in Michaelis parameters when an enzyme is immobilized, compared with the enzyme in free solution. For example,it has been suggested that significant alterations can occur between the apparent K_m of an immobilized enzyme and the K_m of the same enzyme in free solution when the enzyme is immobilized onto a highly charged support and the substrate is also highly charged (Goldman et al.,1972). The second explanation is the effect of pore diffusion into a porous structure. This is due to the rate of an enzymic reaction, when the enzyme is sited predominantly in the pores of a solid, being strongly influenced by factors affecting the diffusion of reactants and products through the pores (Sharp, 1960).

Goldstein et al. (1964) showed that when trypsin was immobilized onto a polyanionic copolymer of ethylene and maleic anhydride, the apparent K_m of the immobilized enzyme was an order of magnitude lower than the K_m value for the enzyme in free solution, when a positively charged substrate was used. This effect was found to occur at low ionic

strengths ($\Gamma/2 \simeq 0.01$) and on increasing the ionic strength the difference between the apparent K_m for the immobilized enzyme and the K_m for the enzyme in free solution decreased. These results have been explained by charge-charge interactions between substrate and support. The unlike charges on the substrate and support result in an enhancement of the substrate concentration in the microenvironment of the bound enzyme relative to the bulk solution. This would result in a localised increase in substrate concentration within the matrix of the support where the enzyme is surrounded by the charged groups.

The opposite effect has been observed for the enzyme ATP-creatine phosphotransferase immobilised onto CM-cellulose 90 (Hornby et al., 1968). In this case the support had a residual negative charge and the substrate, ATP was also negatively charged. An apparent K_m value for the immobilised enzyme an order of magnitude higher than the K_m for the enzyme in free solution was observed. Again this effect was observed at low ionic strengths and decreased with increasing ionic strengths. The like charges on the support and ATP result in a decrease in the substrate concentration in the microenvironment of the bound enzyme relative to the bulk solution. Therefore, there would be a localised decrease in substrate concentration within the matrix of the support.

Axén et al. (1970) studied the effects of diffusion of substrate into a swollen matrix on the apparent K_m of chymotrypsin immobilized onto Sepharose. The immobilized chymotrypsin preparations acting on acetyl-L-tyrosine ethyl ester had apparent K_m values about tenfold higher than that obtained for the enzyme in free solution.

Both the diffusional effects and the microenvironmental effects are dependent on the enzyme being immobilized within and around the matrix of the support. In both cases it is envisaged that the enzyme is buried within a highly charged matrix (Goldstein et al., 1964) and this creates an environment which is markedly different for the enzyme than when it is in free solution. However, as previously stated for the nylon tube derivatives, the enzyme is probably only immobilized on the surface of the nylon matrix, since only the peptide bonds on the surface of the matrix are cleaved. Therefore, the enzyme will not be buried within the nylon, but will probably be distributed solely on the surface of the structure and consequently any effects due to either pore diffusion or electrostatic interactions will be quite small. If this is the case then significant differences would not be expected between the apparent K_m value of the immobilized enzyme and the K_m of the enzyme in free solution. Even for the penicillinase, which has a charged substrate, there is no significant change between the apparent K_m of the immobilized enzyme and the K_m of the enzyme in free solution.

3.3.3. Effects of pH

Fig.37 shows the pH profiles of pyruvate kinase immobilized onto nylon tube and also free in solution. It may be seen that both preparations have a pH optimum at 7.0 and also that there are no significant differences between the shape of the pH profiles of the two preparations. These results, together with those obtained from similar studies with lactate dehydrogenase and urease, both free in solution and supported on nylon tube, are summarised in Table 9.

