NYLON TUBE IMMOBILIZED ENZYMES IN CONTINUOUS-FLOW ANALYSIS

George A. Noy

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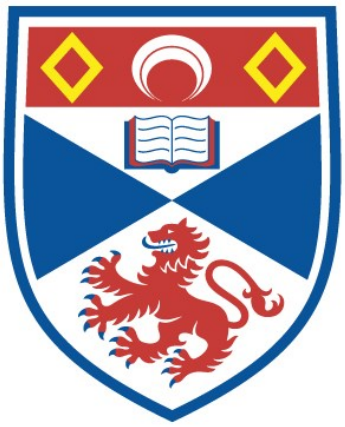
at the
University of St Andrews



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NYLON TUBE IMMOBILIZED ENZYMES IN CONTINUOUS-FLOW ANALYSIS.

by

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

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January, 1978.

DECLARATION.

I hereby declare that the following thesis is based on work carried out by me and that no part of it has been presented previously for a higher degree. The research was carried out in the Biochemistry Department of the University of St. Andrews under the direction of Dr. W.E. Hornby.

CERTIFICATE

I hereby certify that George A. Noy has spent at least nine terms carrying out research under the direction of Dr. W. E. Hornby and that he has fulfilled the conditions of the Resolution of the University Court (1977), No. 1, and is qualified to submit this thesis for the degree of Doctor of Philosophy.

ACKNOWLEDGEMENTS.

I should like to thank my supervisor, Dr. W.E. Hornby for his advice and criticism throughout this work, the Clinical Chemistry Dept., Ninewells Hospital, Dundee for supplying blood samples and the S.R.C. who financed this project.

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ABSTRACT.

The chemistry required for the immobilization of enzymes onto the inner surface of nylon tubing is demonstrated and conditions are optimised. Nylon tube immobilized enzymes are then introduced into continuous-flow systems so that their flow characteristics can be determined and the chemistry of immobilization best suited to continuous-flow analysis ascertained. The practical use of immobilized enzyme derivatives is evaluated in four separate methodologies and their stability and pH dependence is assessed.

1. General Introduction

Enzymes are proteins which exhibit control over the vast number of integrated chemical reactions which occur in living organisms. They are biological catalysts possessing a remarkable degree of specificity and efficiency, enabling them to catalyse particular reactions without the occurrence of undesirable side-reactions. In order to study the nature of reaction mechanisms in living systems, workers have isolated and purified enzymes and introduced them into controlled model systems. This has not only increased knowledge about reaction mechanisms and enzyme chemistry in general, but has led to an enormous growth in the number of known enzymes (Barman, 1969, 1974). With the increased availability of pure commercial preparations, enzymes are now being more widely used for the assay of metabolites.
(A comprehensive treatise on the subject of enzymatic analysis has been compiled by Bergmeyer, 1974, and there are several review articles by Guilbault, 1966, 1968, 1969, 1970.)

Enzyme - based analyses have been developed in order to replace the existing non-specific chemical methods which often measure groups of related, or sometimes non-related, substances rather than one specific compound. With the advent of these methods, a higher degree of accuracy is possible because of the specificity implicit in the use of enzymes. Thus in enzyme-based analysis, the substrate or analyte is chemically modified by the enzyme to produce a product which then can be specifically measured, either directly or indirectly, with no interference from other compounds present in the assay mixture.

Of the many possible enzyme-based analytical methods, only a handful have been fully exploited. Although the use of enzymes as analytical tools is generally considered commendable, there are certain obstacles to their large-scale use. The first of these relates to the economics of the operation. In order to use an enzyme for analytical purposes, the enzyme preparation must not be contaminated by other enzyme activities or substances which might interfere. Isolation of analytical enzymes, therefore, necessarily involves many purification steps, all of which contribute considerably to the expense of the final enzyme product. It follows from this that the use of enzymes as analytical reagents results in more costly analyses than the corresponding ones using conventional chemical analytical reagents. This "cost factor" increases in importance with the growing numbers of analyses that are done routinely in analytical laboratories and the automated methods required to cope with these large workloads.

In the performance of a single assay, soluble enzyme is mixed with its substrate, the amount of product formed is measured and the whole assay mixture is discarded. However, although the enzyme reaction might have reached completion with the substrate depleted, active enzyme is being thrown away. This enzyme is capable of converting more substrate and could perform many additional analyses if it was recovered. Unfortunately the separation and purification steps necessary for recovery are not economically practical.

Another problem associated with enzyme-based analysis emanates from the limited stability of some enzyme reagents.

It is extremely important that an analytical method should be reliable and provide reproducible results. If this is to be achieved, the analytical reagents should be stable throughout the period of analysis - this is not always the case with purified enzymes which often have a restricted stability when diluted - a property which can manifest itself under operational conditions as a loss of reproducibility which detracts from the enzyme's performance.

From the above, it would appear that to enhance the appeal of enzymatic techniques and to make them more readily adopted, cheaper and more stable enzymes are required. These considerations are the principal reasons for the application of immobilized enzymes as analytical tools.

The immobilization of an enzyme refers to the physical confinement or localization of enzyme molecules in a way that renders them operationally insoluble. Thus the enzyme can be readily recovered from solutions containing its reactants and re-used in further reactions. The methods used to accomplish the enzyme immobilization are discussed in several excellent review articles (Silman and Katchalski, 1966; Goldman et al., 1971; Zaborsky, 1973; Salmona et al., 1974).

One of the simplest methods of immobilizing enzymes, is by direct adsorption of the enzyme onto the surface of a water-insoluble support. An early demonstration of this technique is illustrated by the work of Nelson and Griffin (1916) who adsorbed β- fructofuranosidase onto charcoal and aluminium hydroxide. However, although immobilization by adsorption has been employed for many enzymes with as many adsorbents, its use is limited. The disadvantage of the technique is the liability

of enzyme desorption through changes in pH, ionic strength and temperature of the assay media (Zittle, 1958). Consequently, the method is unreliable unless these conditions can be strictly controlled.

An alternative method is that of gel entrapment (Bernfeld and Wan, 1963; Hicks and Updike, 1966), fibre entrapment (Dinelli et al., 1975) or micro-encapsulation (Chang, 1972) where the enzyme is physically enclosed within an insoluble matrix. These insoluble matrices are usually polyacrylamide and starch (gels), cellulose triacetate (fibres) and collodian, nylon and silicone (microcapsules). The entrapment is brought about by the formation of a highly cross-linked network of polymer in the presence of enzyme molecules. As with the adsorption method, the enzyme is not modified and therefore the percentage retention of activity tends to be high. Loss of activity with lattice-entrapped enzymes comes primarily as a result of enzyme leakage from within.

Probably the best method of immobilizing enzymes is by covalent attachment to an insoluble support (Silman and Katchalski, 1966). Clearly, this involves a chemical modification of the enzyme molecule, although to retain activity, the covalent bonds formed between enzyme and support must not involve functional groups at the biologically active site of the enzyme. The amino acid residues, present in enzyme molecules, which are capable of covalent bonding include, lysine, arginine, histidine, tryptophan, tyrosine, cysteine, aspartic and glutamic acids, serine and terminal amino acids. Inevitably such groups will be duplicated many times in the enzyme molecule, some with non-essential roles, so that random chemical reaction will

result in the inactivation of only a proportion of the enzyme molecules.

Most early support materials for covalent attachment of enzymes were natural polymers eg. cellulose (Kay and Crook, 1967) and agarose (Porath et al., 1967) but these have been superseded by synthetic polymers, particularly nylon (Hornby and Filipusson, 1970) and polystyrene (Filipusson and Hornby, 1970) which reduce the risk of microbial attack and degradation. The synthetic polymers, manufactured under standard conditions, have a constant composition and structure, are more easily obtained and are presented in various configurations eg. nylon can be obtained as a powder, a tube or even a membrane.

Support polymers will not covalently bind enzyme molecules unless there are reactive groups present although these may be incorporated into the backbone of the support polymer by chemical modification. However, not only should the introduced group be able to react with enzyme molecules under mild conditions but its introduction should leave the basic structure of the polymer intact eg. agarose can be cyanogen bromide-activated to give imido-carbonate groups (Axen et al., 1967) and nylon can be O-alkylated by triethyloxonium salts to give imidate groups (Hornby and Morris, 1974 a and b) each of these groups reacts readily with primary amine groups.

When an enzyme is immobilized on an insoluble support, or entrapped within a lattice structure, the restrictions imposed upon the enzyme molecule often lead to changes in its chemical and physical properties. The main factors responsible for these changes are diffusion limitations, steric and micro - environmental effects and enzyme modification, although no one factor can account for a specific change (Goldman et al., 1971).

Diffusion limitation effects occur at the interface of the matrix-bound enzyme and the reaction mixture where the unstirred layer of solvent forms a diffusion barrier, the thickness of which is dependent upon the rate of stirring (Nernst, 1904). During the course of an enzymic reaction a concentration gradient of substrate is established across this diffusion barrier as substrate is consumed at the enzyme site. This gradient will be more pronounced with lattice-entrapped enzymes where diffusion of substrate also occurs through the gel matrix (Axen et al., 1970). Saturation of a matrix-bound enzyme will then occur at a higher substrate concentration than that normally required to saturate the soluble enzyme. This results in higher K_m values for immobilized enzymes although the magnitude of the increase will vary with the conditions used for their determination (Kay and Killy, 1970).

The magnitude of K_m values also varies with the hydrophobicity of the support matrix due to partitioning effects. Studies carried out by Johansson and Mosbach (1974) show that when alcohol dehydrogenase is immobilized within matrices of different hydrophobicity (various acrylamide/methylmethacrylate copolymers), the apparent K_m values decrease as the enzyme is bound to more hydrophobic support matrices.

Marked changes in the kinetic behaviour of enzymes with charged substrates are seen when they are immobilized on, or within, highly charged support matrices (polyelectrolytes). K_m values will vary according to whether substrate and support are like-charged (increased K_m) or opposite-charged (decreased K_m) (Goldstein et al., 1964). This displacement of the apparent K_m for polyelectrolyte derivatives of enzymes is abolished at

high ionic strength, which masks electrostatic interaction or when the substrate is uncharged. (Goldstein and Katchalski, 1968; Goldstein, 1970).

Another electrostatic effect is the displacement of pH-activity profiles. Polyanionic derivatives of chymotrypsin have been shown to displace the normal soluble enzyme profile towards more alkaline pH values by 1 - 2.5 pH units whereas polycationic derivatives of chymotrypsin have the reverse effect (Goldstein and Katchalski, 1968). The phenomenon is the result of unequal distribution of hydrogen and hydroxyl ions between solution at the enzyme site and the bulk external solution. Hydrogen ions are attracted to negatively charged matrices but repelled from positively charged matrices, such that the local pH at the enzyme site is lower or higher, respectively, than the pH of the bulk solution. Again, the effect can be minimised at high ionic strength when the pH-activity profile of the immobilized enzyme approaches that of the soluble enzyme (Goldstein et al., 1964).

Apart from diffusional and microenvironmental limitations on substrate availability, which are distributional effects rather than total restrictions, there can be steric hindrance of substrate. This will occur when the active site of the enzyme inaccessible to substrate because of (a) covalent attachment near the active site, (b) small lattice-pore-size. Clearly, this will be a more common occurrence with enzymes which have large molecular weight substrates eg. proteases such as papain (Axen and Ernback, 1971). The enzymes are effectively removed from the reaction mixture and low specific activities result when the enzymes are assayed with high molecular weight substrates.

There appears to be no way of predicting the activity an enzyme will have on immobilization - the activities vary from zero to 100% that of the native soluble enzyme. The retention of enzyme activity is dependent upon the particular enzyme, the support matrix and the conditions used in the immobilization technique. No one immobilization technique can be recommended to give a higher percentage retention of activity, instead each enzyme must be treated in isolation and immobilized in a manner suitable to that enzyme and its proposed use.

Obviously, if an immobilized enzyme derivative is to be useful, it must retain its activity over long periods of storage and use. In fact, there is much evidence in the literature to support a general increased stability with immobilized enzymes. It must be stressed, however, that this enhanced stability is usually engineered by careful choice of support matrix (ie. hydrophobic or hydrophilic support) and the method of immobilization. Consideration of these factors can provide the immobilized enzyme with a favourable environment and consequently reduce the risk of denaturation of the protein structure. Since denaturation of proteins can occur in hydrophobic solvents, it is possible that denaturation of some immobilized enzymes could be promoted by the proximity of a hydrophobic support (Manecke et al., 1970). The effect need not be immediate, but might impose a certain strain on the enzyme molecule resulting in reduced stability and increased sensitivity to external denaturing agents. A hydrophilic support, on the other hand, could stabilize the bound protein. In either case, the enzyme can be further stabilized by cross-linking the enzyme molecules with bifunctional reagents and thus retain the molecules in an active conformation eg. Chang (1972) cross-linked microencapsulated asparaginase with glutaraldehyde.

Where the reaction of an immobilized enzyme is diffusion controlled, complete stability will be observed, although this may not be a true reflection of the rate of denaturation of enzyme (Korus and O'Driscoll, 1975). All substrate presented to the enzyme is converted and the maximum reaction velocity is controlled by the rate of diffusion of substrate to the enzyme. As denaturation of enzyme progresses, a point is reached where the immobilized enzyme can no longer convert all the substrate presented to it and the rate of diffusion is greater than the maximum reaction velocity. The reaction ceases to be diffusion controlled and a fall in the enzyme activity will be observed.

The enhanced stability of certain enzymes on immobilization has made their widespread use an attractive proposition in industry, medicine and chemical analysis. Their insolubility means that they can be readily recovered from their respective reaction mixtures, by simple separation techniques, and re-used many times. Thus the economics of an enzymatic technique can be greatly improved by replacing the soluble enzyme with its immobilized counterpart. In industry, the availability of low cost immobilized enzymes, with reasonable re-use capacities, means that enzymes can be used in large-scale chemical processes, yet still remain economically competitive with alternative processes. Complex reactions can now be carried out using the specificity implicit in the use of enzymes without resorting to involved separation of product and enzyme eg. immobilized penicillin amidase has been used to hydrolyse pencillins to 6-aminopenicillinic acid, (Self et al., 1969) and immobilized glucose isomerase has been used in the production of high fructose syrups (Havewala and Pitcher, 1974).

Further applications lie in the medical field where immobilized enzymes can be used therapeutically to treat metabolic disorders. Micro-encapsulated enzymes are especially useful in this area because they induce no immunological response. Chang and Poznansky (1968) have demonstrated, by experiments on mice, the possible use of microencapsulated catalase in the treatment of acatalasaemia in man. In addition, microencapsulated urease has been proposed as the basis of an articial kidney (Chang, 1966) and microencapsulated asparaginase has been used to suppress the growth of certain asparagine-dependent tumours (Chang, 1971).

In chemical analysis, increasing use is being made of immobilized enzymes, particularly in automated methods where large numbers of analyses are performed (Guilbault, 1972; Weetall, 1974). As a result of improved economics, enzyme-based analysis can now be adopted in preference to alternative non-enzymatic techniques. There are two distinct approaches to the use of immobilized enzymes in analysis. They can be either incorporated directly into existing analytical systems, with no fundamental changes in instrumentation, or completely novel analytical systems can be designed around them. The approach taken is determined by the configuration of the immobilized enzyme derivative eg. packed beds, thermistors, electrodes, open-ended tubular reactors, membranes, fibres and stirrers.

The use of columns of packed beds has been very popular in proposed analytical procedures, probably because they are a simple means for containment of all forms of particulate immobilized enzymes and allow easy incorporation into flow systems. In addition, many units of enzyme activity can be introduced into a packed bed with enzymes immobilized on powders and beads by virtue of the large surface area involved. Thus lactate dehydrogenase

and pyruvate kinase, immobilized on glass beads, have been used to determine pyruvate, adenosine diphosphate and phosphoenolpyruvate levels (Newirth et al., 1973). Glucose oxidase immobilized on glass beads (Kunz and Stastny, 1974) and entrapped in polyacrylamide gel granules (Hicks and Updike, 1966) has been used to measure glucose in serum. Columns of hexokinase and urease immobilized on glass beads have been built into thermistor units and used for the enthalpimetric determination of glucose (Bowers and Carr, 1976) and of urea (Bowers et al., 1976).

The use of open-ended tubular reactors has been demonstrated. Hornby et al. (1970) described a method for the assay of glucose using glucose oxidase chemically attached to the inside surface of polystyrene tubes. Nylon tube immobilized dehydrogenases (Hornby et al., 1972) and urease (Filipusson et al., 1972) have also been used in analysis.

With the advent of ion-selective electrodes has come a very useful analytical tool - the enzyme electrode (Clark, 1972; Guilbault, 1975). Enzyme electrodes are prepared by immobilization of enzymes over the surface of an appropriate ion-selective electrode in the form of a membrane, a gel layer or by direct attachment to the electrode surface. The ion-selective electrode measures either the product of the reaction (eg. urease produces NH_4^+ ion from urea) or the substrate (eg. glucose oxidase uses oxygen).

Other forms of immobilized enzyme derivatives which have been used in analysis include enzyme membranes (Inman and Hornby, 1972), enzyme stirrers (Kiang et al., 1976) and fibre-entrapped enzymes (Marconi et al., 1974; Saronio et al., 1974).

In this work, the use of immobilized enzymes in continuous-flow automated analysis systems will be considered. The principle of the automated continuous-flow system was first described by

Skeggs (1957), later developed by Technicon Instruments Corporation, Tarrytown, New York as the "AutoAnalyzer" and can now be found in the majority of hospital chemical pathology laboratories. Methods which have been automated have been found to be more reliable and less susceptible to operator error than are the corresponding manual techniques. Furthermore, automation has allowed laboratories to cope with increased daily workloads and release technicians for more exacting tasks.

The system of continuous-flow is basically a very simple procedure. Samples and reagents are aspirated by a multiple proportioning pump and propelled through a series of tubes and coils where they are mixed and processed by a number of different functional modules. The resultant stream is directed through the flowcell of a colorimeter where the optical density change, which is proportional to the concentration of the analyte in question, is measured and recorded continuously. By aspirating unknown samples and standard solution under identical conditions and comparing the responses, the values of the unknowns can be determined.

Sample integrity in this system is brought about by the introduction of an air-wash solution - air barrier between each sample. The air forms a bubble which fills the lumen of the sample tube, mechanically separating aspirates and scouring the walls of droplets of fluid which might contaminate following samples. The integrity of the sample is maintained by addition to a liquid stream which is also air-segmented. Thus a sample is divided into a larger number of individual liquid segments by air bubbles which are present throughout the entire analytical sequence until the colorimeter is reached where the stream is de-bubbled and monitored.

Each segment in the stream passing through the flow circuit receives, via the pump, an accurately proportioned amount of each

of the reagents required for the reaction. A fixed rate proportioning pump is used and pump rates are determined using pump tubing of various internal diameters. Any deviation from the fixed proportions results in irregular colour development which manufests itself as noisy pen traces on the recorder and bears no relation to sample concentration. The best results are achieved when the "bubble-pattern" throughout the system is regular.

Once a reagent has been added to the assay mixture, mixing is accomplished by passing the air-segmented stream through tightly wound glass coils. In this way, as the stream flows through the turns of the coil, each segment is inverted a number of times and liquids of different specific gravities are mixed by means of the heavy layer falling through the light layer.

In methods where protein might interfere with the reaction or colour development, its removal is brought about by use of the continuous dialyzer module. The continuous-flow dialyzer comprises two perfectly matched, spirally grooved plates with a semi-permeable membrane sandwiched between them, the whole being immersed in a constant temperature water-bath. Thus, there is a continuous channel through the dialyzer which is divided into an upper and a lower half by the membrane. The segmented sample stream flows through the upper half and the diffusible constituents pass through the membrane into the lower half containing a segmented recipient stream which flows at a similar rate in a concurrent fashion. The recipient stream emerges from the dialyzer containing the diffusible constituents of the sample in concentrations proportional to their concentration in the sample stream.

For optimum sensitivity, delay coils can be incorporated so that the reactions are taken to completion, although since all samples are treated identically this delay is not obligatory as with manual methods. After colour development, the air bubbles are removed from the segmented stream by gravity separation in a T-piece and the integrated liquid stream is pulled through the flowcell of the colorimeter with the minimum of delay. The optical density is thus monitored by the colorimeter and recorded as a series of peaks. The shape of these peaks will depend upon the rate of analysis, which to give the greatest precision should generally allow for the momentary attainment of the steady state value.

Clearly, for inclusion of an immobilized enzyme reactor into a continuous-flow system, its configuration must be of a design which allows for the maintenance of the air segmentation and permits reasonable sampling rates. One such configuration, probably the most suitable structurally, is the open-ended tubular reactor which can be easily inserted into the flow system. The use of enzyme tubes has already been demonstrated in continuous-flow systems using polystyrene supported glucose oxidase (Hornby et al., 1970) and other enzymes immobilized on nylon tubes (Hornby et al., 1972; Filipusson, 1972; Morris et al., 1975).

If automated enzyme-based analysis is to be considered, then the preparation of the immobilized enzyme reactor should be cheap and the product stable. To compensate for enzyme loss during immobilization, the immobilized enzyme must be able to perform many assays with no appreciable loss of activity. Furthermore, the support for the enzyme should be physically and chemically inert to reagents flowing through it.

Whatever immobilization technique is used, the properties of enzymes are usually changed or modified in some way. This change can be due to diffusional, microenvironmental and/or steric effects. Since the magnitude of some effects will undoubtedly vary with the conditions of assay, it is worthwhile studying immobilized enzyme derivatives under the conditions of their proposed use. This work takes a look at several dehydrogenases immobilized on the inner surface of nylon tubing and considers various factors which influence their performance in continuous-flow analyzer systems.

2. Immobilization of enzymes on nylon.

2.1 Introduction.

Nylon is a generic term applied to long-chain unbranched polymeric amides - chains of methylene groups interspaced with secondary amide groups at regular intervals. Each member of the nylon family is named according to the number of carbon atoms in the basic repeating units of the polymer. For example, nylon-6 is the polymerisation product of caprolactam and has a single repeating 6-carbon unit of five methylene groups and an amide group. Nylon-66, on the other hand, is synthesised by the polycondensation of adipic acid with hexamethylene diamine which gives the polymer two alternate repeating 6-carbon units with either four or six methylene groups respectively.

Nylon polymers make ideal support matrices for enzyme immobilization because they are mechanically strong, resistant to microbial attack and readily available at moderate cost in the form of mesh, powder, membrane and tube. Enzymes can be immobilized by covalently linking them to reactive groups on the nylon surface although this does necessitate pretreatment of the nylon. High molecular weight nylon has very few free amine or carboxyl groups available for covalent attachment of enzyme molecules but it does possess many potentially reactive centres in the form of its secondary amide groups. These reactive centres can be generated by (a) cleavage of the secondary amides to liberate their component amine and carboxyl groups, (b) N-substitution of the secondary amide, (c) O-alkylation of the secondary amide.

Secondary amide bonds can be cleaved either hydrolytically (Inman and Hornby, 1972) under acid conditions to yield free amine and carboxyl groups or non-hydrolytically (Hornby et al., 1972) with an amine, eg. N,N-dimethyl-1,3-propane-diamine under non-aqueous conditions, to yield free primary and tertiary amine groups (Fig. 1). The carboxyl groups can be converted to acid azides (Fig. 2) and diazonium salts (Fig. 3) and used for the direct attachment of enzymes (Inman and Hornby, 1972). Coupling of enzymes through primary amine groups can be achieved by employing bifunctional reagents such as glutaraldehyde (Fig. 1). After the amine groups have been blocked by the bifunctional reagent, hydrolytically cleaved nylon will carry a net negative charge when the carboxyl groups are ionised and non-hydrolytically cleaved nylon will carry a net positive charge when the tertiary amine groups are protonated. This means that enzymes can be immobilized on nylon supports carrying a favourable charge.

The use of cleavage methods to generate reactive centres on nylon polymers results in a loss of structural integrity and hence mechanical strength. This can be overcome by using the process of N-substitution of the secondary amide bonds (Goldstein et al., 1974) which involves mild acid hydrolysis followed by a four component condensation reaction between amine, carboxyl, aldehyde and isocyanide which results in the restoration of an intact nylon backbone and the formation of an N-substituted amide (Fig. 4). By using a bifunctional isocyanide, the same type of four component condensation reaction can be used for the coupling of enzymes through their amine or carboxyl groups.