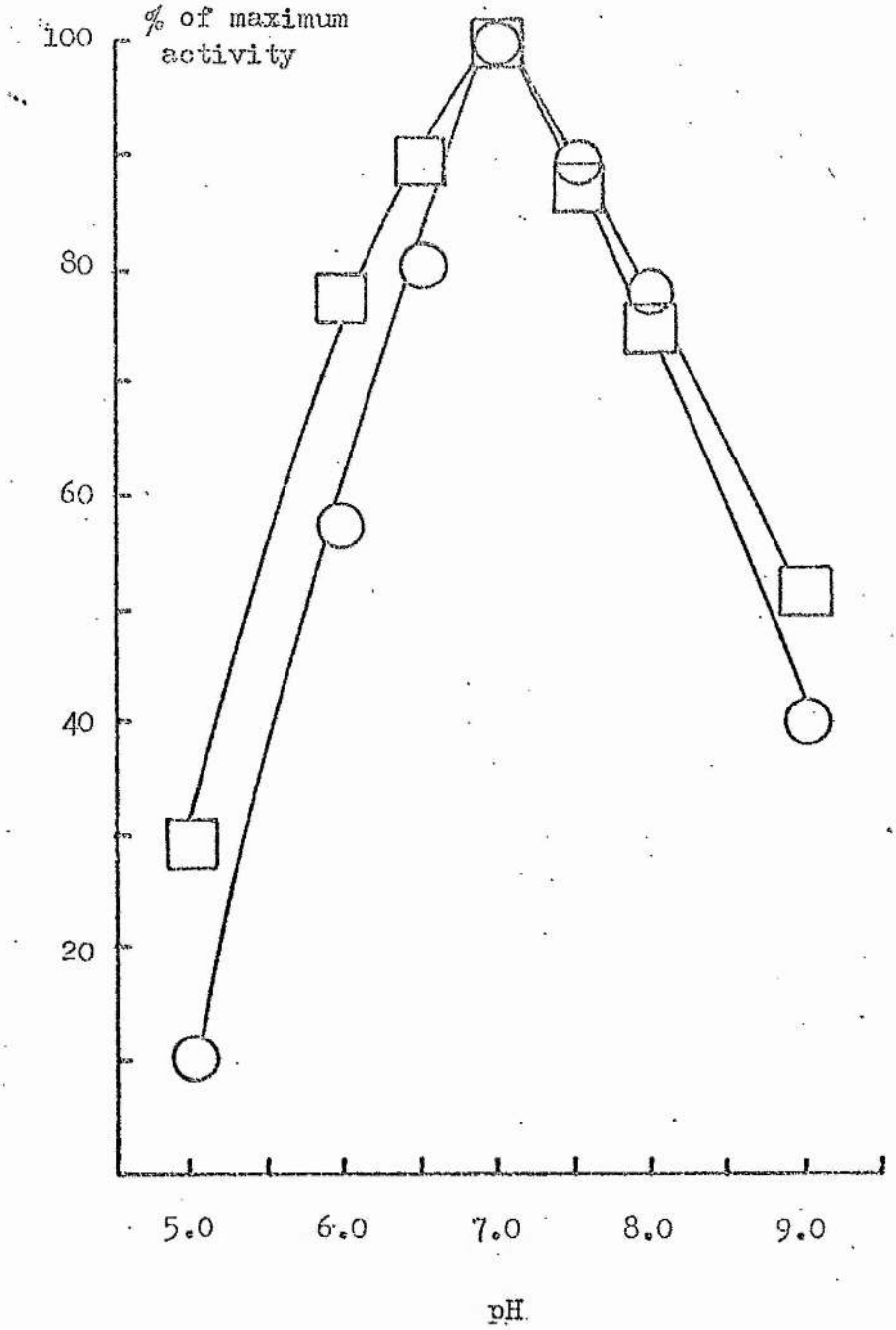


Fig.37. Effect of pH on the formation of pyruvate by pyruvate kinase in free solution(○) and chemically attached to nylon tube(□). All assays were performed in the presence of borate-acetate-phosphate buffer at 30°C as described in the text.

Table 9

pH optima for nylon tube-supported enzyme derivatives

Enzyme	pH optimum for nylon tube-supported enzyme	pH optimum for enzyme in free solution
Pyruvate kinase	7.0	7.0
Lactate dehydrogenase	5.5	5.5
Urease	7.5	7.5

3.3.4. General Considerations on the Properties of Nylon Tube-Supported Enzymes

It can be an advantage if no significant changes occur in the kinetic properties of an enzyme when it is immobilised. This is because the optimal operating conditions in terms of substrate concentration, pH and ionic strength are less arduous to define. Significant changes in the kinetic properties of the enzymes upon immobilisation might have occurred, if much lower ionic strengths were used ($\Gamma/2 \simeq 0.01$). However, the ionic strengths of the substrate solutions used for the assay of the nylon tube-supported enzyme derivatives were not less than 0.1 and consequently they might have masked changes in the kinetic properties.

General Considerations

General Considerations

Many methods have been described for the preparation of immobilized enzyme derivatives (Melrose, 1971). The chemistry involved in some of these preparations is often quite complex, involving several different steps; for example, Inman & Dintzis (1969) and Marshall et al., (1972). It may be argued that the degree of complexity of a preparative procedure controls to a certain extent the reproducibility and efficiency of the method. For example, the greater the number of unit operations in the procedure then the greater is the chance of experimental error. Likewise, the overall yield of the method is dependant on the number of unit operations and finally, the longer the procedure takes, the higher are the costs involved. Therefore, in the preparation of an immobilised enzyme the more steps involved, the longer the process takes and the higher the costs.

A number of compounds have been used for the preparation of immobilized enzyme derivatives, which are dangerous to handle. For example, some of these reagents are carcinogenic whereas others are highly toxic. Benzidine derivatives (Silman et al., 1966) and 1,5-difluoro-2,4-dinitrobenzene (Marfey & King, 1965) have been used as bifunctional reagents and cyanogen bromide (Porath et al., 1967) and phosgene (Horvath & Solomon, 1972) have been used for support activation. If immobilized enzyme derivatives are to be prepared routinely and on a large scale then it is desirable that the use of reagents such as these is avoided. In general any

compound that reacts with a protein and causes cross-linking of the protein is potentially dangerous. However, the level of toxicity of such compounds will be dependent clearly on the physical properties of the compound; for example, whether it is a liquid, a solid or a vapour and its chemical properties, such as the method by which it reacts with the protein and also the nature of its reactive groups.

Reagents come in a variety of forms, for example, solids (e.g. benzidine), gases (e.g. phosgene) and liquids (e.g. glutaraldehyde). With powders it is easy to generate reagent dusts which propagate the material and likewise gases by virtue of their physical form are also easily propagated. Probably the most convenient form in which to handle a toxic compound is in the form of a liquid with a very low vapour pressure, preferably already in the form of a dilute aqueous solution.

Some of the bifunctional reagents, which have been used for the preparation of immobilized enzyme derivatives are quite expensive. For instance, compounds such as the 1,5-difluoro-2,4-dinitrobenzene (Manfroy & King, 1965) and N-ethyl-5-phenyl isoxazolium-3'-sulphonate (Patel et al., 1967) cost approximately £1/g and £3/g respectively. Since one of the main advantages derived from using enzyme in an immobilized form emanates from economic considerations, then it is obviously best to avoid using expensive compounds such as these. For the preparation of many immobilized enzyme derivatives an excess of bifunctional reagent relative to enzyme is required. Therefore it is

possible in certain situations, where the enzyme is inexpensive (e.g. commercial grade glucose oxidase), that the major cost involved in the preparation of the immobilized enzyme derivative is not the enzyme itself, but the reagent used for linking the enzyme to the support.

In conclusion therefore, the preparation of immobilized enzyme derivatives should be as simple as possible and involve as few steps as possible. The cross-linking reagent should have low toxicity, be easy to handle and finally it should be readily available at low cost. It has already been stated in section 3.1.1 that glutaraldehyde is relatively non-toxic, easy to handle and readily available at low cost as well as affording a facile method for the preparation of immobilised enzyme derivatives. Taking into account the above considerations the advantages from using this material as a bifunctional reagent for the preparation of immobilized enzyme derivatives are obvious.