Reactive groups can also be generated by O-alkylation without depolymerisation of the nylon structure. When the

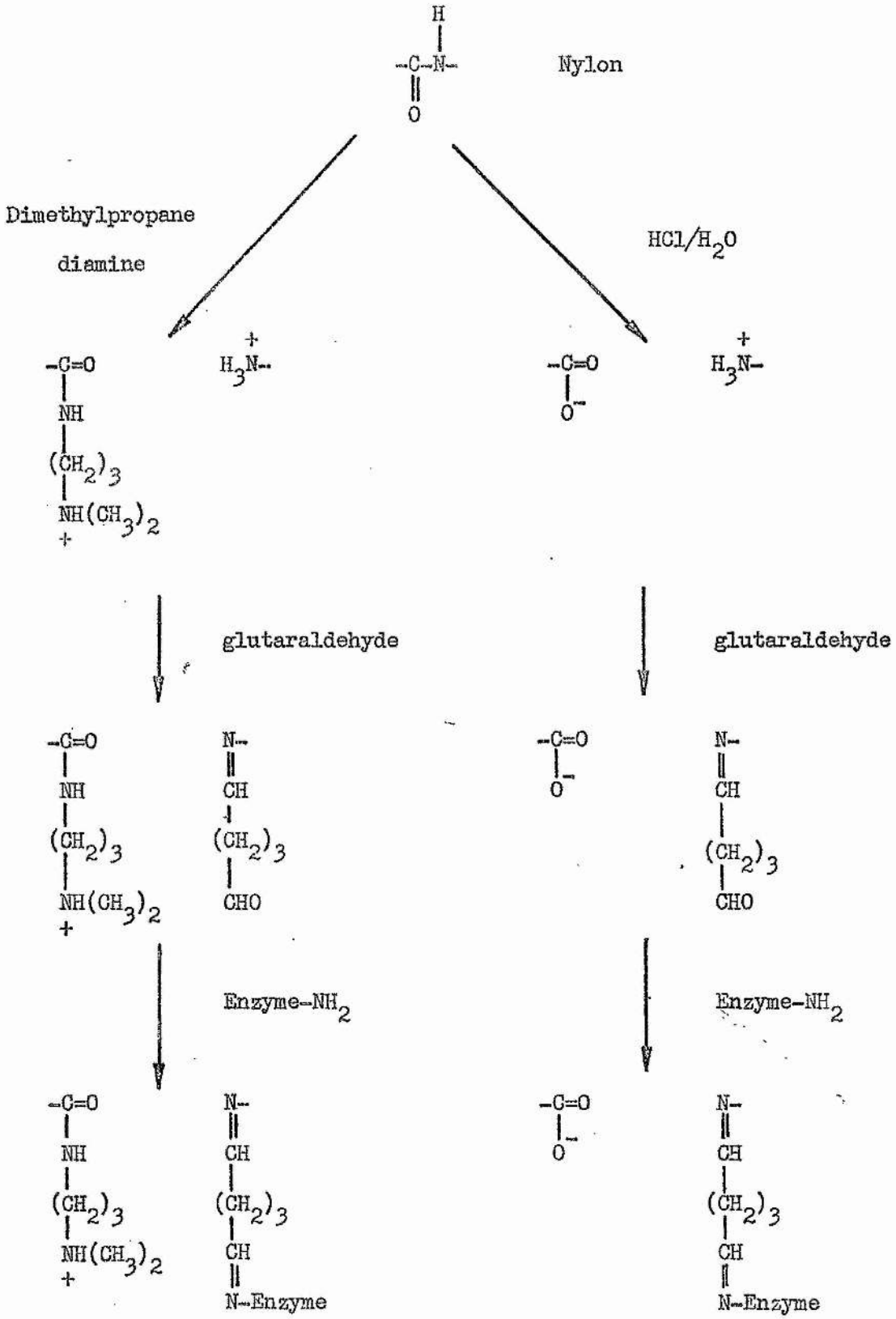


Fig. 1. Cleavage of secondary amide bonds of nylon and covalent binding of enzymes through the liberated amine groups.

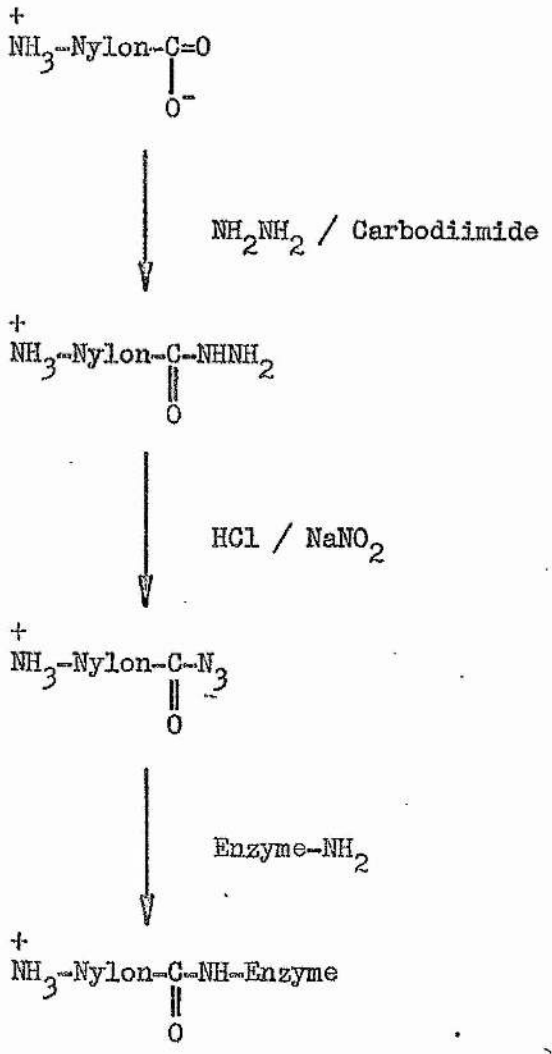


Fig. 2. Direct attachment of enzyme to the liberated carboxyl groups from hydrolytically cleaved secondary amide bonds (Inman and Hornby, 1972).

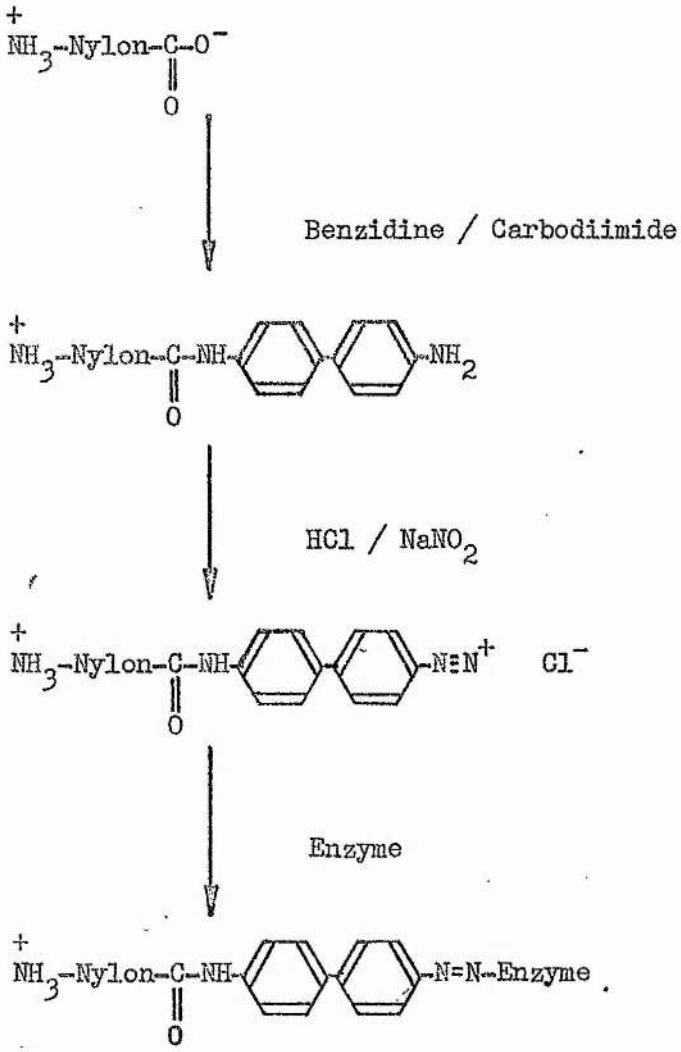


Fig. 3. Attachment of enzyme to the liberated carboxyl groups from hydrolytically cleaved secondary amide bonds through benzidine (Inman and Hornby, 1972).

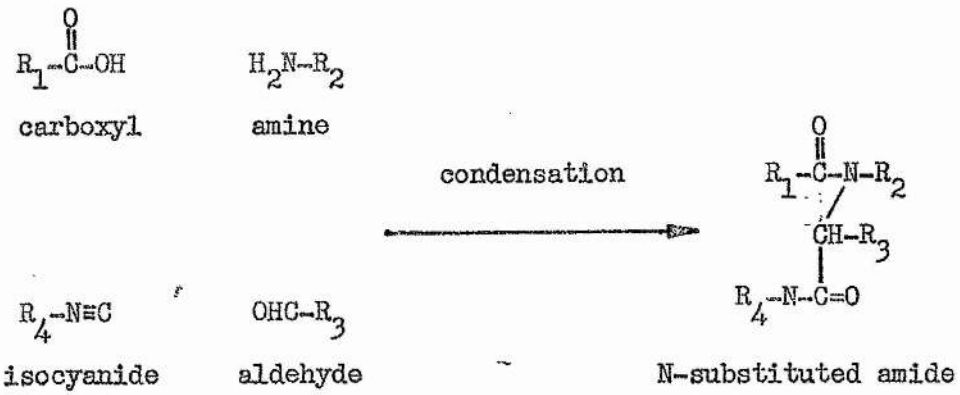


Fig. 4. Four component condensation of amine, carboxyl, aldehyde and isocyanide.

secondary amide groups of nylon are O-alkylated by strong alkylating agents, reactive imidate salts of nylon are formed. Campbell et al., (1975) alkylated nylon by incubation with dimethylsulphate at 100°C for a period of 3 min. These were rather harsh alkylating conditions which led to total disintegration of the nylon structure after prolonged treatment. Milder conditions of O-alkylation have been described by Morris et al., (1975) using triethyloxonium salts as alkylating agents (Fig.5). At room temperature, the extent of O-alkylation using triethyloxonium salts can be more easily controlled than with dimethyl sulphate.

Enzymes can be coupled directly to the imidate salt of the nylon through their free amine groups although the proximity of the hydrophobic support does not always give the most active immobilized enzyme derivatives (Campbell et al., 1975). Often it has been found necessary to place bifunctional amines and hydrazides between support and enzyme. These molecules have been termed spacer molecules. Imidate salts readily react with primary amine and hydrazine derivatives to give the corresponding amidines and amidrazones respectively (Fig.5). O-alkylated nylon which has been treated with bifunctional amine or hydrazide is referred to as amine- or hydrazide-substituted nylon. Enzymes can be covalently bound to these substituted nylons by using coupler molecules, bifunctional agents such as glutaraldehyde and the bisimidates (Fig. 6).

The coupling of enzymes through bisimidate molecules is brought about by the well-characterised reaction of free amine groups with imidates to form amidines as shown in Fig.6 (Hunter and Ludwig, 1972).

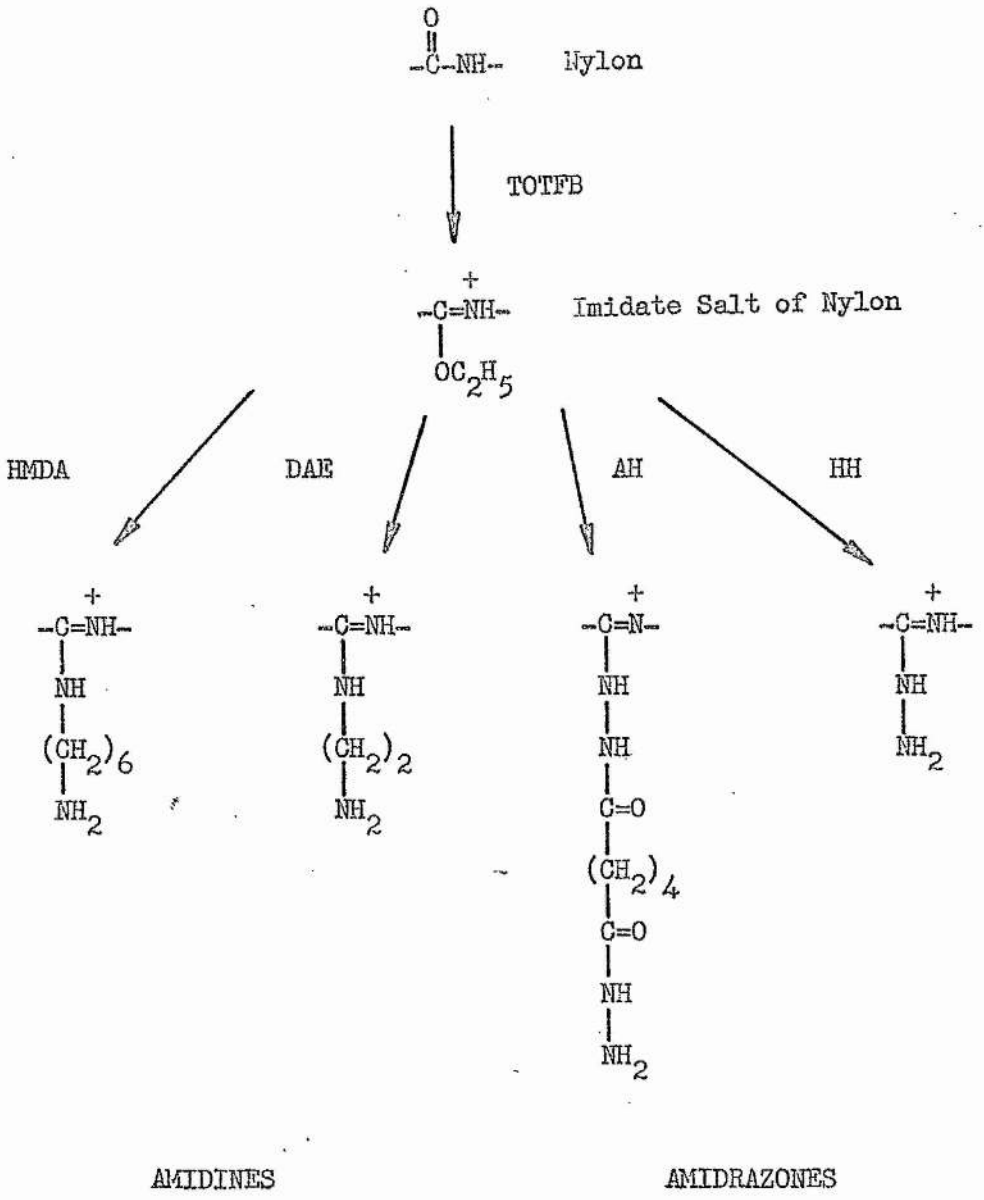


Fig. 5. O-Alkylation of nylon with triethyloxonium tetrafluoroborate (TOTFB) and the reaction of the imidate salt with diamines and dihydrazides. (HMDA=hexamethylene diamine; DAE=diaminoethane; AH=adipic dihydrazide; HH=hydrazine)

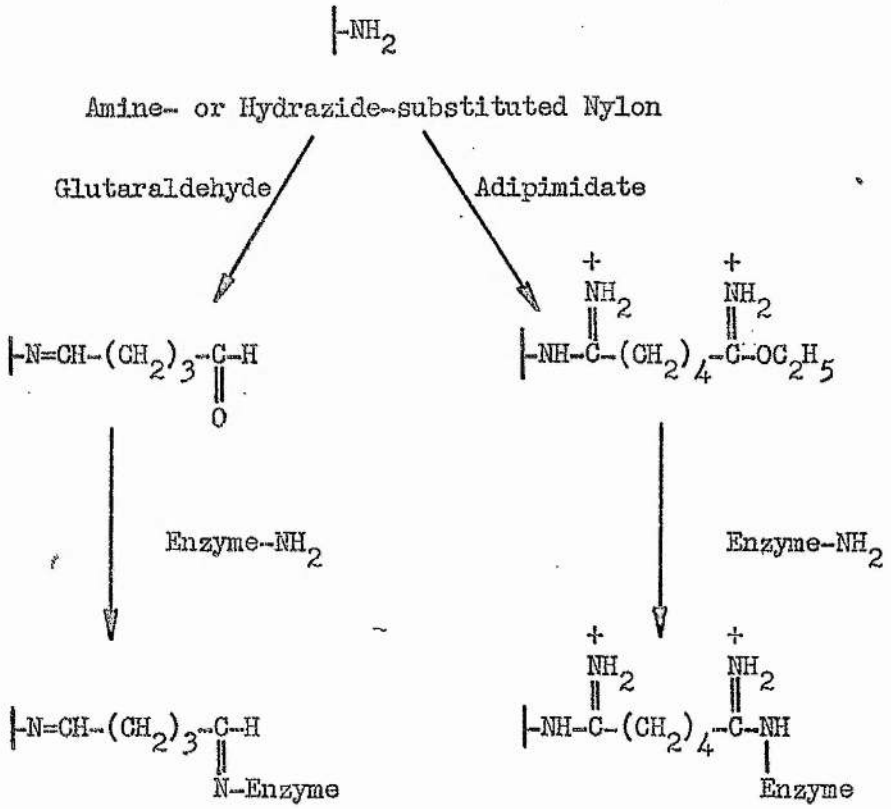


Fig. 6. The coupling of enzymes to amine- or hydrazide-substituted nylon by the use of the bifunctional agents glutaraldehyde and adipimidate.

There is some doubt as to the exact mechanism of the glutaraldehyde coupling reaction since the structure of glutaraldehyde in aqueous solution has yet to be established. Commercial preparations of glutaraldehyde are often used for coupling or cross-linking proteins without further purification. Aqueous solutions have been shown to contain significant amounts of α,β-unsaturated aldehydes and polymeric materials derived from glutaraldehyde itself (Richard and Knowles, 1968). Consequently, it was proposed that the glutaraldehyde reaction was a Michael-type addition of amine groups of the protein to α,β-unsaturated aldehydic polymers (Fig.7). Hardy et al., (1969) suggest that these α,β-unsaturated aldehydes are only minor components and that in neutral solution glutaraldehyde exists essentially as an equilibrium mixture of the dialdehyde and its hydrates (Fig. 8) and thus the glutaraldehyde coupling reaction would involve a monomeric form of glutaraldehyde. This falls into line with the generally accepted idea of glutaraldehyde reaction involving the formation of a Schiff's base between aldehyde and amine group (Fig. 6). In reality, the glutaraldehyde coupling step may well include both of the mechanisms described.

2.2 Preparation of intermediate compounds.

a. Preparation of triethyloxonium tetrafluoroborate (TOTFB).

Materials.

1. Diethyl ether: Redistilled and stored over calcium hydride.
2. Dichloromethane: Redistilled and stored over calcium hydride.
3. 1-Chloro-2:3-epoxypropane: Used as supplied by Aldrich Chemical Co.
4. Boron trifluoride diethyletherate: Used as supplied by Aldrich Chemical Co. This reagent was stored under sulphur dioxide.

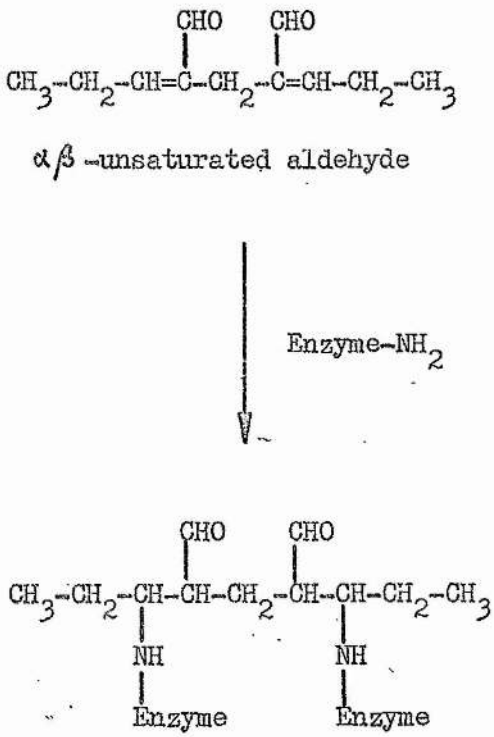


Fig. 7. Michael-type addition proposed by Richard and Knowles (1968) as the basis for the glutaraldehyde coupling of enzymes.

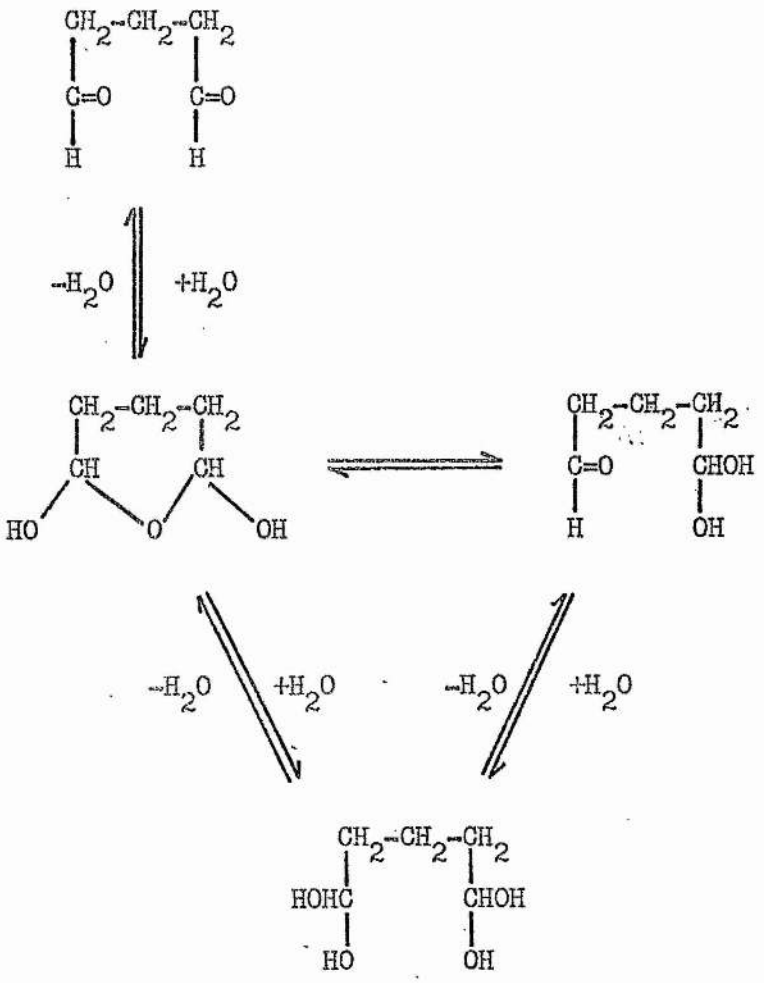


Fig. 8. Equilibrium mixture of glutaraldehyde and its hydrates (Hardy et al., 1969).

Method.

The method used is based on that described by Meerwein (1966). The reaction is illustrated in Fig.9. 25g. of boron trifluoride diethyletherate are added to 250ml. of dry diethyl ether in a round-bottomed flask and brought to reflux. 12.5g. of 1-chloro-2:3 -epoxypropane in 25ml. diethyl ether are slowly added to this solution and the mixture is stirred under reflux for a period of 2h. The triethyloxonium tetrafluoroborate (TOTFB) precipitates out as a white solid, which is washed thoroughly with dry diethyl ether, dissolved in dry dichloromethane and stored as a solution in dichloromethane.

b. Preparation of diethyladipimidate.

Materials.

1. Adipamide: Obtained from Cambrian Chemicals Ltd.
2. Triethyloxonium tetrafluoroborate (TOTFB): This material is prepared by the method of Meerwein (1966) and used as a 10% (w/v) solution in dichloromethane.
3. Diethyl ether: Redistilled and dried over calcium hydride.
4. Dichloromethane: Redistilled and dried over calcium hydride.

Method.

Diethyl adipimidate is prepared by the synthesis of Mocks et al. (1975).* The reaction is illustrated in Fig.10. 10g. of adipamide is added to 160ml. of the 10% (w/v) TOTFB in dichloromethane and the mixture is stirred vigorously at room temperature for 12h. The diethyladipimidate tetrafluoroborate forms a viscous light-brown oil which is separated and washed, first with dichloromethane and then with diethyl ether. Finally, excess diethyl ether in the preparation is removed under vacuum and the product is stored under vacuum at room temperature over concentrated H_2SO_4.

* see Pinner (1892)

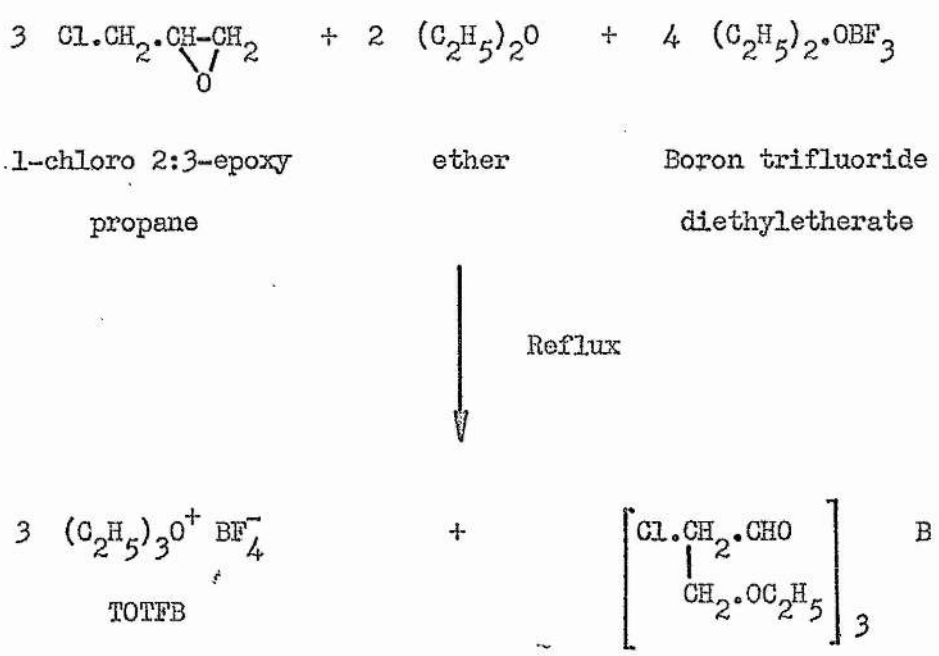


Fig. 9. Preparation of triethyloxonium tetrafluoroborate (TOTFB) as described in section 2.2a.

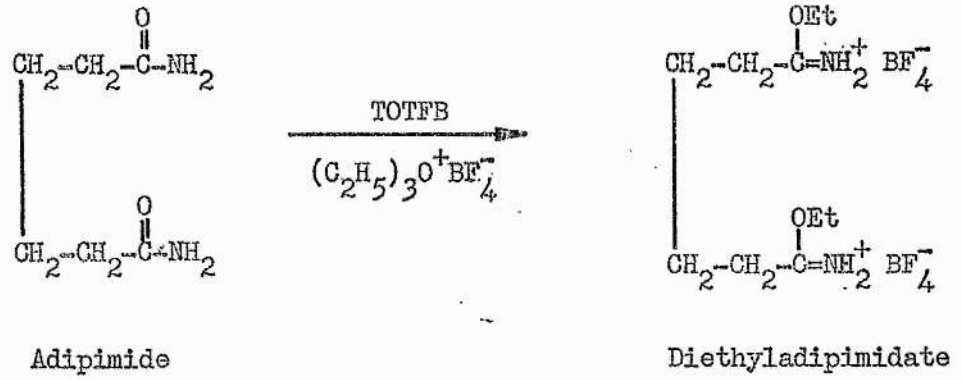


Fig. 10. Preparation of diethyladipimidate as described in section 2 .2b.

2.3 O-Alkylation of nylon tube.

Materials.

1. Nylon tube: Type 6 nylon tube (extruded from polycaprolactam) was obtained from Portex Ltd. All tubes used in this work had a bore of either 1mm. or 1.5mm. and a wall thickness of 0.3mm. or 0.6mm respectively.
2. Triethyloxonium tetrafluoroborate (TOTFB): 12.5% (w/v) solution in dichloromethane prepared as described above (section 2.2a)
3. Dichloromethane: Redistilled and dried over calcium hydride.

Method.

Typically, 3m lengths of nylon tube are filled with a 12.5% (w/v) solution of TOTFB in dichloromethane and incubated at 25°C for 15 min. (Fig. 5). The tubes are then washed with dry dichloromethane and immediately used to prepare amine- and hydrazide-substituted nylon tube.

2.4 Preparation of amine- and hydrazide-substituted nylon tube.

Materials.

1. O-Alkylated nylon tube: 3m. lengths prepared as described above (section 2.3).
2. Methanol: Redistilled and dried over molecular sieve (Type 3A potassium aluminosilicate pellets, BDH Chemicals Ltd.)
3. Dichloromethane: Redistilled and stored over calcium hydride.
4. Diaminoethane (DAE): Used as supplied by BDH Chemicals Ltd.
5. Hexamethylene diamine (HMDA): Material obtained from BDH Chemicals Ltd. and used as a 10% (w/v) solution in dry methanol.

6. Adipic dihydrazide (AH): Material obtained from Cambrian Chemicals Ltd. was used as a 4% (w/v) solution in formamide.

7. Hydrazine hydrate (HH): Used as supplied by BDH Chemicals Ltd.

Method.

Amine- and hydrazide-substituted nylon tube is prepared by filling 3m. lengths of O-alkylated nylon tube with appropriate solutions of diamine or dihydrazide (DAE, HMDA, AH and HH) and incubating for a period of 2h. at room temperature (Fig. 5). Thereafter, excess diamine or dihydrazide is removed by washing the tubes through with 5 l. distilled water overnight.

2.5 Preparation of nylon tube immobilized enzymes.

a. Preparation of glutaraldehyde activated nylon tube.

Materials.

1. Amine- or hydrazide-substituted nylon tube: prepared as described above (section 2.4).
2. 0.2M-Borate buffer, pH 8.5: 0.2M-boric acid is titrated to pH 8.5 with 2M-NaOH.
3. 25% (w/v) Glutaraldehyde: Electron microscopy grade glutaraldehyde was obtained from BDH Chemicals Ltd. This reagent was stored refrigerated at 4°C. 5% (w/v) solutions of glutaraldehyde in 0.2M-borate buffer, pH 8.5 prepared immediately before use.
4. 0.5M-NaCl in 0.2M-borate buffer, pH 8.5.

Method.

A 5% (w/v) solution of glutaraldehyde in 0.2M-borate buffer, pH 8.5 is pumped through the amine- or hydrazide-substituted nylon tube for 15 min. at room temperature. The tube is then washed free from excess glutaraldehyde by perfusion with 0.5M-NaCl in

0.2M-borate, pH 8.5 for a further 15 min. The glutaraldehyde activated nylon tube is immediately filled with a solution of the enzyme in 0.2M-borate buffer, pH 8.5.

b. Preparation of bisimidate activated nylon tube.

Materials.

1. Amine- or hydrazide-substituted nylon tube: Prepared as described above (section 2.4).
2. Methanol: Redistilled and dried over molecular sieve (Type 3A potassium aluminosilicate pellets, BDH Chemicals Ltd.)
3. N-Ethylmorpholine (NEM): This material was obtained from BDH Chemicals Ltd. 20% (w/v) solutions of NEM in methanol are used within 24h. of preparation.
4. Diethyl adipimidate: This material is prepared as described above (section 2.2b) 4% (w/v) solutions of diethyladipimidate in 20% (v/v) NEM in methanol are used within 2h. of preparation.
5. Dimethyl suberimidate: This material was obtained from Aldrich Chemical Co. 4% (w/v) solutions of dimethyl suberimidate in 20% (v/v) NEM in methanol are used within 2h. of preparation.
6. 0.1M-N-ethylmorpholine buffer, pH 8.5: 0.1M-NEM is titrated to pH 8.5 with 2M-HCl.

Method.

Bisimidates are very prone to hydrolysis (Rodger and Neilson, 1961). Therefore the amine- and hydrazide-substituted nylon tube are initially dried by perfusing with methanol for 10 min. Thereafter a 4% (w/v) solution of the appropriate bisimidate in NEM and methanol is recycled through the tube for 10 min. at room temperature. Finally the tube is rapidly washed through with dry methanol and immediately filled with a solution of the enzyme in 0.1M-NEM buffer, pH 8.5.

c. Coupling of the enzyme to activated nylon tube.

Method.

Nylon tube immobilized enzymes are prepared by filling glutaraldehyde or bisimidate activated nylon tube with solutions of the enzymes in the appropriate buffer. Typically nylon tube immobilized lactate dehydrogenase is prepared by filling a 3m. length of glutaraldehyde activated nylon tube with a 1mg/ml. solution of the enzyme in 0.2M-borate buffer, pH 8.5 and storing at $4^{o}C$ for 2 - 3h. The lactate dehydrogenase solution is then washed from the tube with a few ml. of 0.2M-borate buffer, pH 8.5 collected and the volume measured. Since direct measurement of the amount of protein attached to the inner surface of the nylon tube is not practical, the disappearance of lactate dehydrogenase from the coupling solution is measured instead. This is achieved by assaying the enzyme activity contained in pre- and post-coupling solutions. These results are confirmed by measuring the decrease in absorption at 280nm. of the lactate dehydrogenase post-coupling solution ($E^{1\%}_{280nm.} = 14.9$; Hakala et al., 1950).

d. Reduction of the Schiff's base using borohydride.

Materials.

1. 0.1M-Phosphate buffer, pH 7.5: 0.1M-NaH_2PO_4 is titrated to pH 7.5 with 2M-NaOH.
2. Sodium borohydride: Material was obtained from Sigma London Chemical Co.Ltd. and used as a 1% (w/v) solution in 0.1M-phosphate buffer, pH 7.5.

Method.

When nylon tube immobilized enzymes are prepared using glutaraldehyde as the coupling agent, the covalent attachment can be stabilized by reduction of the double bond of the Schiff's base.

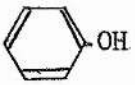
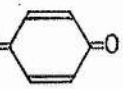
A 1% (w/v) solution of sodium borohydride in 0.1M-phosphate buffer, pH 7.5 is perfused through the enzyme tube until the yellow colouration, formed during the glutaraldehyde coupling step, disappears. At this point it is assumed that the reduction is complete.

2.6 Variation of time and temperature of the O-alkylation step.

The effect of time and temperature on the O-alkylation incubation step is demonstrated by preparing a series of immobilized enzyme tubes from nylon tube which has been O-alkylated at temperatures between 0 - 35°C for different incubation times. The activity of the enzyme tubes are assessed by incorporating them into a standard Technicon continuous-flow system where they are perfused with substrate at a fixed flow rate and the amount of product formed is measured. At low percentage conversion this is proportional to the enzymic activity of the enzyme tube. The method is a convenient way of rapidly assessing relative enzyme tube activity although not as good as the recycling method of Ford et al., (1972) which gives specific tube activity (described in section 1.7.2).

In this work, glucose oxidase is used for assessing the effects of time and temperature on the O-alkylation incubation step on subsequent enzyme tube activity. The activity is determined by the method described by Trinder (1969). Glucose oxidase catalyses the oxidation of glucose to yield gluconic acid and hydrogen peroxide. The hydrogen peroxide generated is then allowed to oxidise 4-aminophenazone and phenol in a reaction catalysed by the enzyme peroxidase. A reddish-pink quinone is formed, the amount of which is proportional to the amount of glucose oxidised. The method is summarised by the following equations:

1. D-glucose + O_2 + H_2O $\rightleftharpoons^{GOD}$ D-gluconic acid + H_2O_2

2. H_2O_2 + $CH_3 \cdot C=C \cdot NH_2$ (with $CH_3 \cdot N$ and C=O, N, Ph) +  $\xrightarrow{POD}$ $CH_3 \cdot C=C \cdot N=$  $=O + H_2O$ (with $CH_3 \cdot N$ and C=O, N, Ph)

4-Aminophenazone

Reagents.

1. 0.1M-Phosphate buffer, pH 7.0 : 0.1M-NaH_2PO_4 is titrated to pH 7.0 with 2M-NaOH and contains 0.05% (v/v) Triton-X.
2. Sample wash: 0.15M-NaCl.
3. Colour reagent A: 20mM phenol in 0.1M-phosphate buffer, pH 7.0.
4. Colour reagent B: 5mg.% (w/v) Horse radish peroxidase R.Z. 0.6 (Sigma London Chemical Co.Ltd.), 60mg.% (w/v) 4-aminophenazone (BDH Chemicals Ltd.) dissolved in 0.1M-phosphate buffer, pH 7.0.
5. Glucose standards: 0 - 28mM glucose solutions in saturated benzoic acid.
6. Coupling solution: 1mg/ml. glucose oxidase in 0.2M-borate buffer, pH 8.5 (section 2.5a). The enzyme was obtained from Boehringer Mannheim GmbH as a lyophilised powder.

Method.

Two series of glucose oxidase tubes are prepared via the diaminoethane/glutaraldehyde linkage (sections 2.4 and 2.5a). In one series the time of TOTFB alkylation is varied and in the other the temperature of TOTFB alkylation is varied (section 2.3). The amount of protein bound in each case is measured spectrophotometrically by reading the decrease in the absorption of the coupling solutions at 280nm. (Gibson et al., 1964). These results are then compared with the corresponding activities of the nylon tube immobilized enzymes which are assayed using an automated method of Trinder (1969). The flow system is illustrated in Fig.11.

The glucose standards are sampled at a rate of 60/h. with a 2:1 sample:wash ratio, diluted by an air-segmented stream of buffer and passed through a 25cm. length of the glucose oxidase tube. Peroxidase is then added to the effluent stream where it catalyses the transfer of oxygen from the hydrogen peroxide formed to 4-aminophenazone and phenol, producing a reddish-pink coloured compound. After debubbling the reagent stream, the absorption of this compound is measured at 515nm. and recorded as a series of peaks which are proportional to the glucose concentrations.

The flow rates of pump tubes on the manifold are as follows:

Line	Flow rate ml/min.
1. Sample	0.1ml.
2. Buffer	1.4ml.
3. Air	1.0ml.
4. Reagent A	0.42ml.
5. Reagent B	0.42ml.
6. Sample wash	2.0ml.
7. Waste	1.6ml.

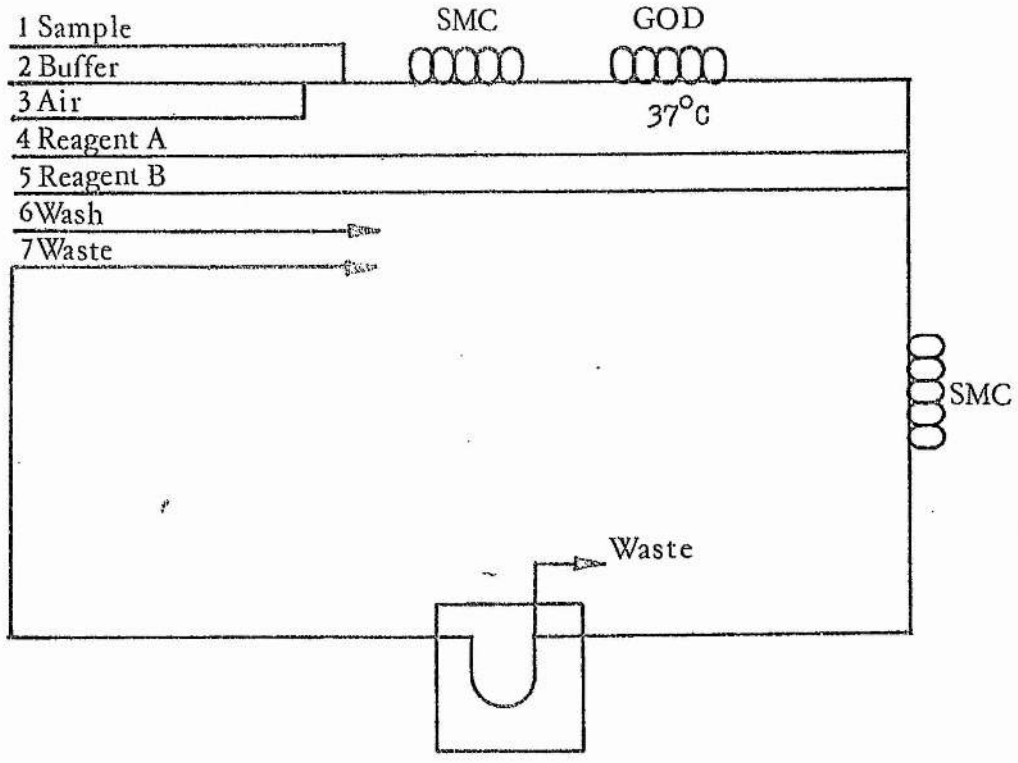


Fig. 11. Flow diagram of the analyser system used to assay nylon tube immobilized glucose oxidase derivatives by the method of Trinder (1969). (GOD=glucose oxidase tube; SMC=single mixing coil.)

2.7 Measurement of enzyme activity

2.7.1 Measurement of the activity of soluble enzymes.

a. Alcohol dehydrogenase.

Reagents.

1. 0.1M-Pyrophosphate buffer, pH 9.0 : 0.1M-$Na_2P_2O_7$, 0.1M-semicarbazide is titrated to pH 9.0 with 2M-HCl.
2. Ethanol.
3. Nicotinamide-adenine dinucleotide: 80mM-NAD^+ solution in water is prepared and stored at 4°C. Grade III NAD^+ was obtained from Sigma London Chemical Co.Ltd.
4. Enzymes: Horse liver alcohol dehydrogenase and yeast alcohol dehydrogenase were obtained from Sigma London Chemical Co.Ltd. as lyophilised powders. Solutions of these enzymes in the appropriate coupling buffer are stored at 4°C for a maximum of 24h.

Method

The activity of alcohol dehydrogenase (ADH) is determined by measuring the rate of increase in absorption at 340nm. due to the production of NADH in the enzyme-catalysed oxidation of ethanol (Bücher and Redetzki 1951). The acetaldehyde formed in the reaction is effectively removed by condensation with semicarbazide. All assays are performed at 25°C in :

$$\underset{\text{Ethanol}}{C_2H_5OH} + NAD^+ \xrightleftharpoons{ADH} \underset{\text{acetaldehyde}}{CH_3CHO} + NADH + H^+$$

reaction mixtures of the following composition:

	Test	Blank
Buffer / semicarbazide	2.5ml.	2.6ml.
Ethanol	0.1ml.	0.1ml.
NAD^+	0.1ml.	0.1ml.
Enzyme	0.1ml.	-

In each case the reaction is initiated by the addition of the enzyme and the change in absorption is measured relative to the blank solution. One unit of enzyme activity corresponds to the oxidation of 1μmol. of ethanol to acetaldehyde per min. under the above conditions.

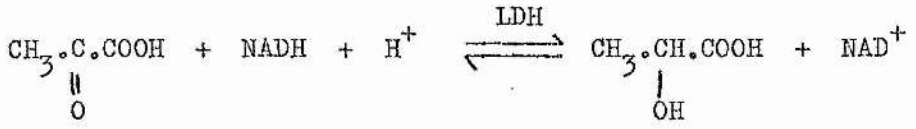
b. Lactic dehydrogenase.

Reagents.

1. 0.1M-Phosphate buffer, pH 7.5 : 0.1M-NaH_2PO_4, 0.15mM-EDTA, 5mM-dithiothreitol is titrated to pH 7.5 with 2M-NaOH.
2. Reduced nicotinamide-adenine dinucleotide: 6mM-NADH solution in 1mM-tris is freshly prepared and stored at 4°C. Grade III disodium-NADH was obtained from Sigma London Chemical Co.Ltd.
3. Sodium pyruvate: Material obtained from Sigma London Chemical Co.Ltd. A 30mM solution in water is prepared and used within 24h.
4. Enzyme: Rabbit muscle lactic dehydrogenase, Type II, was obtained from Sigma London Chemical Co.Ltd. as a lyophilised powder. The enzyme is dissolved in the appropriate coupling buffer and stored at 4°C for a maximum of 24h.

Method.

The activity of lactic dehydrogenase (LDH) is determined by measuring the rate of decrease in absorption at 340nm. due to the depletion of NADH in the enzyme-catalysed reduction of pyruvate (Reeves and Timognari, 1963).



pyruvate lactate

All assays are performed at 25°C in reaction mixtures of the following composition:

Buffer	2.8ml.
NADH	0.1ml.
Pyruvate	0.1ml.
Enzyme	0.01ml.

In each case the reaction is initiated by the addition of the enzyme. One unit of enzyme activity corresponds to the oxidation of 1umol. of NADH to NAD^+ per min. under the above conditions.

c. Aldehyde dehydrogenase.

Reagents.

1. 0.1M-Tris buffer, pH 8.5: 0.1M-Tris is titrated to pH 8.5 with 2M-HCl.
2. Dithiothreitol: Material obtained from Sigma London Chemical Co.Ltd. A 7.5mM solution in water is freshly prepared, stored at 4°C and used within 6h.
3. Nicotinamide-adenine dinucleotide: 15mM-NAD^+ solution in water is prepared and stored at 4°C. Grade III NAD^+ was obtained from Sigma London Chemical Co.Ltd.
4. 0.1M-Phosphate buffer, pH 6.5: 0.1M-KH_2PO_4, 1M-KCl, 2mM-dithiothreitol is titrated to pH 6.5 with 2M-NaOH.
5. Acetaldehyde: 18mM-acetaldehyde solution in water is prepared from freshly distilled acetaldehyde, stored at 0°C and used within 24h.

6. Enzyme: Yeast aldehyde dehydrogenase (AlDH) was a gift from Boehringer Mannheim GmbH. The enzyme preparation was a lyophilised powder containing 1.0mg. of glutathione per mg. of enzyme. Whenever necessary glutathione-free AlDH is prepared by gel filtration of the enzyme on a column (20 x 1 cm. approx.) of Sephadex G10 pre-equilibrated with 0.1M-phosphate buffer, pH 6.5.

Method.

The activity of aldehyde dehydrogenase (AlDH) is determined by measuring the rate of increase in absorption at 340nm. due to the formation of NADH in the enzyme-catalysed oxidation of acetaldehyde (Black, 1951).

$$CH_3CHO + NAD^+ + H_2O \xrightleftharpoons{AlDH} CH_3COOH + NADH + H^+$$

acetaldehyde — acetate

All assays are performed at 25°C in stoppered cells containing reaction mixture of the following composition:

	Test	Blank
Buffer	2.6ml.	2.7ml.
Dithiothreitol	0.1ml.	0.1ml.
NAD^+	0.1ml.	0.1ml.
Enzyme	0.1ml.	0.1ml.
Acetaldehyde	0.1ml.	-

In each case the reaction is initiated by the addition of the acetaldehyde and the change in absorption is measured relative to the blank solution. One unit of enzyme activity corresponds to the oxidation of 1μmol. of acetaldehyde to acetate per min. under the above conditions.

2.7.2 Measurement of the activity of nylon tube immobilized enzymes.

The activity of nylon tube immobilized enzymes can be measured in one of two ways. One method involves the use of an integrated form of the Michaelis-Menten equation in a single-pass technique.

$$FS - K.\ln(1-F) = k_t LQ^{-1}$$

where F = fractional conversion of substrate to product.

S = initial substrate concentration (mM).

K = substrate concentration required to produce half the maximum reaction velocity (mM).

k_t = tube specific activity (μmol/min/cm.)

L = length of tube (cm.)

Q = rate of flow of substrate through tube (cm^3/min.)

A length of nylon tube immobilized enzyme is perfused with a series of substrate solutions of different concentrations at a fixed flow rate and FS is plotted as a function of ln(1-F). The tube specific activity, k_t, is determined by the value of the intercept on the ordinate.

The second method, which is the one adopted in this work, is the recycling method described by Ford et al. (1972). This method of assessing the activity of nylon tube immobilized enzymes is analogous to the soluble enzyme assay methods and is therefore to be preferred. The apparatus, illustrated schematically in Fig.12, comprises a stirred reaction vessel at 25°C, a spectrophotometer with flowcell, a peristaltic pump and a length of nylon tube immobilized enzyme linked together with narrow bore tubing to form a continuous flow circuit. The assay mixture is continuously pumped around the circuit at a rate of 40ml. per min. and changes in absorbance are monitored by the spectrophotometer. The activity of the nylon tube immobilized enzyme is measured from the rate of change in absorption.

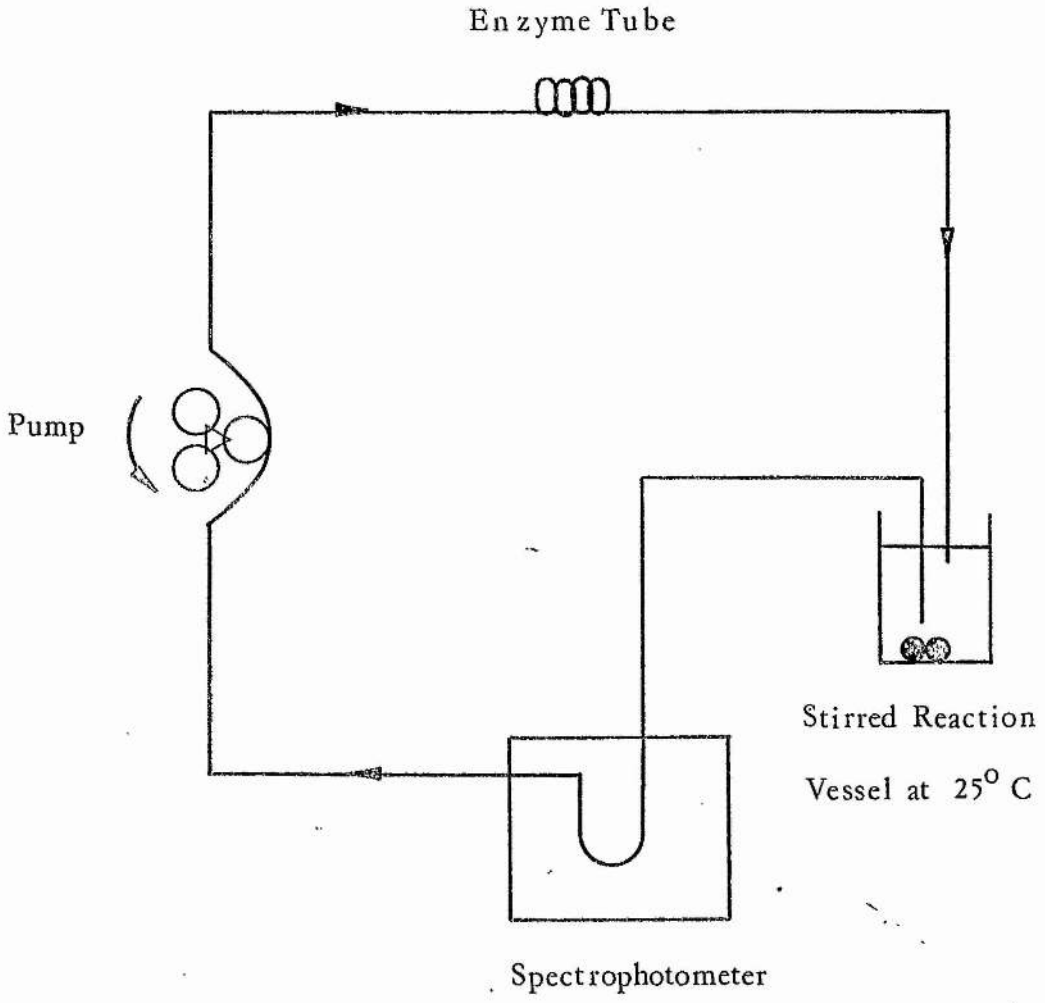


Fig. 12. Diagramatic representation of the recycling method of assaying nylon tube immobilized enzymes (Ford et al.,1972).