During the last twenty years a large number of different materials have been used as support structures for the preparation of immobilized enzyme derivatives (Goldman et al., 1971). These materials may be roughly divided into two different categories; those which are commercially readily available such as cellulose, polystyrene and nylon and those which have to be specially made, such as porous glass and amino acid copolymers. Any support which has to be specially made for the preparation of immobilized enzyme derivatives, will be expensive and involve time consuming processes. Therefore, on economic

grounds it is obviously best to try and use a material which is produced industrially in large quantities at low cost.

As previously mentioned, one of the principal reasons for using an enzyme in an immobilized form is the improved economics of enzyme utilisation. The idea is that the immobilised enzyme derivative is used repeatedly, over an extended period of time. Therefore it is essential that the support material remains inert, while maintaining its structural integrity during use, and is resistant to microbial attack. For instance, porous glass has been used as a support material for the preparation of many immobilized enzyme derivatives (Weetall, 1969). However, it has been observed that prolonged use of preparations results in a deterioration in the structural integrity of the glass (Weetall & Havewala, 1972). Naturally occurring polymers such as cellulose and related dextrans have also been used extensively as support materials, but it has been found that they are susceptible to microbial attack.

Hydrophobic materials have been used as support structures for the preparation of immobilized enzyme derivatives. Polystyrene, for instance, has been used for the preparation of polystyrene bead-supported invertase (Filippusson & Hornby, 1970) and for the preparation of polystyrene tube-supported glucose oxidase (Hornby et al., 1970). However, it has been suggested that support materials containing hydrophobic groups might denature a protein by processes similar to that whereby a hydrophobic solvent denatures a protein (Goldman et al.,1971).

Therefore, very hydrophobic materials might have limited use as support structures for immobilized enzyme derivatives simply because of the unfavourable nature of the support on the enzyme protein.

Protein molecules are composed of repeating units held together by peptide bonds. Therefore, it is not unreasonable to suppose that a support material, which contains this basic feature, might offer a very attractive environment for enzyme immobilization. Support materials, which fall into this category, are polyglutamic acid (Patel et al., 1967), polyaspartic acid (Patel et al., 1969) and nylon (Sundaram & Hornby, 1970). However, as already stated some of these polymeric amino acid supports are very expensive, for instance, polyglutamic acid and polyaspartic acid cost approximately £36/g and £78/g respectively and consequently the use of materials such as these greatly increases the cost of an immobilized enzyme preparation derived from them.

The choice of a support and type of support structure for the preparation of an immobilized enzyme derivative will be dependant on the way in which the derivative is to be used. For instance, beads, powders and fibres are used in packed beds and stirred tank reactors, membranes are used in enzyme electrodes and in dialyser units and tubes in continuous flow-through analysis. Therefore, it is advantageous to be able to obtain a support which can assume a variety of different structures, since the type of structure required for one particular application might be different from the structure required for a second application. For instance, cellulose and related dextrans

have been used in the form of powders (Porath et al., 1967) and in the form of sheets (Kay et al., 1967). Although this represents a reasonable diversification of structure the sheets are very fragile and great care has to be taken when they are handled. Porous glass may only be obtained in the form of beads and so the use of this material is restricted to packed beds and stirred tank reactors.

Finally, the choice of support material will be dependent on the specific properties of the enzyme such as its isoelectric point and also on the nature of the substrate. Charge-charge interactions between an enzyme and a support may facilitate the uptake of the enzyme on the support surface, if the charges are opposite in nature, when for example the enzyme is above its isoelectric point and the support is positively charged at the pH of the coupling. Since enzymes differ markedly in their isoelectric points it is obviously advantageous to be able to obtain a support, which can be converted readily into a form with either a residual positive or a residual negative charge. Cellulose may be obtained in a form carrying either a residual negative charge (CM-cellulose) or a residual positive charge (DEAE-cellulose). As was described in section 2.1, nylon may be hydrolytically-cleaved to yield residual negative charges in the form of dissociated carboxyl groups and non-hydrolytically-cleaved to yield residual positive charges in the form of positively charged tertiary amino groups.