Let A = activity of the enzyme derivative (μmol/min/m)

L = length of tube (m).

s = change in substrate concentration across tube (μmol/cm^3).

S = substrate concentration (μmol/cm^3)

Q = rate of flow of substrate through tube (cm^3/min.)

TV = total volume of assay mixture (cm^3).

$-\frac{ds}{dt}$ = observed rate of change of substrate concentration (μmol/min/cm^3)

For a mass balance across the enzyme tube

$$sQ = AL \quad \ldots\ldots\ldots \quad (1)$$

and for a mass balance in the total system

$$-\frac{ds}{dt}(TV) = AL \quad \ldots\ldots\ldots \quad (2)$$

From equations (1) and (2)

$$A = -\frac{ds}{dt}.\left(\frac{TV}{L}\right) \quad \ldots\ldots\ldots \quad (3)$$

An essential condition of this assay is that the fractional substrate conversion per pass through the enzyme tube, s/S, is less than 0.02. If this condition is satisfied, then the enzyme appears to be equally distributed throughout the reaction mixture. For any given length of nylon tube immobilized dehydrogenase, the fractional substrate conversion per pass is calculated according to equation (4).

$$\frac{s}{S} = \frac{AL}{SQ} \quad \ldots\ldots\ldots \quad (4)$$

In practice, the fractional substrate conversion per pass is made less than 0.02 by controlling the length of the enzyme tube and the flow rate in the system. For reactions using or liberating NADH (ie. immobilized dehydrogenases) the activity is measured by monitoring the absorption at 340nm.

In this case, equation (3) becomes:

$$\text{Activity } A = \frac{x}{6.22} \cdot \frac{(TV)}{L} \quad \mu\text{mol/min/m.}$$

where $6.22 \times 10^{-3} M^{-1} cm^{-1}$ is the molar extinction coefficient of NADH at 340nm. (Siegel et al., 1959) and x is the rate of change in absorption/min. at 340nm.

a. Nylon tube immobilized alcohol dehydrogenase.

Reagents.

1. 0.1M-Pyrophosphate buffer, pH9.0 : 0.1M-$Na_2P_2O_7$, 0.1M-semicarbazide is titrated to pH 9.0 with 2M-HCl.
2. Ethanol.
3. Nicotinamide-adenine dinucleotide: 300mM-NAD^+ solution in water is prepared and stored at 4°C. Grade III NAD^+ was obtained from Sigma London Chemical Co.Ltd.
4. Enzyme tube: Length of tube used varies with activity.

Method.

This is basically the same as that used for the soluble enzyme (section 2.7.1a) with concentrations of substrates adjusted for the greater reaction volume. All assays are performed at 25°C in reaction mixtures of the following composition:

Buffer/semicarbazide	9.5ml.
Ethanol	0.4ml.
NAD^+	0.1ml.

In each case the reaction is initiated by the addition of NAD^+ and the increase in absorption at 340nm. is measured. One unit of enzyme activity corresponds to the oxidation of 1umol. of ethanol to acetaldehyde per min. under the above conditions.

b. Nylon tube immobilized lactic dehydrogenase.

Reagents.

1. 0.1M-Phosphate buffer, pH 7.5: 0.1M-NaH_2PO_4, 0.15mM-EDTA, 5mM-dithiothreitol is titrated to pH 7.5 with 2M-NaOH.
2. Reduced nicotinamide-adenine dinucleotide: 10mM-NADH solution in 1mM-tris is freshly prepared and stored at 4°C. Grade III disodium-NADH was obtained from Sigma London Chemical Co. Ltd.
3. Sodium pyruvate: Material obtained from Sigma London Chemical Co.Ltd. A 100mM solution in water is prepared and used within 24h.
4. Enzyme tube: Length of tube used varies with activity.

Method.

This is basically the same as that used for the soluble enzyme (section 2.7.1b) with concentrations of substrates adjusted for the greater reaction volume. All assays are performed at 25°C in reaction mixtures of the following composition:

Buffer	9.7ml.
NADH	0.2ml.
Pyruvate	0.1ml.

In each case the reaction is initiated by the addition of the pyruvate solution and the decrease in absorption at 340nm. is measured. One unit of enzyme activity corresponds to the oxidation of 1μmol. of NADH to NAD^+ per min. under the above conditions.

c. Nylon tube immobilized aldehyde dehydrogenase.

Reagents.

1. 0.1M-Tris buffer, pH 8.5: 0.1M-Tris, 30mM-KCl is titrated to pH 8.5 with 2M-HCl.

2. Dithiothreitol: Material obtained from Sigma London Chemical Co.Ltd. A 25mM solution in water is freshly prepared, stored at 4°C and used within 6h.

3. Nicotinamide-adenine dinucleotide: 50mM-NAD^+ solution in water is prepared and stored at 4°C. Grade III NAD^+ was obtained from Sigma London Chemical Co.Ltd.

4. Acetaldehyde: 60mM-acetaldehyde solution in water is prepared from freshly distilled acetaldehyde, stored at 0°C and used within 24h.

5. Enzyme tube: Length of tube used varies with activity.

Method.

This is basically the same as that used for the soluble enzyme (section 2.7.1c) with concentrations of substrates adjusted for the greater reaction volume. All assays are performed at 25°C in reaction mixtures of the following composition:

Buffer	9.7ml.
Dithiothreitol	0.1ml.
NAD^+	0.1ml.
Acetaldehyde	0.1ml.

In each case the reaction is initiated by the addition of the acetaldehyde and the increase in absorption at 340nm. is measured. One unit of enzyme activity corresponds to the oxidation of 1μmol. of acetaldehyde to acetate per min. under the above conditions.

2.8 Results.

a. Optimisation of the O-alkylation step.

The O-alkylation of nylon tube has been optimised by studying the effect of time and temperature of the alkylation reaction on the activity of nylon tube immobilized glucose oxidase. Glucose oxidase activity has been used as an index of the number of reactive centres generated in the O-alkylation step. In this experiment (section 2.6) GOD-tubes were prepared using diaminoethane as the spacer (section 2.4) and glutaraldehyde as the coupler (section 2.5a). The activity of each enzyme tube, assayed as described in section 2.6, is expressed as a percentage of the maximum tube activity ie. as the relative activity.

Fig. 13 and 14 show that both the amount of protein bound and the relative activity increase with the incubation time of alkylation at 25°C until a point is reached, after 15 min., where the increase in bound protein is small and the relative activity actually decreases. Fig. 15 shows that nylon tubes incubated for short periods have greater specific activities (measured as relative activity/ protein bound) than those incubated for long periods. Tubes which are incubated for periods between 7.5 and 20.0 min. give enzyme derivatives of uniform specific activity.

Fig. 16 and 17 show that both the amount of protein and the relative activity also increase with the incubation temperature of a 15 min. alkylation until a point is reached, at 25°C, where again the relative activity decreases with further increases in bound protein. Fig. 18 shows that tube specific activity increases sharply with temperature of alkylation reaching a maximum at 17.5°C. Above this temperature the tube specific activity falls, then plateaus between 22 - 30°C and finally falls above 30°C.

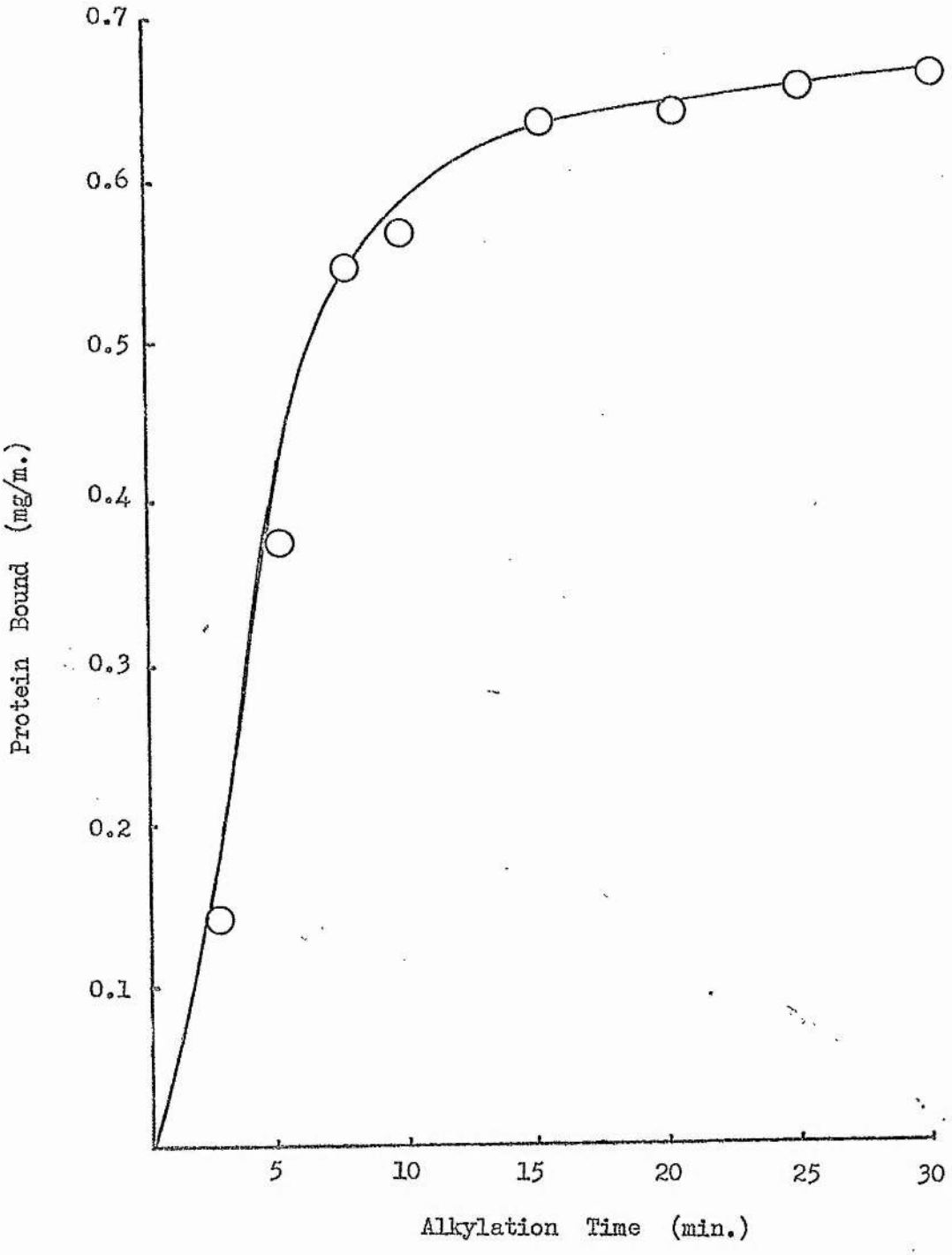


Fig. 13. The effect of alkylation time on the amount of protein bound. Nylon tube was O-alkylated at 25°C for increasing incubation times (section 2.3), then substituted with DAE (section 2.4), activated with glutaraldehyde (section 2.5a) and finally coupled with GOD (section 2.6).

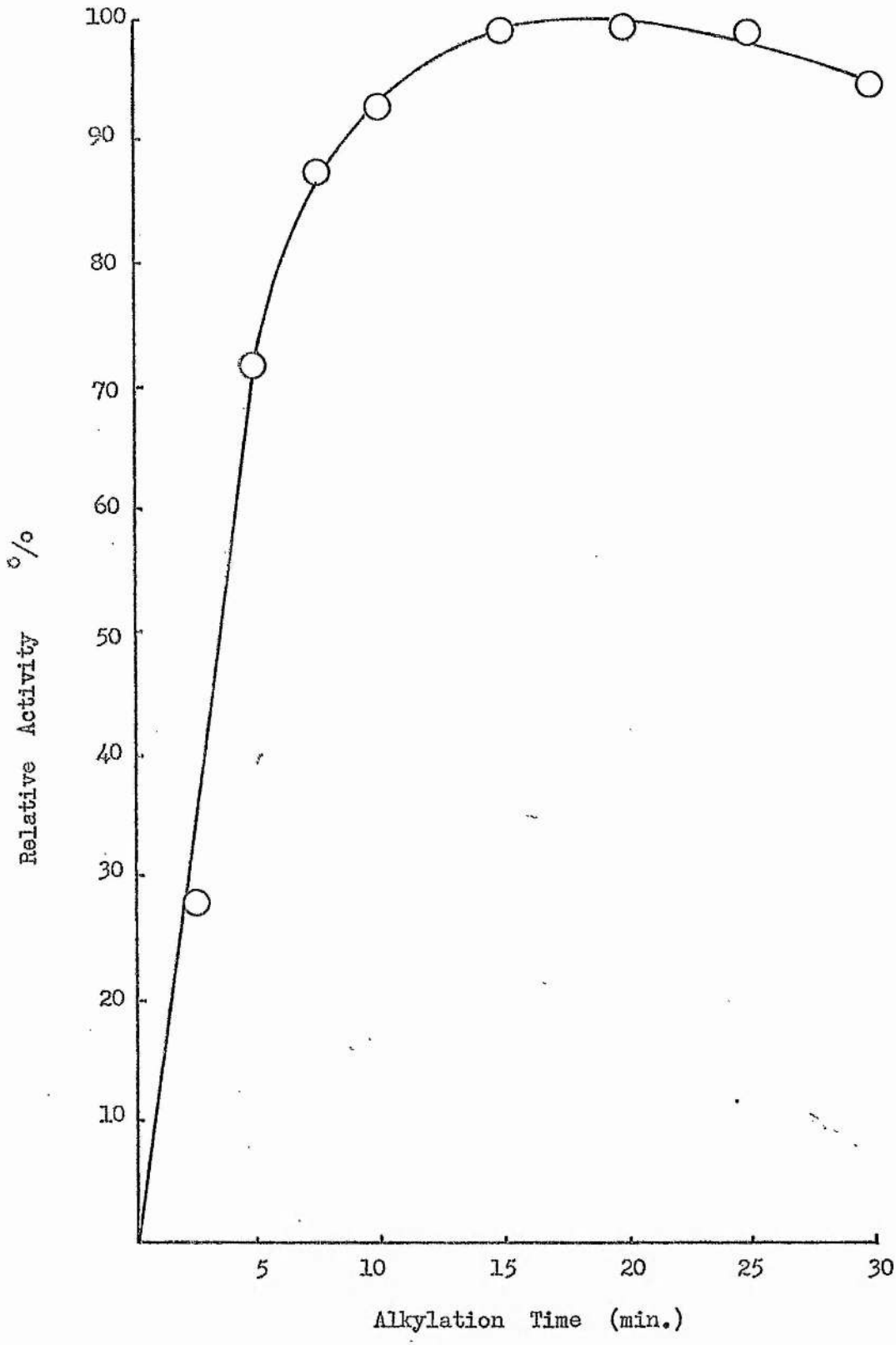


Fig. 14. The effect of alkylation time on the relative activity. Nylon tube was O-alkylated at 25°C for increasing incubation times (section 2.3), then substituted with DAE (section 2.4), activated with glutaraldehyde (section 2.5a) and finally coupled with GOD (section 2.6).

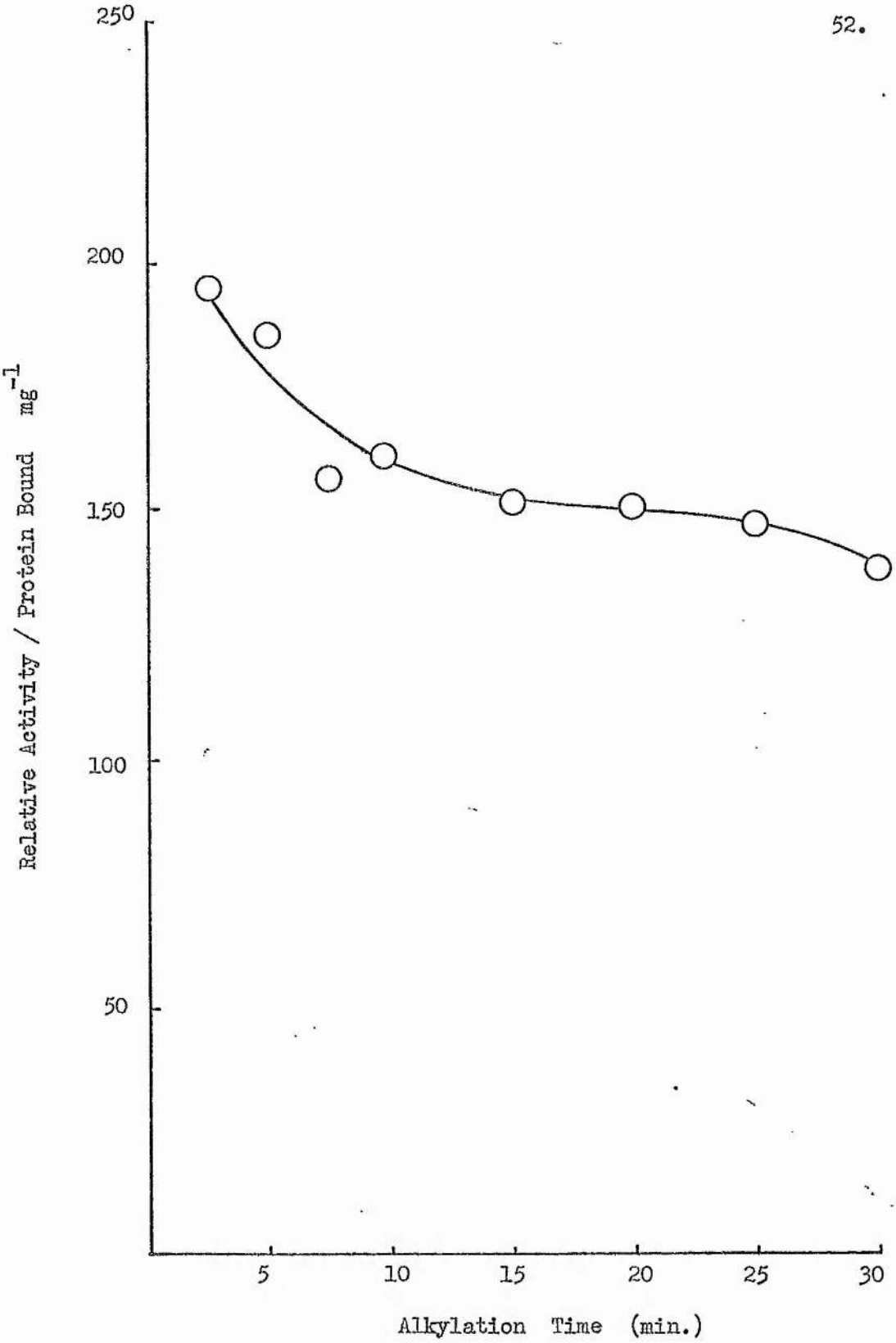


Fig. 15. The effect of alkylation time on the tube specific activity (measured as relative activity/protein bound). Nylon tube was O-alkylated at 25°C for increasing incubation times (section 2.3), then substituted with DAE (section 2.4), activated with glutaraldehyde (section 2.5a) and finally coupled with GOD (section 2.6).

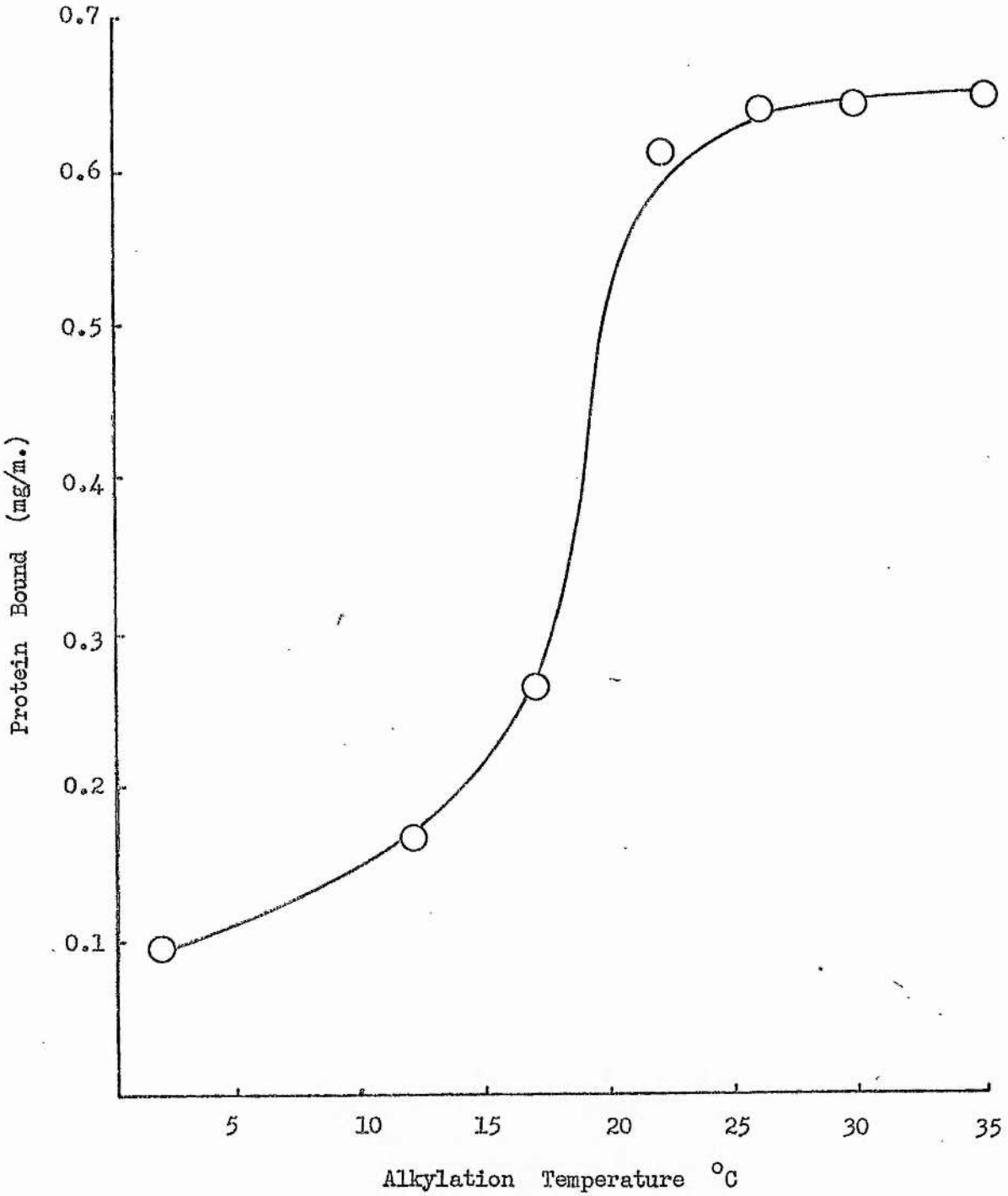


Fig. 16. The effect of alkylation temperature on the amount of protein bound. Nylon tube was O-alkylated for 15min. at increasing temperatures (section 2.3), then substituted with DAE (section 2.4), activated with glutaraldehyde (section 2.5a) and finally coupled with 30D (section 2.6).

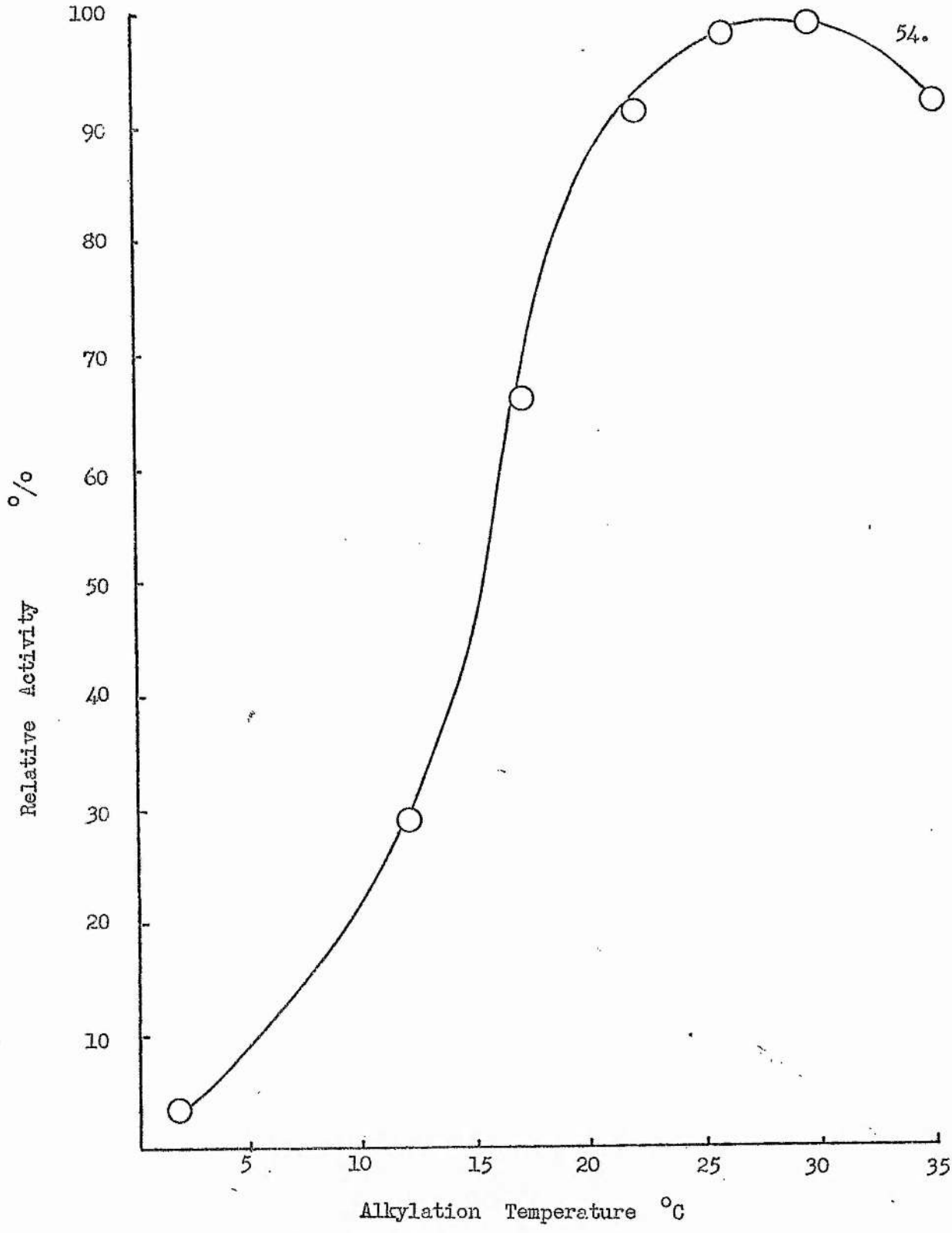


Fig. 17. The effect of alkylation temperature on the relative activity. Nylon tube was O-alkylated for 15min. at increasing temperatures (section 2.3), then substituted with DAE (section 2.4), activated with glutaraldehyde (section 2.5a) and finally coupled with GOD (section 2.6).

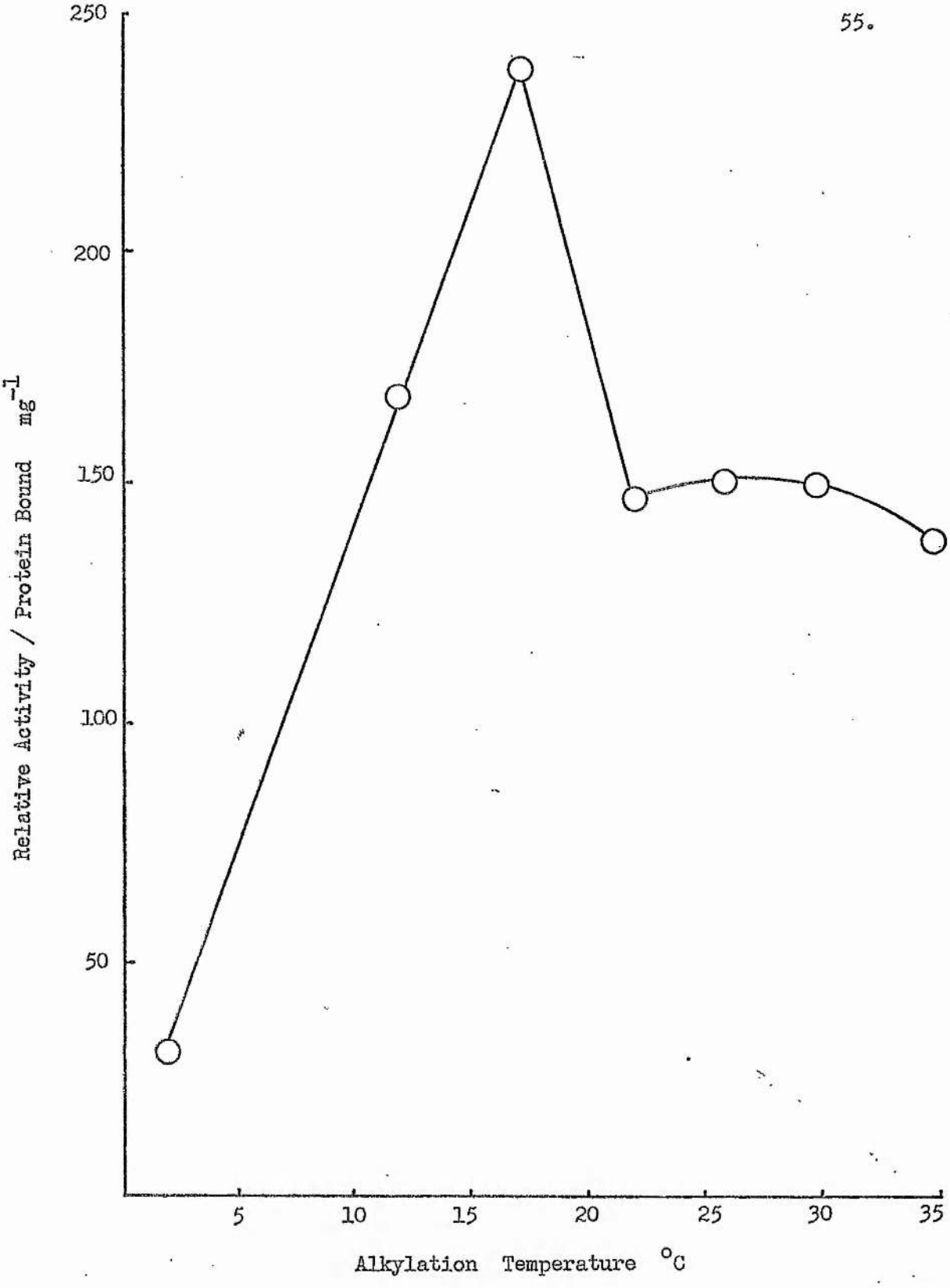


Fig. 18. The effect of alkylation temperature on the tube specific activity (measured as relative activity/protein bound). Nylon tube was O-alkylated for 15min. at increasing temperatures (section 2.3), then substituted with DAE (section 2.4), activated with glutaraldehyde (section 2.5a) and finally coupled with GOD (section 2.6).

The results above have been used to determine the final optimum conditions to be employed in all subsequent work found in this thesis ie. O-alkylation at 25°C for a period of 15 min. (section 2.3)

b. Effect of spacers on the activity of nylon tube immobilized enzymes.

Nylon tube was first O-alkylated (section 2.3), then substituted with a variety of amine and hydrazide spacers (section 2.4), activated with glutaraldehyde (section 2.5a) or bisimidate (section 2.5b) and finally coupled with enzyme (section 2.5c). The effect of these different chemistries on the subsequent activity of nylon tube immobilized enzymes is summarised in Table I.

When LDH is coupled to substituted nylon with glutaraldehyde, the diamine derivatives have a greater activity (U/m.) than the dihydrazide derivatives. Also, the hexamethylene diamine (HMDA) and adipic dihydrazide (AH) derivatives have slightly greater activities than the diaminoethane (DAE) and hydrazine (HH) derivatives respectively (Table II). Again, when LADH is coupled to substituted nylon with glutaraldehyde, the diamine derivatives have greater activities than the dehydrazide derivatives. Such differences are not obvious when bisimidate is used to couple LADH (Table II). Within each group of enzyme derivatives, no great differences are found in the tube specific activities (U/mg. protein bound) although the HH-derivatives have the lowest tube specific activities. (Table III)

Type of derivative	Tube activity U/metre	Protein bound mg/metre	Tube specific activity U/mg.	Percentage* retention of	Tube bore
	A	P	A/P		
HMDA.G.LDH	0.79	0.39	2.03	0.44	1.0
DAE.G.LDH	0.73	0.34	2.15	0.46	1.0
AH.G.LDH	0.57	0.21	2.71	0.51	1.0
HH.G.LDH	0.35	0.21	1.67	0.31	1.0
HMDA.A.LDH	0.29	0.90	0.32	0.07	1.0
DAE.A.LDH	0.26	0.85	0.31	0.06	1.0
DAE.S.LDH	0.27	0.84	0.32	0.06	1.0
HMDA.G.LADH	0.72	0.76	0.95	66	1.5
DAE.G.LADH	0.51	0.61	0.84	58	1.5
AH.G.LADH	0.37	0.42	0.88	61	1.5
HH.G.LADH	0.36	0.43	0.84	58	1.5
HMDA.S.LADH	0.53	0.52	1.02	71	1.5
DAE.S.LADH	0.27	0.31	0.87	60	1.5
AH.S.LADH	0.40	0.37	1.08	75	1.5
HH.S.LADH	0.19	0.25	0.76	53	1.5
HMDA.G.YADH	4.2	1.54	2.73	0.7	1.5
HMDA.A.YADH	4.0	1.44	2.78	0.6	1.5
DAE.G.AlDH	0.08	1.70	0.05	2.5	1.5
DAE.A.AlDH	0.07	1.61	0.04	2.3	1.5

*Percentage retention of activity $= \frac{\text{Tube Specific Activity}}{\text{Specific Activity of Soluble Enzyme}} \times 100$

Table I A summary of the effects of chemically modifying nylon tube on the activity of immobilized enzymes. Nylon tube was alkylated (section 2.3), then substituted with a variety of amine and hydrazide spacers (section 2.4), activated with glutaraldehyde (section 1.5a) or bisimidate (section 2.5b) and finally coupled with enzyme (section 2.5c). All tubes were assayed as described in section 2.7.2

	Activity U/m.		
	glutaraldehyde-LDH	glutaraldehyde-LADH	suberimidate-LADH
HMDA	0.79	0.72	0.53
DAE	0.73	0.51	0.27
AH	0.57	0.37	0.40
HH	0.35	0.36	0.19

Table II. The effect of spacer molecules on the activity of nylon tube immobilized enzymes. Nylon tube was alkylated (section 2.3), then substituted with a variety of amine and hydrazide spacers (section 2.4), activated with glutaraldehyde (section 2.5a) or bisimidate (section 2.5b) and finally coupled with enzyme (section 2.5c). All tubes were assayed as described in section 2.7.2.

	Activity U/m.		
	glutaraldehyde-LDH	glutaraldehyde-LADH	suberimidate-LADH
HMDA	2.03	0.95	1.02
DAE	2.15	0.84	0.87
AH	2.71	0.88	1.08
HH	1.67	0.84	0.76

Table III. The effect of spacer molecules on the specific activity of nylon tube immobilized anzymes., Nylon tube was alkylated (section 2.3), then substituted with a variety of amine and hydrazide spacers (section 2.4), activated with glutaraldehyde (section 2.5a) or bisimidate (section 2.5b) and finally coupled with enzyme (section 2.5c). All tubes were assayed as described in section 2.7.2.

c. Effect of couplers on the activity of nylon tube immobilized enzymes.

Nylon tube immobilized enzymes were prepared as above and the effect of couplers on the subsequent activity of enzyme derivatives was compared. Table IV shows that when LDH, LADH and YADH are attached to diamine-substituted nylon, the glutaraldehyde coupled derivatives have greater activities than the corresponding bisimidate derivatives. This trend is not obvious with dihydrazide-substituted nylon derivatives of LADH which have been coupled with glutaraldehyde and bisimidate. (Table I).

The choice of coupler has a more marked effect on the activity of LDH derivatives because although bisimidate binds more protein, glutaraldehyde gives more active tubes (Table I). This is emphasised by the variation of tube specific activities (Table V). With the alcohol dehydrogenases, differences in the tube specific activities are less pronounced.

2.9 Discussion.

The method of O-alkylation described in this work allows reactive centres to be generated on the nylon surface under controlled conditions. The results suggest that the number of reactive groups generated increases with both time and temperature of the alkylation step. This is reflected in the activity of immobilized enzyme derivatives prepared from nylon tube which has been a) alkylated for different incubation times, b) alkylated at different temperatures. In each case, a point is reached where the activity is optimal, thereafter increased alkylation of the nylon leads only to reduced tube activity with increased bound protein. The higher concentration of bound protein, together with increased disruption of the nylon surface, will undoubtedly cause an increase in the thickness of the diffusion layer which occurs at the interface of

Activity U/m.

	HMDA/LDH	HMDA/LADH	DAE/LDH	DAE/LADH	HMDA/YADH
Glutaraldehyde	0.79	0.72	0.73	0.51	4.2
Adipimidate	0.29	-	0.26	-	4.0
Suberimidate	-	0.53	0.27	0.27	-

Table IV. The effect of coupler molecules on the activity of nylon tube immobilized enzymes. Nylon tube was alkylated (section 2.3), then substituted with a variety of spacers (section 2.4), activated with glutaraldehyde (section 2.5a) or bisimidate (section 2.5b) and finally coupled with enzyme (section 2.5x). All tubes were assayed as described in section 2.7.2.

Tube Specific Activity U/m/mg.

	HMDA/LDH	HMDA/LADH	DAE/LDH	DAE/LADH	HMDA/YADH
Glutaraldehyde	2.03	0.95	2.15	0.84	2.73
Adipimidate	0.32	-	0.31	-	2.78
Suberimidate	-	1.02	0.32	0.87	-

Table V. The effect of coupler molecules on the specific activity of nylon tube immobilized enzymes. Nylon tube was alkylated (section 2.3), then substituted with amine spacers (section 2.4), activated with glutaraldehyde (section 2.5a) or bisimidate (section 2.5b) and finally coupled with enzyme (section 2.5c). All tubes were assayed as described in section 2.7.2.

the nylon-bound enzyme and the reaction mixture (Nernst, 1904). The increased diffusion barrier will in turn restrict the transport of substrate to the active site of the enzyme forming a substrate concentration gradient. The enzyme derivative will appear to be less active because it is diffusion controlled.

The results show that the chemistry chosen for enzyme attachment to nylon tube has an effect on the subsequent activity of that enzyme. By using the appropriate spacers and couplers, nylon tube immobilized enzymes can be tailor-made to suit the individual enzyme. Thus, enzymes not stable in the hydrophobic environment of the nylon backbone can be held away from the nylon surface by means of long spacer molecules eg. this appears to be the case with LDH where the more active amine- and hydrazide-derivatives are found with HMDA and AH respectively (Table II).

One of the basic differences between the two forms of coupling used is that, at normal working pHs, the lysine residues remain protonated with the bisimidates but not with the dialdehyde. This property can be of importance if lysine residues, involved in the linking of the enzyme to the support, are required in the positively charged form in order to retain an active configuration. The requirement of a non-protonated lysine residue could explain the differences found in the tube specific activities of the bisimidate and glutaraldehyde coupled LDH derivatives (Table V).

Low percentage retention of enzyme activity could be due to the nature and strength of binding of the enzyme to the nylon support. Each enzyme molecule will contain more than one lysine residue and therefore the orientation of the molecules and the number of points of attachment will vary. Thus loss of activity

may be due to single-point attachment at or near the active site, or to multi-point attachment causing conformational stress in the enzyme molecule and consequently deactivation. The two most likely candidates for deactivation by conformational changes are LDH and YADH because both have four subunits which are liable to dissociate with any mechanical stress. The percentage retention of activity found with these particular enzymes appears to support this argument (Table I).

3. Factors influencing interaction between samples when using nylon tube immobilised enzymes in continuous flow analysis systems.

3.1 Introduction

A prerequisite for replacing soluble enzymes with their nylon tube immobilized counterparts in any analytical method is the maintenance, or improvement, of the overall performance displayed by the original method. In the automated continuous flow system of Skeggs (1957) a series of samples are transported through the system by means of an air segmented reagent stream which allows successive samples to be processed with little interaction. The extent of this interaction, or carryover, between samples is basically governed by the design of the analytical system, the physical nature of the reagent solutions, the flow rate and the sample rate. When analytical methods are converted to continuous flow, these parameters are adjusted so that the carryover from one sample to the next lies within acceptable limits ie. within allowable limits of error. Tonks (1963) calculated these limits using the following formula:

$$\text{Allowable limits of error (maximum } \pm 10\%) = \frac{\frac{1}{4} \text{ of normal range}}{\text{mean of normal range}} \times 100\%$$

Clearly, if nylon tube immobilized enzymes are to be included in continuous flow systems, their effect on the carryover should be minimal. By studying the carryover obtained with a variety of immobilized enzyme derivatives, the chemistry of preparation can be chosen such that enzyme derivatives fulfil this obligation when used under certain conditions. Changes in carryover, brought about by nylon tube immobilized enzymes, can be assessed by monitoring the passage of substrates through the tube using either (a) a kinetic

method which measures the rate of attainment of steady state values, or (b) a slug-flow method which measures the peak diffusion of discrete samples.

3.2 Assessment of carryover by the kinetic analysis method.

The kinetic analysis method of assessing carryover proposed by Thiers et al., (1967) is based on the premise that the transition between any two steady state values in continuous flow systems follows, to a close approximation, first order kinetics. When samples are introduced into a continuous flow system they are in the form of square wave profiles. During their passage through the analyser these square wave forms are distorted to give characteristic peaks. It is this distortion that leads to sample interaction.

Prolonged aspiration of sample and blank solutions results in the setting-up of two steady states where the monitored values are constant. Since the transition between these two steady states is exponential the concept of a half-wash time, analogous to the half-life of radioisotopes, can be applied to the rise and fall curves. In fact, Thiers et al., found that the rise and fall curves are inverted images of each other (see shaded areas Fig. 19) and therefore it is necessary to consider only one of these transitions in the study of carryover.

In fall curves, after an initial lag phase, the rate of change of apparent substrate concentration, $-ds/dt$, is directly proportional to the difference between the apparent concentration at any given time and the final steady state. This apparent concentration, s, can be expressed as a fraction of the overall concentration difference between steady state values.

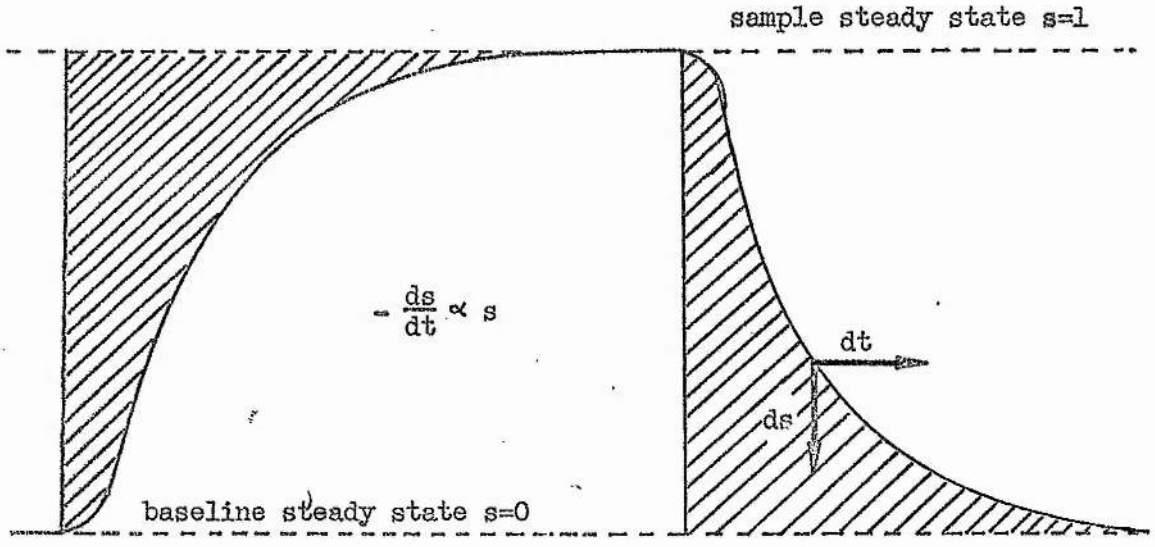


Fig. 19. Transition between two steady states. Diagram of typical rise and fall curves used in the kinetic analysis method of assessing carryover (section 3.2).

Thus

$$-\frac{ds}{dt} = Ks \quad \ldots\ldots \text{ where K is the proportionality constant}$$

Integrating

$$\int -\frac{ds}{s} = \int K.dt$$

gives the following expression;

$$-\ln s = Kt + L \quad \ldots\ldots \quad (1)$$

where L is the constant of integration. Equation 1 can be expressed as

$$-2.3 \log s = Kt + L$$

or

$$-\log s = kt + l$$

where k and l are constants.

A plot of - log s against time from values derived from the chart recording of a transition between two steady state values will give a curve having an initial lag phase followed by a straight line portion (Fig. 20). From this graph the half-wash time, W, can be calculated. This is defined as the time taken for the apparent concentration to fall from one value to half that value. The half-wash time can be used to determine the maximum sampling rate since the percentage interaction can be calculated from the number of half-wash times between samples ie. with x half-wash times there is 50/x per cent interaction.

Reagents.

1. Phosphate buffer, pH 7.4: 0.1M-Na_2HPO_4 is titrated to pH 7.4 with 0.1M-NaH_2PO_4 to give a buffer of ionic strength 0.1 (Datta and Grzybowski, 1961).

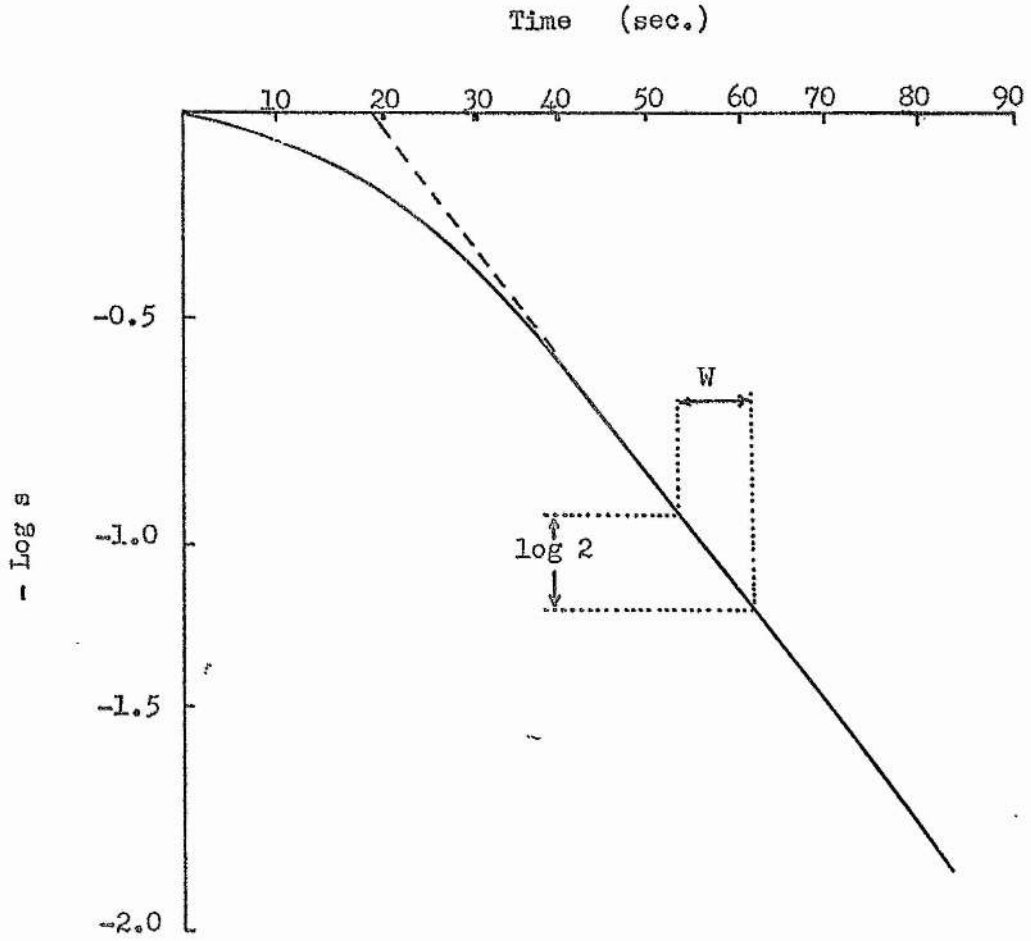


Fig. 20. Transition between two steady states. The fall curve plotted on semilogarithmic coordinates is used to determine the half-wash time, W, in the kinetic analysis method of assessing carryover (section 3.2).

2. Enzyme tube: Rabbit muscle lactate dehydrogenase immobilized on 1mm. bore nylon tube. Nylon tube is alkylated (section 2.3), then substituted with HMDA (section 2.4), activated with glutaraldehyde (section 2.5a) and finally coupled with enzyme (section 2.5c). A 50 cm length is used.
3. Flavin mononucleotide: 0.25 mg/dl. FMN in phosphate buffer, pH 7.4.
4. Riboflavin: 0.25 mg/dl. riboflavin in phosphate buffer, pH 7.4.
5. Reduced nicotinamide-adenine dinucleotide: 10 mg/dl. NADH in phosphate buffer pH 7.4.
6. Nicotinamide-adenine dinucleotide: 1 mg/dl. NAD^+ in phosphate buffer, pH 7.4.
7. Nicotinamide-adenine dinucleotide phosphate: 1 mg/dl. $NADP^+$ in phosphate buffer, pH 7.4.

Compounds 3 - 7 were supplied by Sigma London Chemical Co.Ltd. in their purest forms.

Method.

The flow system is illustrated in Fig.21. Solutions of the compounds 3 - 7 are sampled, diluted with distilled water and passed through the enzyme tube which is incubated at 37°C. The absorption of the effluent is then recorded, after debubbling the air-segmented stream, at wavelengths 340nm. (NADH) and 264nm. (FMN, riboflavin, NAD^+ and $NADP^+$). Each solution is aspirated until the system reaches the sample steady state condition where absorption is constant. At this point the sample probe is transferred to a water blank. The transition between sample steady state and baseline steady state (ie. the fall curve) is then monitored by recording the absorption at a chart drive speed of 10 cm/min. For each compound blank experiments are performed.