In conclusion, therefore, the ideal support material would seem to be one which is cheap and readily available in a variety of mechanically strong forms. It must also be resistant to microbial attack and preferably be obtainable in a form which may be readily converted into a state carrying either a residual negative or residual positive charge. Finally, it would be advantageous if the material had protein like properties so that the environment offered to the immobilized enzyme is quite favourable. Nylon satisfies most of these requirements. It is very cheap and can be obtained either as a tube, a powder or as a membrane, all of which are mechanically strong. All nylon structures are resistant to microbial attack and the tube and powder may be converted into forms with either a residual negative charge or a residual positive charge. Finally, the backbone of the nylon is held together by peptide bonds. Therefore, taking into account all the preceding considerations, nylon offers an excellent support material for the preparation of immobilized enzyme derivatives.

In an earlier section of this work it was stated that more active immobilized enzyme derivatives could be prepared by using enzyme preparations, which had a greater specific activity and a higher degree of purity. This was demonstrated using two different preparations of glucose oxidase, the triazole-treated glucose oxidase and the Boehringer glucose oxidase preparations. The amount of enzyme protein used for the preparation of a 3m length of nylon tube-supported triazole-treated glucose oxidase was 35mg, whereas for the preparation of a 3m length of nylon tube-supported Boehringer glucose oxidase it was 7.5mg. It was

shown that this latter quantity could be further reduced, since a second 3m length of nylon tube-supported Boehringer glucose oxidase was prepared using only 3mg of enzyme protein and this derivative had the same catalytic activity as the comparable derivative prepared from 7.5mg of the same enzyme protein. Therefore, it is possible that further savings in terms of enzyme protein may be realised without significantly affecting the catalytic activity of the structure.

The work in this thesis showed that the tube-supported enzyme was the best way of using an immobilized enzyme for the determination of its substrate in automated analysis. The tube derivatives were the most sensitive structures in terms of the highest conversions of substrate to product and also they could be used for the performance of more assays/unit time than either the powder- or membrane- supported derivatives. The other principal advantage of the tube- supported enzyme derivatives, relative to the powder- and membrane-supported derivatives, is the ease with which they can be incorporated and used in automated flow systems. An autoanalyser is a flexible piece of equipment and may be used for a large number of different types of assay. Therefore, it is essential that if immobilized enzyme structures are to be incorporated into automated flow systems, then their inclusion must not involve gross changes in the existing circuitry, otherwise the time required to change the flow systems between two different types of assay will be increased.

Apart from the determination of its substrate, it was shown that the nylon tube-supported enzyme derivative could be used to

continuously generate an expensive analytical reagent from a cheaper reagent. In this respect yeast alcohol dehydrogenase was used to continuously generate NADH from NAD^+, and the NADH produced was used for two analytical systems requiring this particular coenzyme. The continuous production of NADH from NAD^+ results in a significant financial saving, because there is an approximate two-fold price difference between comparable grades of the two materials. Commercial preparations of NADH usually contain a variable amount of water and also a variety of degradation products. Therefore, unless extensive purification and chemical analysis is carried out the precise concentration of any NADH solution is difficult to establish. This problem is not encountered when the NADH is generated in situ, with the nylon tube-supported alcohol dehydrogenase, because the concentration may be easily calculated from the change in extinction when the coenzyme generating tube is inserted into the flow system. Solutions of NADH are much less stable than NAD^+ solutions, when both are stored under optimal conditions, and so producing the NADH in situ minimises the time available for the formation of degradation products.