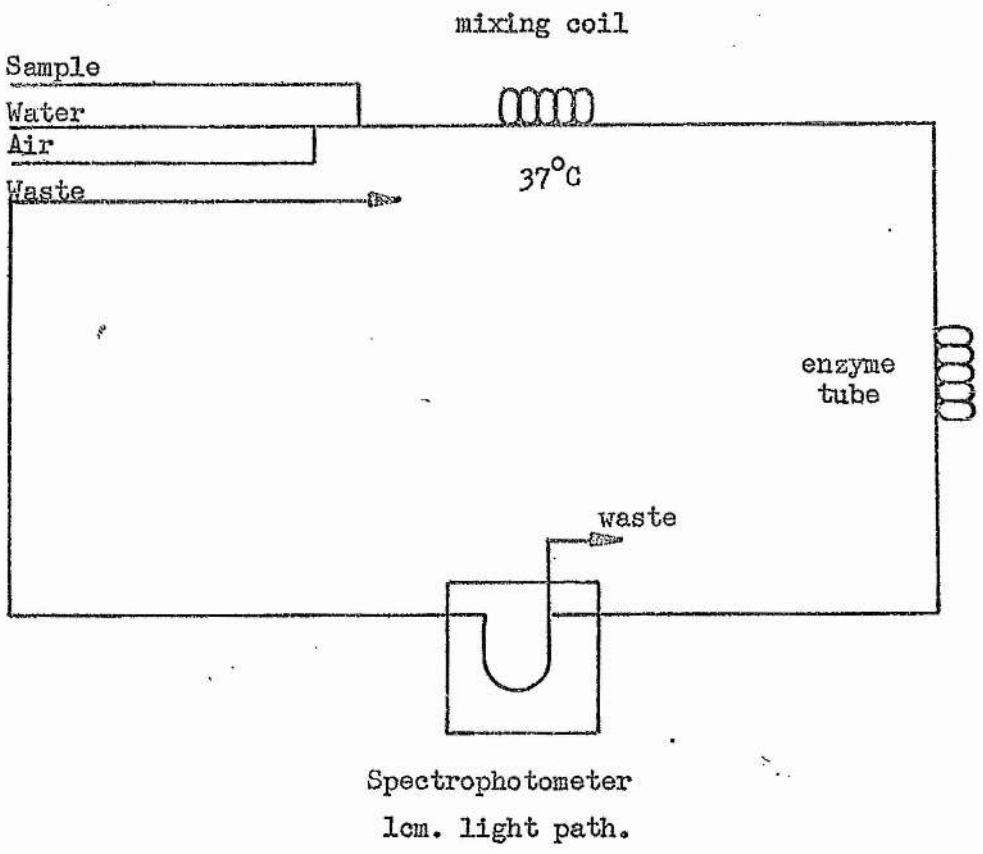


Fig. 21. The flow system used in the kinetic analysis method of assessing carryover.

The flow rates of pump tubing on the manifold are as follows:

Line.	Flow rate ml/min.
1. Sample	0.15
2. Distilled water	1.0
3. Air	0.8
4. Waste	1.0

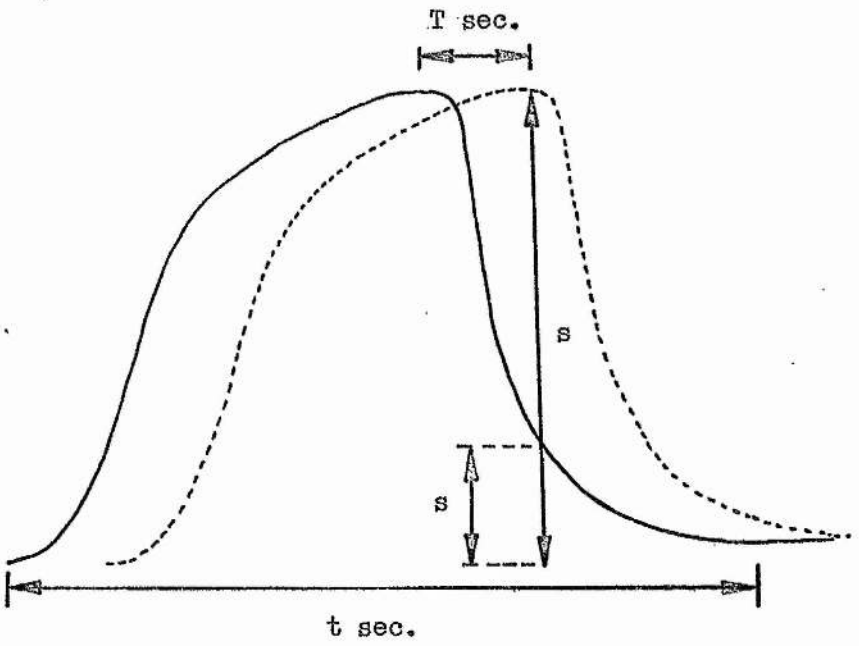
This method is used to determine the effects of alkylation incubation time, length of tube and nature of sample on the carryover in nylon tube immobilized LDH derivatives. The effect of ionic strength is demonstrated by sampling a series of NADH solutions containing 0 - 10 g/l. NaCl.

3.3 Assessment of carryover by the slug-flow analysis method.

The slug-flow analysis method of assessing carryover measures the peak distortion which occurs when samples are introduced into a continuous flow system. By monitoring the complete wave profiles of slug samples padding through the system the degree of interaction between samples can be determined. Broad peak profiles and low percentage sample with time denote increased carryover and reduced maximum sampling rate (Fig. 22). Zero carryover will be seen only when the time interval, T, between sample peaks is greater than, or equal to, half the time, t, taken for the sample to pass completely through the system. If T is less than t/2 then an additive effect is observed with overlapping sample peaks and subsequent peaks are elevated.

Reagents.

1. Buffers: 0.1 ionic strength buffers are prepared from the tables of Datta and Grzybowski (1961).



s = true sample concentration.

Δs = carryover from previous sample.

T = time between samples.

t = duration of sample.

when T t/2

height of second peak = $s + \Delta s$

when T t/2

$\Delta s = 0$

and height of second peak = s

Fig.22. Diagram illustrating the additive effect of overlapping sample peaks.

a. Na_2HPO_4/NaH_2PO_4, pH 6.6

b. Na_2HPO_4/NaH_2PO_4, pH 7.4

c. Na_2HPO_4/NaH_2PO_4, pH 7.9

d. Tris pH 9.0

2. Enzyme tube: Rabbit muscle lactic dehydrogenase immobilized on 1mm. bore nylon tube. Nylon tube is alkylated (section 2.3), then substituted with a variety of spacer molecules (section 2.4), activated with glutaraldehyde (section 2.5a) or bisimidate (section 2.5b) and finally coupled with the enzyme (section 2.5c).

3. Reduced nicotinamide-adenine dinucleotide: 10 mg/dl. NADH solutions in phosphate buffer, pH 7.4 containing a range of NaCl concentrations 0 - 1 g/dl.

Method.

The flow system used is the same as that used in the previous experiment and is illustrated in Fig. 23. In this method the NADH, without NaCl, is sampled for a period of 1 min., diluted with water and then passed through a 50 cm. length of either untreated tube or enzyme tube incubated at 37°C. The absorption is monitored at 340nm. recording from water baseline the rise, fall and return to baseline at a chart speed of 10 cm/min. Carryover is expressed in terms of the percentage sample passed with time.

Factors influencing carryover are determined as follows:

(1) The effect of spacer molecules on carryover is demonstrated by using LDH-tubes which differ only in the spacer molecule used for enzyme attachment. Nylon tube is O-alkylated (section 2.3), then substituted with a variety of spacer molecules (section 2.4), activated with glutaraldehyde (section 2.5a) and finally coupled with LDH (section 2.5c).

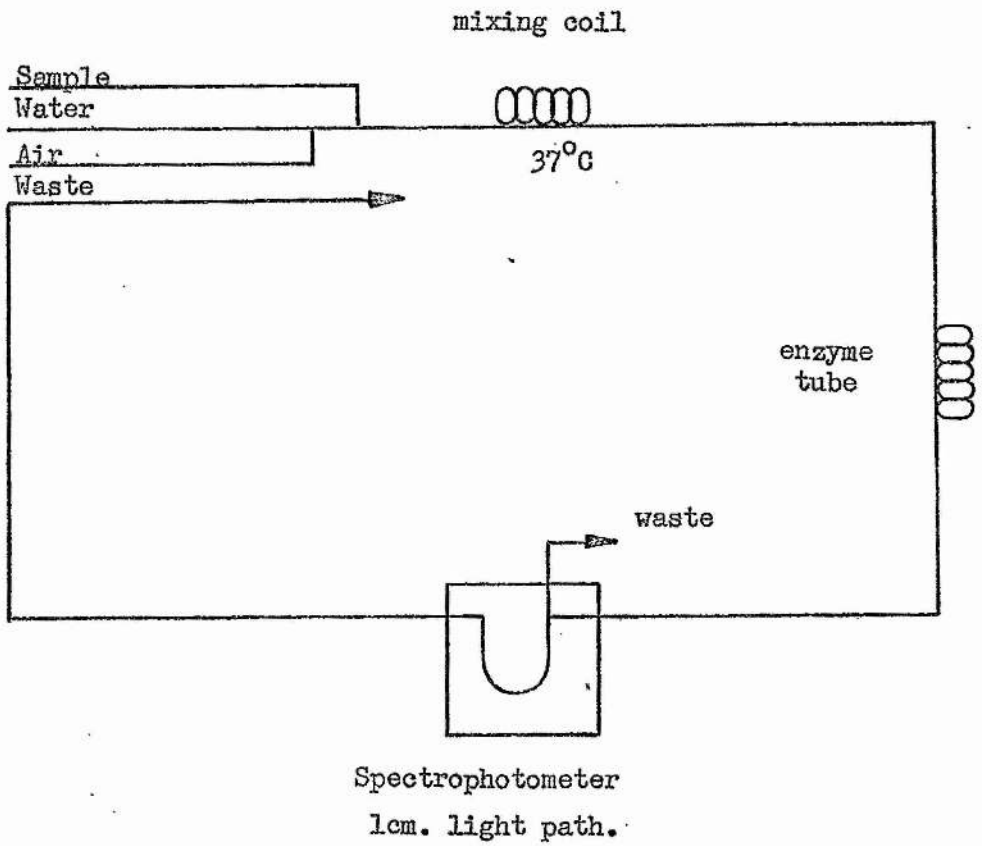


Fig. 23. The flow system used in the slug-flow analysis method of assessing carryover.

(2) The effect of coupler on carryover is demonstrated by using LDH-tubes which differ only in the coupler molecule used to attach the enzyme. Nylon tube is O-alkylated (section 2.3), then substituted with DAE (section 2.4), activated with either glutaraldehyde (section 2.5a) or diethyl adipimidate (section 2.5b) and finally coupled with LDH (section 2.5c).

(3) The effect of ionic strength on carryover is demonstrated by sampling NADH solutions containing increasing amounts of NaCl. These solutions are passed through LDH-tubes which differ only in the spacer molecule used for enzyme attachment. Nylon tube is O-alkylated (section 2.3), then substituted with HMDA or AH (section 2.4), activated with glutaraldehyde (section 2.5a) and finally coupled with LDH (section 2.5c).

(4) The effect of pH on carryover is demonstrated by sampling NADH (without NaCl) and diluting with one of the buffers (a, c, or d). These solutions are passed through LDH-tubes which differ only in the spacer molecule used for enzyme attachment. Nylon tube is O-alkylated (section 2.3), then substituted with HMDA or AH (section 2.4), activated with glutaraldehyde (section 2.5a) and finally coupled with LDH (section 2.5c).

3.4 Results.

3.4.1 Assessment of carryover by the kinetic analysis method.

a) Effect of compound sampled on carryover.

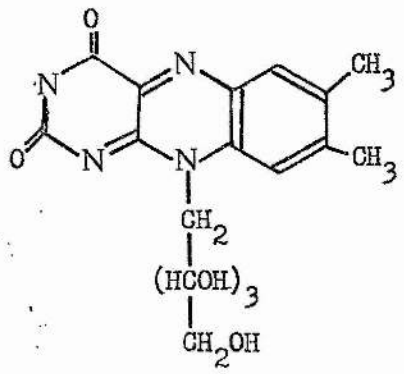
Nylon tube was O-alkylated at 25°C for 15 min. (section 2.3), then substituted with HMDA (section 2.4), activated with glutaraldehyde (section2 .5a) and finally coupled with LDH (section 2.5c).

A 50cm. length of enzyme tube was then inserted into the continuous flow system described in section 2.2. When NAD^+, riboflavin, FMN, $NADP^+$ and NADH samples are introduced into the system they are adsorbed to a greater or lesser extent depending upon their overall charge (see Fig. 24). The more negatively charged molecules FMN (2-), $NADP^+$ (3-) and NADH (2-) are adsorbed more strongly than the less negatively charged NAD^+ (1-) and riboflavin (neutral). The adsorption of sample impedes its passage through the system and leads to longer transition times between steady states and increased carryover. Fig. 25 illustrates the adsorption of NAD^+, riboflavin, FMN, NADH and $NADP^+$ in terms of the percentage attainment of equilibrium, in the transition between two steady states, after a fixed period of 90 sec. (the point at which equilibrium is reached when there is no tube included in the flow circuit).

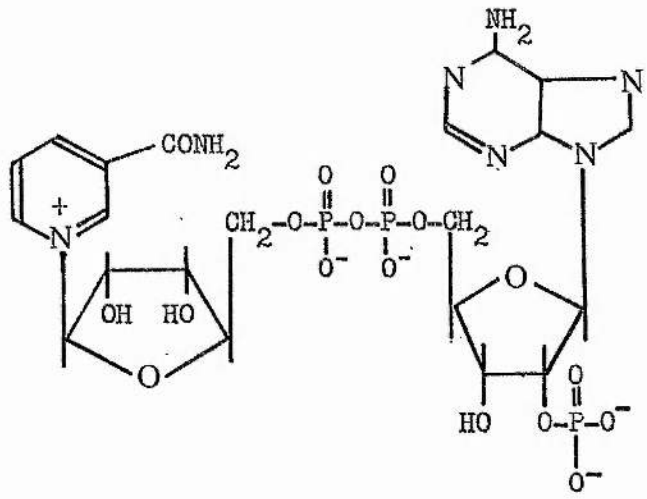
b) Effect of length of tube on carryover.

Nylon tube was O-alkylated at 25°C for 15 min. (section 2.3), then substituted with HMDA (section 2.4), activated with glutaraldehyde (section 2.5a) and finally coupled with LDH (section 2.5c). 10 - 50 cm. lengths of enzyme tube were then inserted into the continuous flow system described in section 3.2. When NAD^+, riboflavin, FMN, NADH and $NADP^+$ samples are introduced into the system, the carryover is seen to increase exponentially with the length of enzyme tube used. This is illustrated in Fig. 25 where carryover is expressed in terms of the percentage attainment of equilibrium, in the transition between two steady states, after a fixed period of 90 sec.

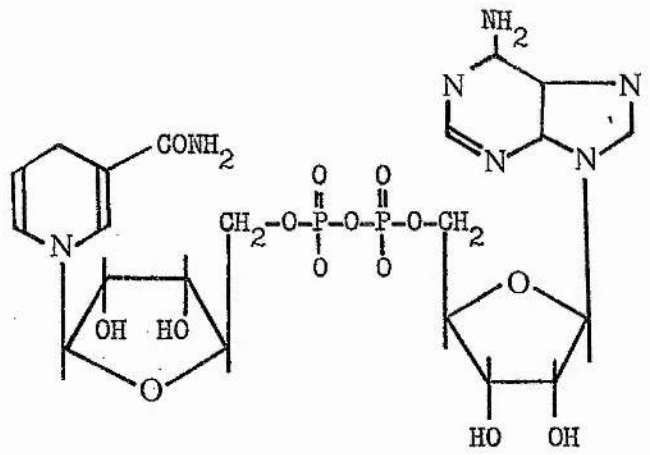
Fig. 26, 27, 28 and 29 show a series of fall curves (-log s against time) obtained when NAD^+, riboflavin, FMN and NADH samples, respectively, are passed through enzyme tubes of increasing length.



Riboflavin (no charge)

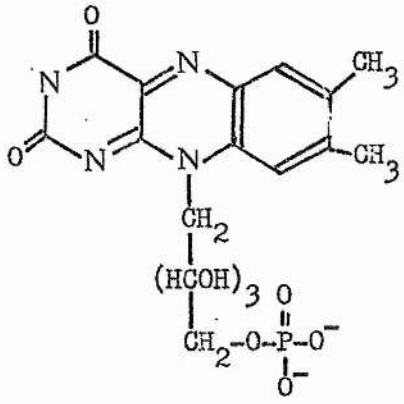


$NADP^+$ (3-)

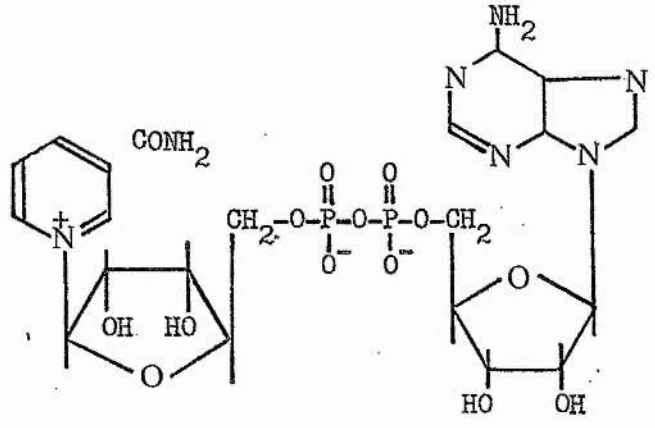


NADH (2-)

Fig. 24. Structures of sample compounds used in carryover studies. (section 3.2).



FMN (2-)



NAD^+ (1-)

Fig. 24. continued.

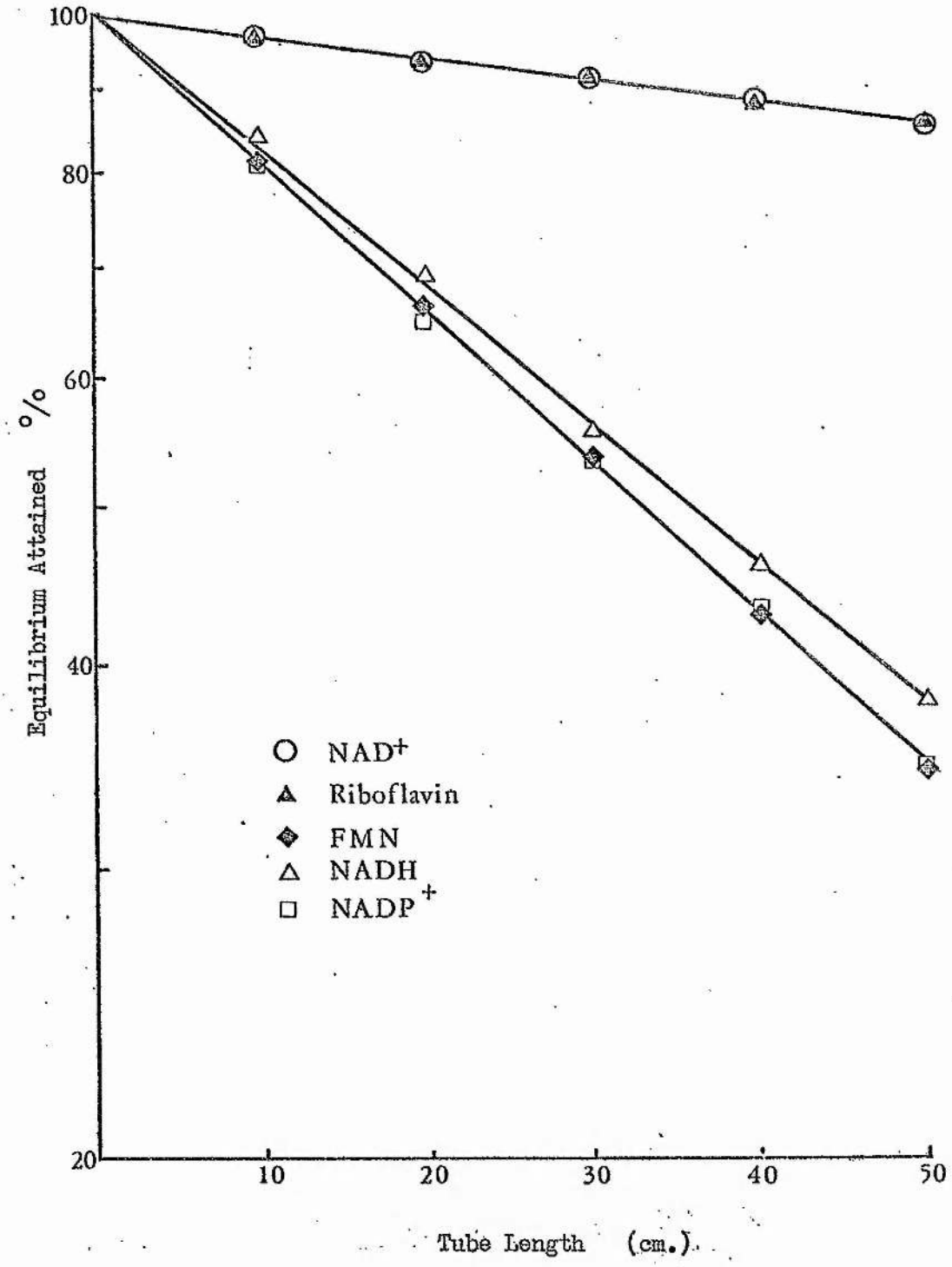


Fig. 25. Adsorption of NAD^+, riboflavin, FMN, NADH and $NADP^+$ in terms of enzyme tube length and the percentage attainment of equilibrium, in the transition between two steady states, after a fixed period of 90 sec. (sections 3.4.1a &3.4.1b).

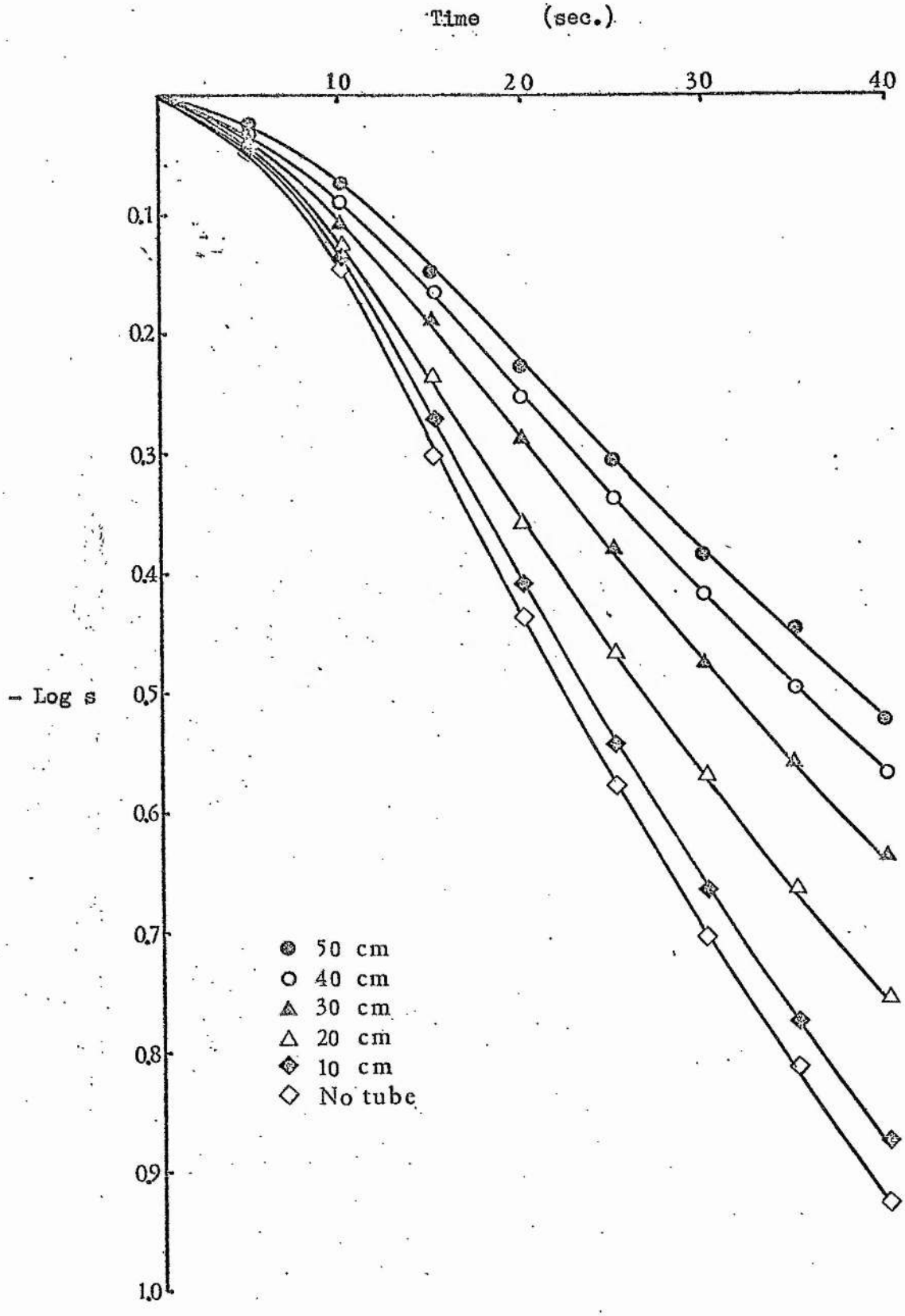


Fig. 26. Fall curves obtained when NAD^+ is passed through enzyme tubes of increasing length (section 3.4.1b). Plotted as - log s against time where s = fractional concentration (section 3.1).

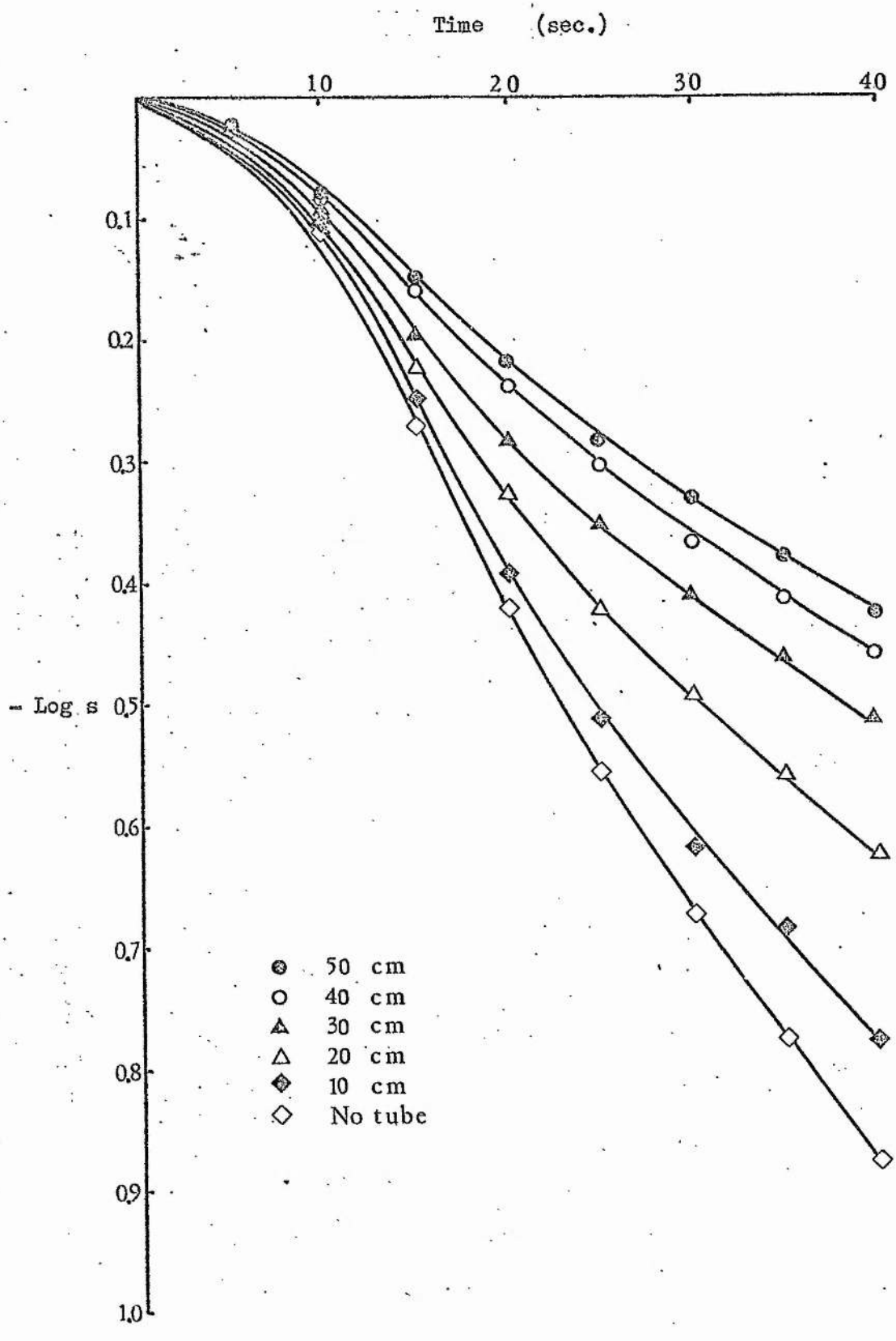


Fig. 27. Fall curves obtained when riboflavin is passed through enzyme tubes of increasing length (section 3.4.1b). Plotted as -log s against time, where s = fractional concentration (section 3.1).

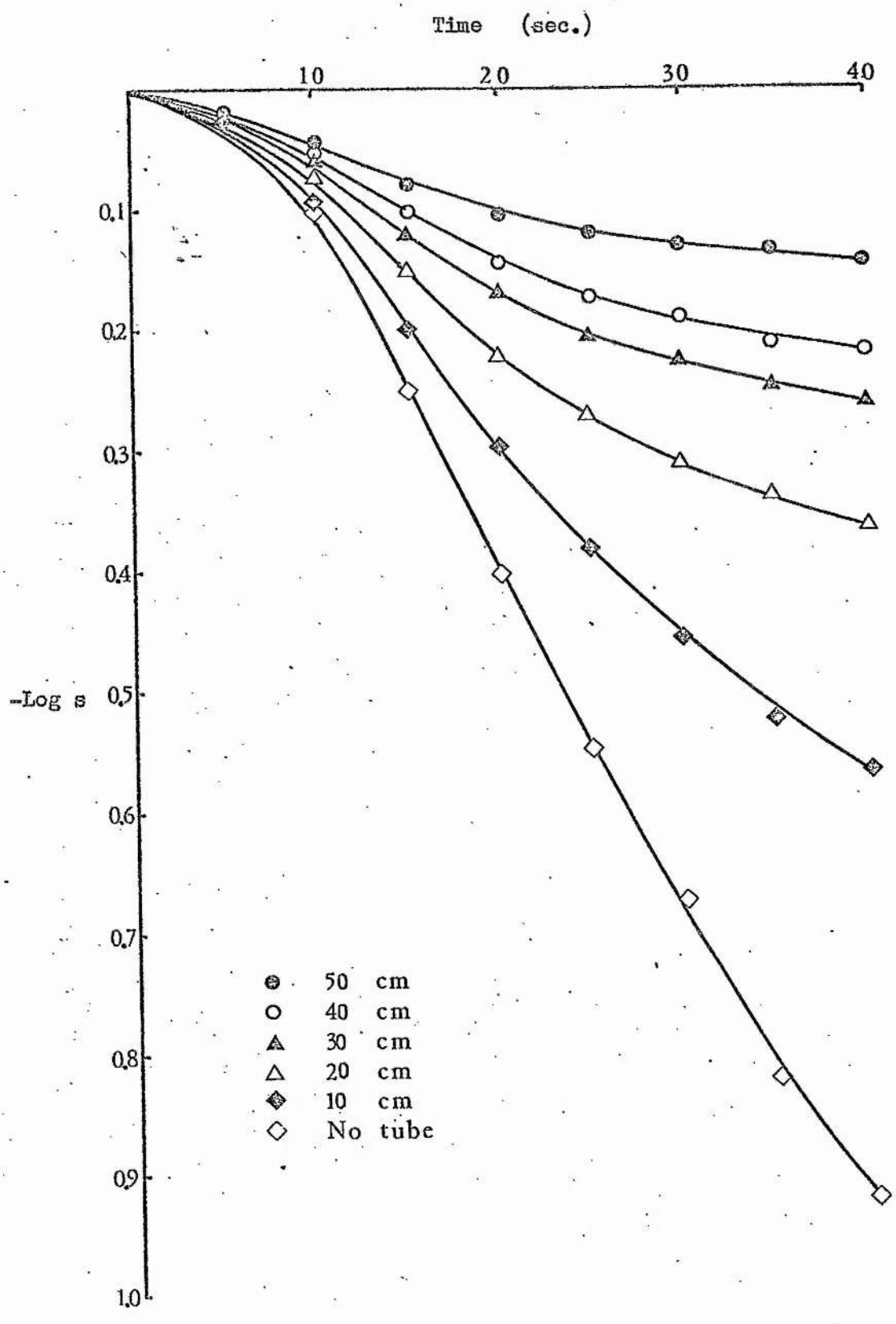


Fig. 28. Fall curves obtained when FMN is passed through enzyme tubes of increasing length (section 3.4.1b). Plotted as -log s against time, where s = fractional concentration (section 3.1).

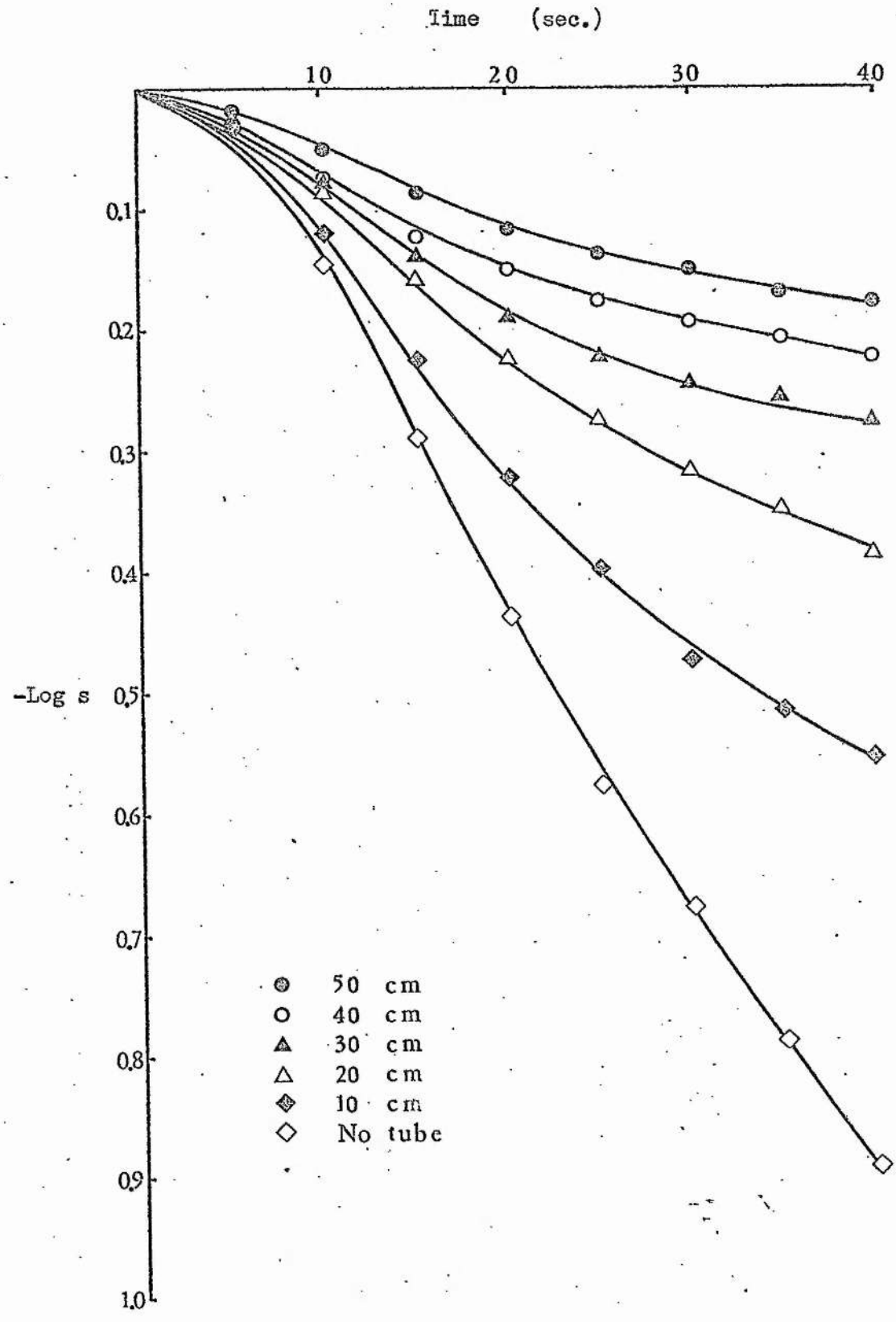


Fig. 29. Fall curves obtained when NADH is passed through enzyme tubes of increasing length (section 3.4.1b). Plotted as -log s against time, where s = fractional concentration (section 3.1).

The W values obtained from these graphs, presented in Table VI, show not only an increase with length of enzyme tube but also confirm the previous finding that molecules having two or more negative charges give poor carryover characteristics eg. a 50 cm, length of enzyme tube gives W = 19.3 sec. with NAD^+(1-) and W = 39.3 sec. with NADH (2-).

c) Effect of alkylation incubation time on carryover.

Nylon tube was O-alkylated at 25°C for increasing incubation times (section 2.3), then substituted with HMDA (section 2.4), activated with glutaraldehyde (section 2.5a) and finally coupled with LDH (section 2.5c). When 50 cm. lengths of these tubes are inserted into the continuous flow system described in section 3.2, an increase in sample carryover is observed with increased alkylation time. This is illustrated in Fig. 30 where carryover expressed as the percentage attainment of equilibrium after a fixed period of 90 sec. is plotted against time of alkylation. Again the effect is more marked with the more negatively charged molecules (NADH and $NADP^+$). Carryover increases sharply with increasing alkylation time until a maximum is reached after 15 min. (ie. the optimum alkylation time - section 2.8a).

The effect of passing (the uncharged) riboflavin through 1m. lengths of non-alkylated tube and 15 min. alkylated LDH-tube is shown in Fig. 31 where the respective fall curves are plotted (-log s against time). From the straight portion of the curves the half-wash time, W, has been calculated as 11 sec. for the non-alkylated tube and 48 sec. for the alkylated tube. This four-fold increase in carryover is not electrostatic in nature but more probably due to impaired laminar flow through the tube.

Sample	Tube length (cm.)					
	0	10	20	30	40	50
NAD^+	10.4	11.0	13.0	16.3	18.0	19.3
Riboflavin	10.3	11.0	13.0	16,5	20.3	22.0
FMN	10.1	11.3	20.3	23.3	32.3	44.4
NADH	10.3	14.0	20.0	25.3	30.6	39.3

Table VI The effect of enzyme tube length and sample compound on the half-wash time (W sec.) calculated from the fall curves illustrated in Fig. 26, 27, 28 and 29 (section 3.4.1b).

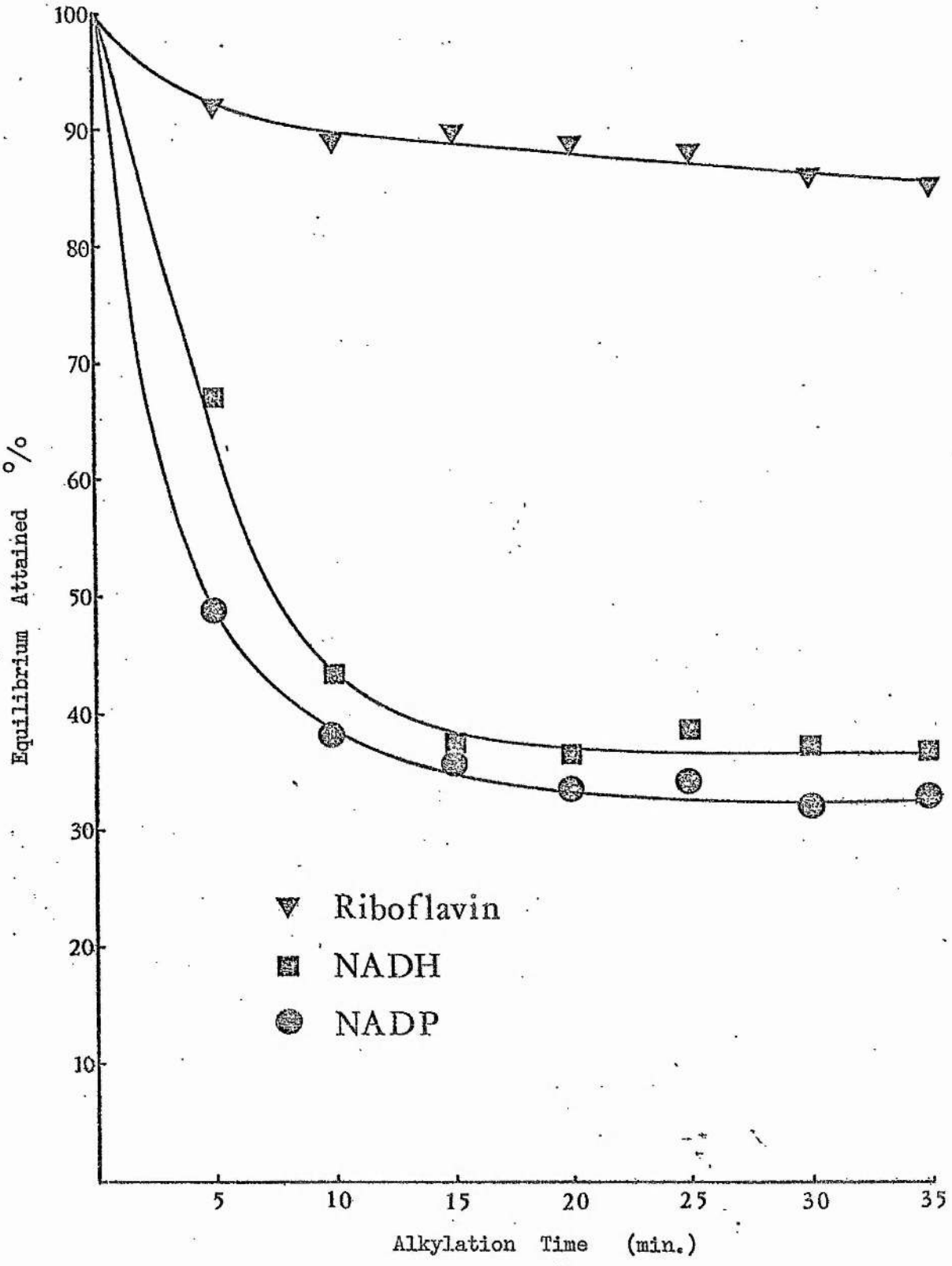


Fig. 30. Adsorption of riboflavin, NADH and $NADP^+$ on 50 cm. lengths of enzyme tube prepared from nylon tube alkylated for increasing incubation times (section 3.4.1c). Adsorption is expressed as the percentage attainment of equilibrium, in the transition between two steady states, after a fixed period of 90 sec.

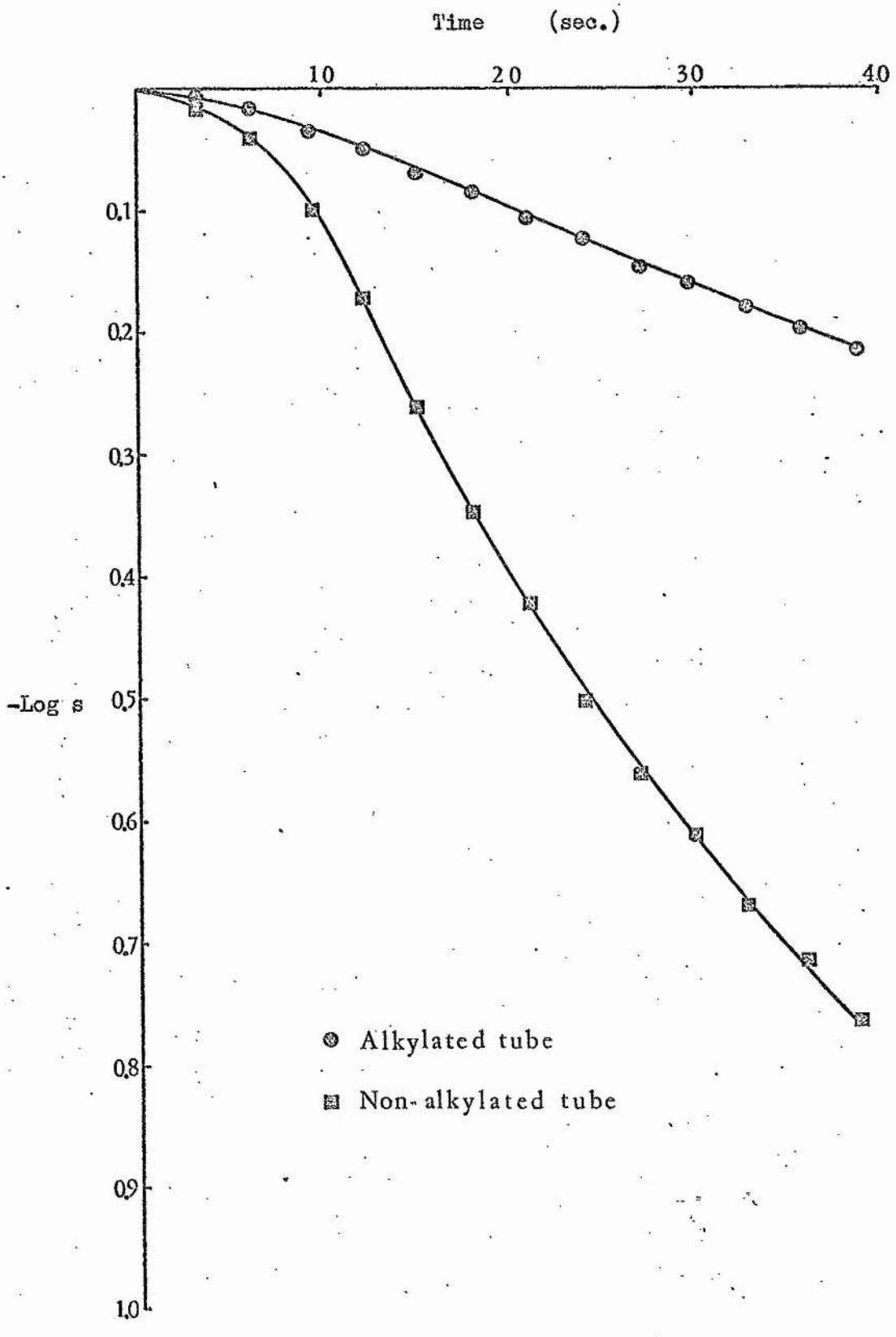


Fig. 31. The fall curves obtained when riboflavin is passed through 1m. lengths of non-alkylated tube and 15 min. alkylated LDH-tube (section 3.4.1c). Plotted as -log s against time, where s = fractional concentration.

d) Effect of ionic strength on carryover.

Nylon tube was O-alkylated at 25°C for 15 min. (section 2.3), then substituted with HMDA (section 2.4), activated with glutaraldehyde (section 2.5a) and finally coupled with LDH (section 2.5c). A 1m. length of LDH tube was then inserted into the continuous flow system described in section 3.2. When NADH solutions containing 0 - 10 g/l NaCl are introduced into the system the carryover is observed to fall with increasing salt concentration. This is illustrated in Fig. 32 where the fall curves are plotted (-log s against time). The half-wash time, W, for each fall curve has been calculated and is presented in Table VII. Results show that the addition of NaCl has a marked effect on carryover such that with increasing NaCl concentration fall curves tend towards that given when the NADH sample is passed through a non-alkylated tube. Again carryover is seen as a charge effect.

3.4.2 Assessment of carryover by the slug-flow analysis method.

a) The effect of spacer molecules on carryover.

Nylon tube was O-alkylated at 25°C for 15 min. (section 2.3), then substituted with a variety of spacer molecules (section 2.4), activated with glutaraldehyde (section 2.5a) and finally coupled with LDH (section 2.5c). 50 cm. lengths of these tubes were then inserted into the continuous flow system used in section 3.3. When 1 min. NADH samples at pH 7.4 are introduced into the system more carryover is observed with enzyme tubes prepared from amine-substituted nylon than those prepared from hydrazide-substituted nylon. This is illustrated in Fig.33 where the percentage sample passed has been plotted against time. No difference in carryover is observed between the two members of each group of LDH derivatives (ie. DAE and HMDA derivatives and HH and AH derivatives have identical carryover characteristics at pH 7.4).