The monitoring of fermentation processes is very important, because fine control is essential over a fermentation to ensure optimal utilisation of medium and optimal product formation. Since enzyme based assays are highly specific, they would be very useful for determining the concentration of a particular compound in a fermentation medium. However, due to the protracted nature of fermentationsand the need for continuous analysis, the quantities of enzymerequired would be quite

considerable. Therefore, the monitoring of fermentations with tube-supported enzymes is a field where the potential of these derivatives may be exploited. A vast majority of fermenter media will be complex and so it would be necessary to determine the substrate in the presence of many other compounds. It was shown for both the P.chrysogenum and the yeast fermentations that the efficiency of determination is not impaired by having other compounds present, apart from the substrate. Linked enzyme systems could be especially useful in this particular field of analysis, since it has already been described how monosaccharides and disaccharides may be determined in the same sample.

Table 10 presents all the nylon tube-supported derivatives studied in this work and lists the possible applications of these different preparations. It may be seen from this table that the main areas of application would seem to be clinical analysis and quality control in the food and fermentation industries. This type of analysis involves the determination of one particular compound in a large number of samples, using an automated enzyme-based assay. As the work in this thesis has shown, the nylon tube-supported enzyme derivative is particularly suited to this type of analysis.

Table 10

The possible applications of nylon tube-supported enzyme derivatives

Derivative	Applications
Nylon tube-supported glucose oxidase	Measurement of glucose in blood, urine and fermentation broths.
Nylon tube-supported urease	Measurement of urea in urine and blood.
Nylon tube-supported penicillinase	Removal of penicillin from milk and measurement in fermentation broths.
Nylon tube-supported invertase	Measurement of sucrose in food materials and fermentation broths.
Nylon tube-co-supported glucose oxidase and peroxidase	Measurement of glucose in blood, urine and fermentation broths.
Nylon tube-supported lactate dehydrogenase	Measurement of pyruvate and determination of glutamate-pyruvate transaminase activity.
Nylon tube-supported malate dehydrogenase	Measurement of oxalacetate and determination of glutamate-oxalacetate transaminase activity.
Nylon tube-supported alcohol dehydrogenase	Continuous generation of NADH and measurement of ethanol in fermentations.
Nylon tube-co-supported pyruvate kinase and lactate dehydrogenase	Measurement of ADP

Table 10 (Contd.)

Derivative	Applications
Nylon tube-supported invertase and nylon tube-supported glucose oxidase	Measurement of sucrose and glucose in food materials and fermentation broths.
Nylon tube-supported amyloglucosidase and nylon tube-supported glucose oxidase	Measurement of maltose in food materials, fermentation broths and in the brewing industry.
Nylon tube-supported β-galactosidase and nylon tube-supported glucose oxidase	Measurement of lactose in milk, food materials and fermentation broths.

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Abstract

1. Enzymes have been immobilized onto the inside surface of positively charged and negatively charged nylon tube using a novel pretreatment process for the nylon tube.

2. Glucose oxidase (EC 1.1.3.4) and urease (EC 3.5.1.5) were immobilized onto nylon tube, nylon powder and nylon membrane.

3. 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.

4. The efficiencies for the three immobilized enzyme structures as reagents for the automated determination of their substrates were compared.

5. Nylon tube-supported derivatives of malate dehydrogenase (EC 1.1.1.37), lactate dehydrogenase (EC 1.1.1.27) and yeast alcohol dehydrogenase (EC 1.1.1.1) were prepared and automated analytical procedures for the determination of their respective substrates are described.

6. Yeast alcohol dehydrogenase was used to continuously generate NADH from NAD^+ for the automated determination of pyruvate and oxaloacetate using nylon tube-supported derivatives of lactate dehydrogenase and malate dehydrogenase respectively.

7. Immobilized linked enzyme systems of invertase (EC 3.2.1.26) and glucose oxidase, amyloglucosidase (EC 3.2.1.20) and glucose

oxidase, β-galactosidase (EC 3.2.1.23) and glucose oxidase have been prepared and used for the determination of sucrose, maltose and lactose by their incorporation into a common flow system.

8. Nylon tube-supported derivatives of yeast alcohol dehydrogenase, glucose oxidase and penicillinase (EC 3.5.2.6) were used for the monitoring of fermentation processes.

9. The kinetic properties of some of the immobilized enzyme derivatives were studied.

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