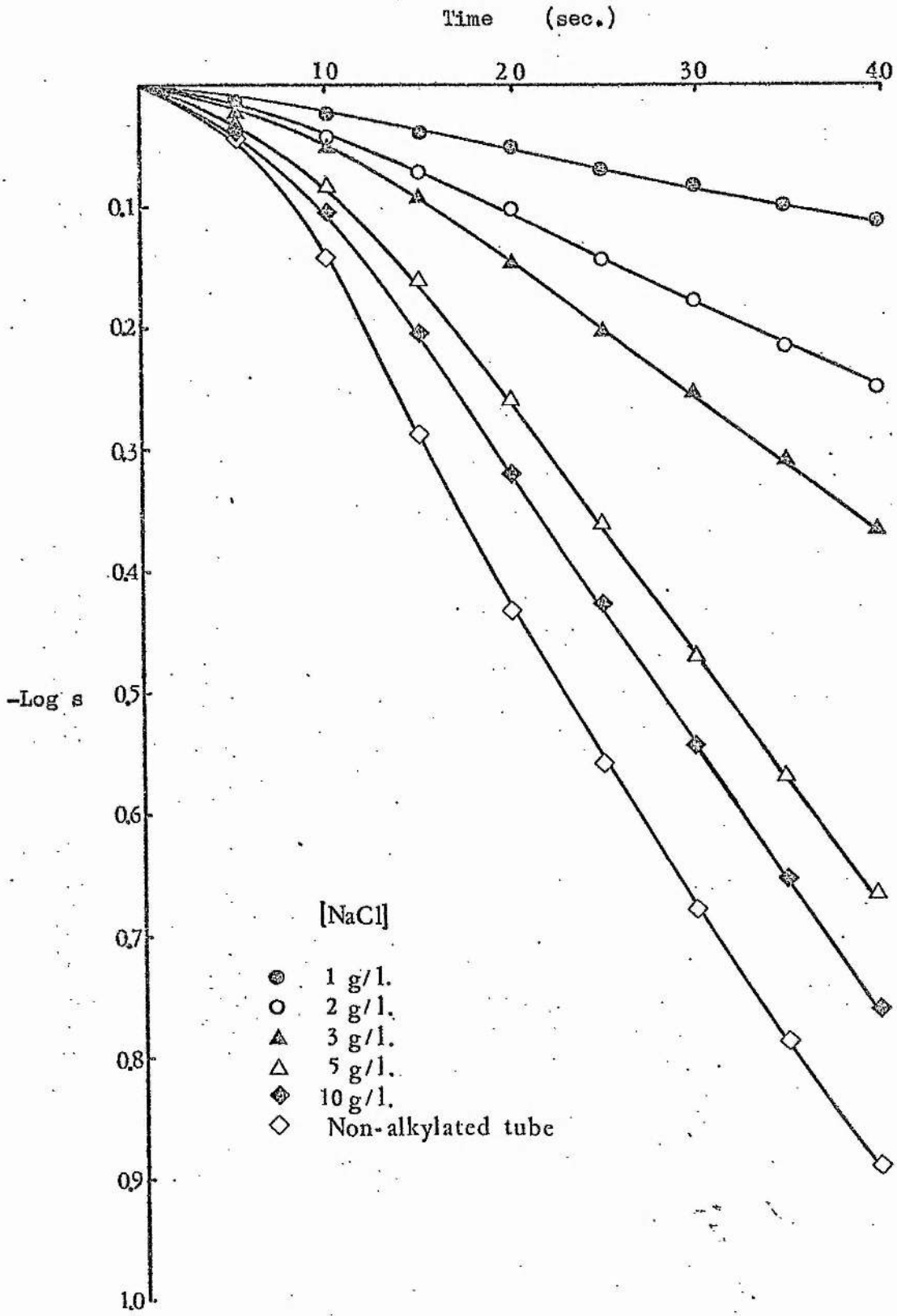


Fig. 32. The fall curves obtained when NADH solutions containing 0 - 10 g/l NaCl are passed through a 1m. length of enzyme tube (section 3.4.1d). Plotted as -log s against time, where s = fractional concentration.

	NaCl g/l.	W sec.
LDH tube	1	46.0
LDH tube	2	39.5
LDH tube	3	27.0
LDH tube	5	15.0
LDH tube	10	12.5
Non-alkylated tube	-	10.3

Table VII The effect of salt concentration on the half-wash time, W, calculated from the fall curves illustrated in Fig. 32 (section 3.4.1d).

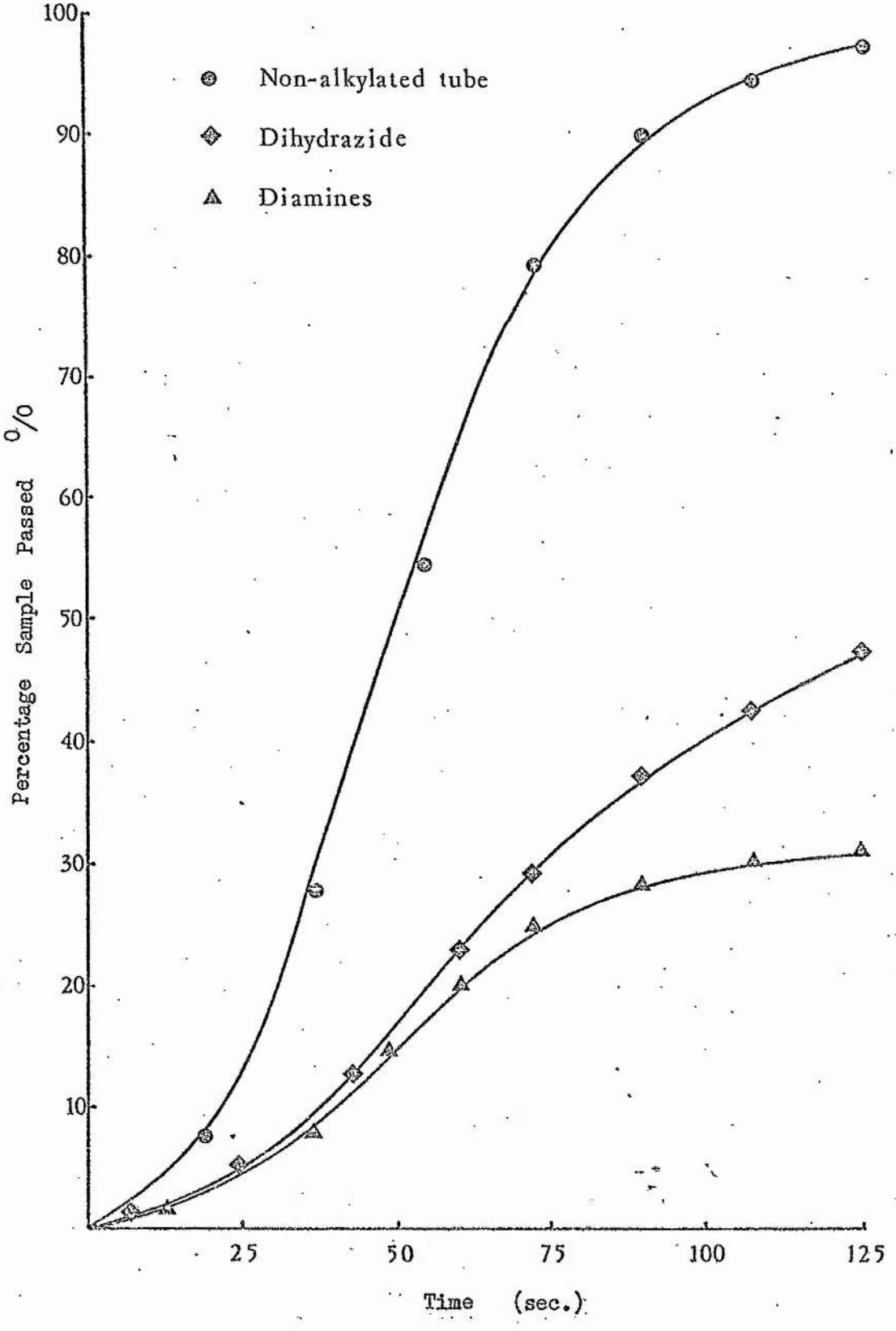


Fig. 33. Variation of carryover with spacer molecule when NADH samples at pH 7.4 are introduced into the continuous flow system (section 3.4.2a). Carryover is expressed as the percentage sample passed with time (section 3.2).

b) The effect of coupler molecules on carryover.

Nylon tube was O-alkylated at 25°C for 15 min. (section 2.3), then substituted with DAE (section 2.4), activated with either glutaraldehyde (section 2.5a) or diethyl adipimidate (section 2.5b) and finally coupled with LDH (section 2.5c). 50 cm. lengths of these two derivatives were then inserted into the continuous flow system used in section 3.3. When 1 min. NADH samples at pH 7.4 are introduced into the system no significant difference in carryover is observed between the two LDH derivatives. This is illustrated in Fig. 34 where carryover expressed as percentage sample passed is plotted against time.

c) The effect of ionic strength on carryover.

Nylon tube was O-alkylated at 25°C for 15 min. (section 2.3), then substituted with HMDA or AH (section 2.4), activated with glutaraldehyde (section 2.5a) and finally coupled with LDH (section 2.5c). 50 cm. lengths of these derivatives were then inserted into the continuous flow system used in section 3.3. When a series of 1 min. NADH samples containing NaCl are introduced into the system the carryover decreases with increasing NaCl concentration. This is illustrated in Fig. 35 for the AH derivative and Fig. 36 for the HMDA derivative where carryover expressed as percentage sample passed is plotted against time.

In each case, as the NaCl concentration increases the curves approach that given when the sample is passed through non-alkylated nylon tube. However, the effect is more pronounced with the AH derivative. Whereas the AH derivative gives minimum possible carryover (maximum percentage sample passed) when 4g/l NaCl is included in the NADH sample, the HMDA derivative does not achieve minimum carryover with 10g/l NaCl in the NADH sample (Table VIII). These results imply that at pH 7.4 the two derivatives are not charged to the same extent.

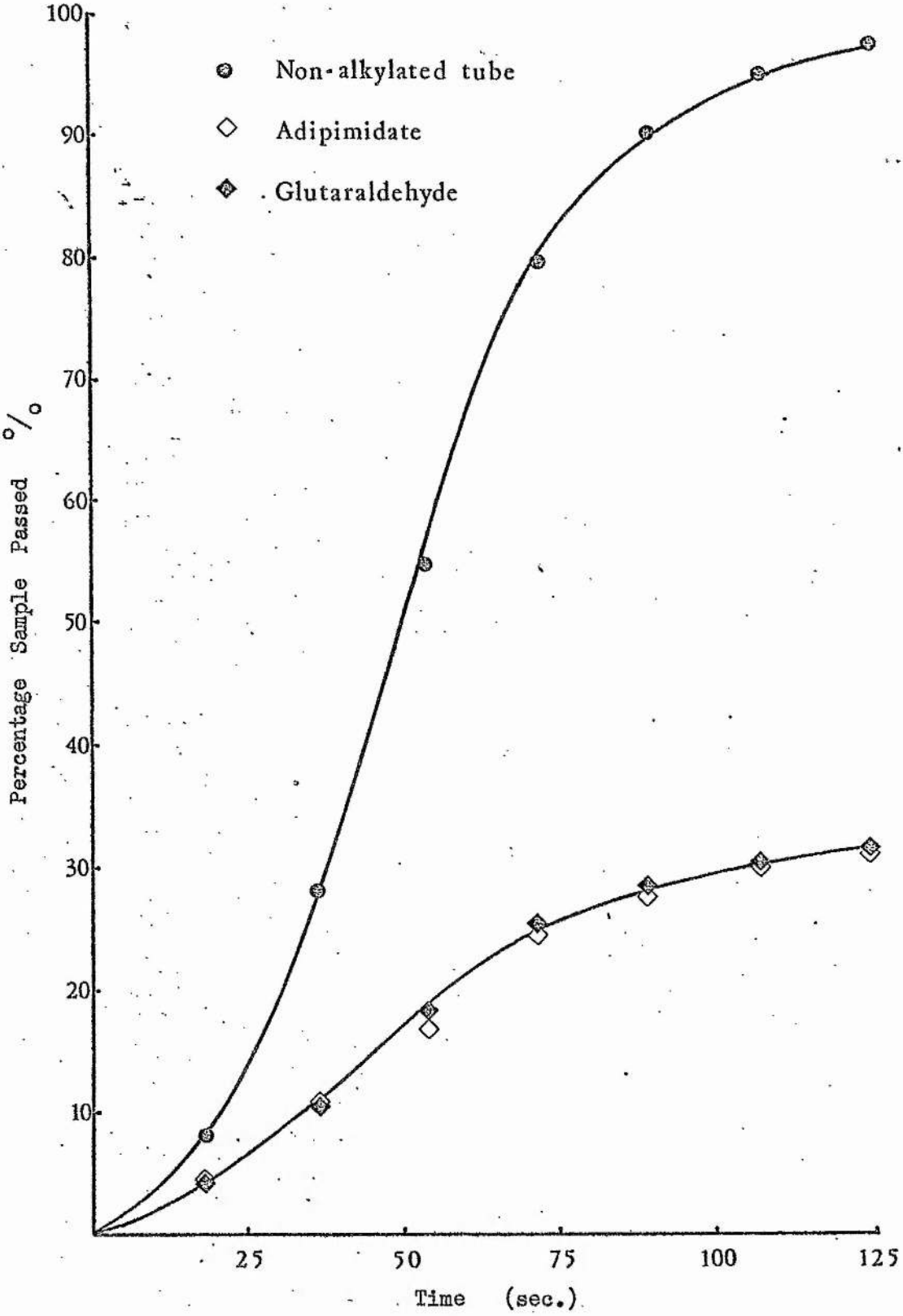


Fig. 34. Variation of carryover with coupler molecule when NADH samples at pH 7.4 are introduced into the continuous flow system (section 3.4.2b). Carryover is expressed as the percentage sample passed with time (section 3.2).

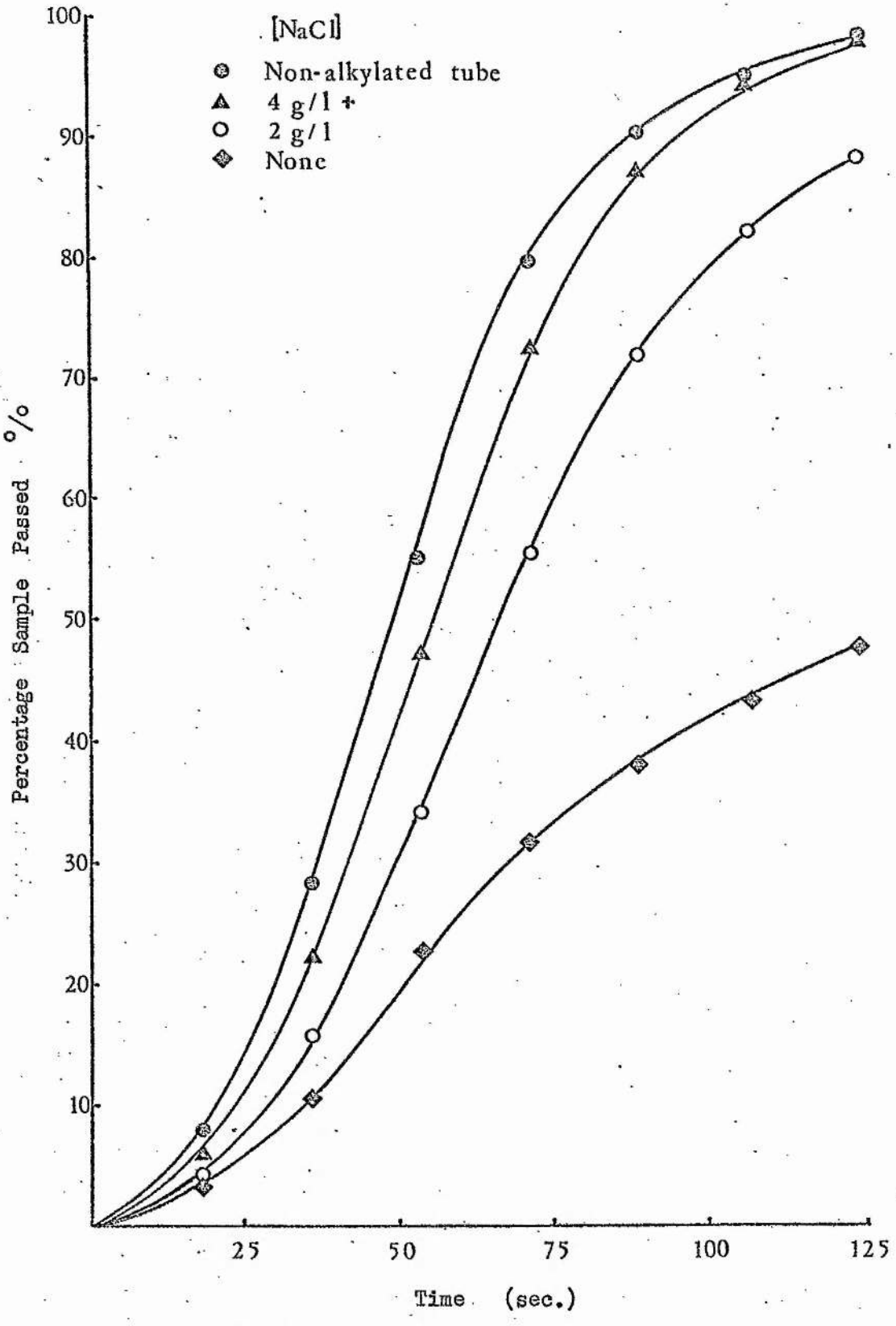


Fig. 35. The effect of increasing salt concentrations on the carryover of NADH solution, pH 7.4, in the continuous flow system when the AH--enzyme derivative is used (section 3.4.2c). Carryover is expressed as the percentage sample passed with time (section 3.2).

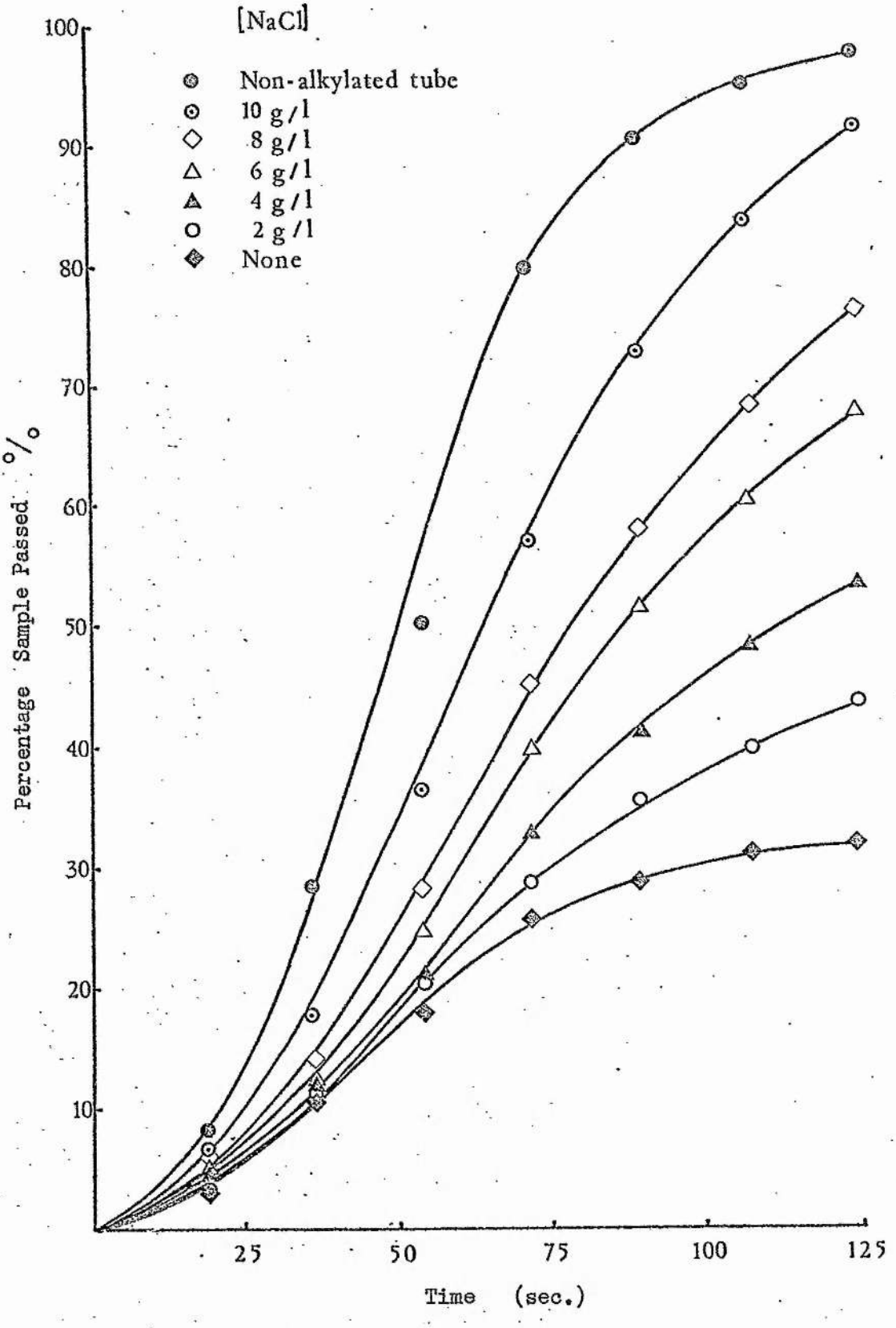


Fig. 36. The effect of increasing salt concentration on the carryover of NADH solution, pH 7.4, in the continuous flow system when the HMDA-enzyme derivative is used (section 3.4.2c). Carryover is expressed as the percentage sample passed with time (section 3.2).

NaCl g/l	AH derivative %	HMDA derivative %
10	98	93
8	98	77
6	98	68
4	98	54
2	89	44
0	48	32

Table VIII The effect of salt concentration on the carryover of NADH solutions, pH 7.4, in the continuous flow system when HMDA and AH enzyme derivatives are used (section 3.42c). Carryover is expressed as the percentage sample passed after 125 sec. (Time taken for 100% sample passed with non-alkylated tube in the system).

d) The effect of pH on carryover.

Nylon tube was O-alkylated (section 2.3), then substituted with HMDA or AH (section 2.4), activated with glutaraldehyde (section 2.5a) and finally coupled with LDH (section 2.5c). 50 cm. lengths of these derivatives were then inserted into the continuous flow system used in section 3.3. When NADH samples introduced into the system are diluted with buffers of different pH, the carryover falls with increasing pH. This is illustrated in Fig. 37 (AH derivative) and Fig. 38 (HMDA derivative) where carryover expressed as percentage sample passed is plotted against time. Fig. 38 shows that with the HMDA derivative carryover is marked even at high pH whereas Fig. 37 shows that with the AH derivative carryover is appreciable only at low pH. Table IX shows that at pH 9.0 98% sample passes after 125 sec. (time taken for 100% sample passed with non-alkylated tube in system) with AH derivative compared with 79% sample passed with HMDA derivative. At pH 6.6 the AH derivative still gives 77% sample passed whereas the HMDA derivative gives only 49% sample passed. Two facts become apparent from this experiment (1) the charge on each enzyme derivative is reduced by increasing pH (2) each enzyme derivative is charged to a different extent at any one pH value.

3.5 Discussion

In this section two methods for assessing the carryover in continuous flow systems are described. One method is a kinetic analysis of the rate of transition between two steady states. This method produces a specific constant, the half-wash time, the calculation of which is dependent upon the premise that the transition is first order with respect to sample concentration.

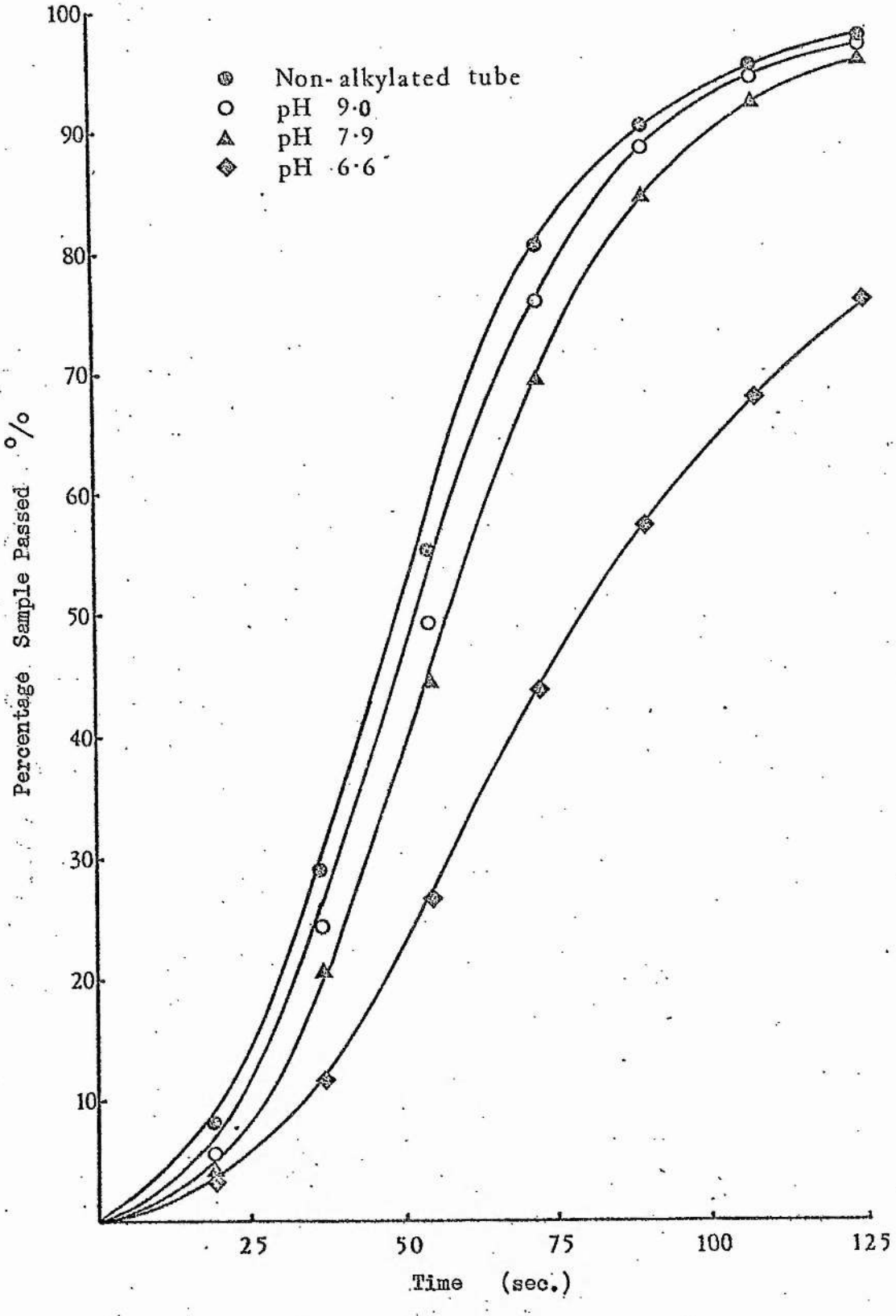


Fig. 37. The effect of pH on the carryover of NADH solution in the continuous flow system when the AH-derivative is used (section 3.4.2d). Carryover is expressed as the percentage sample passed with time (section 3.2).

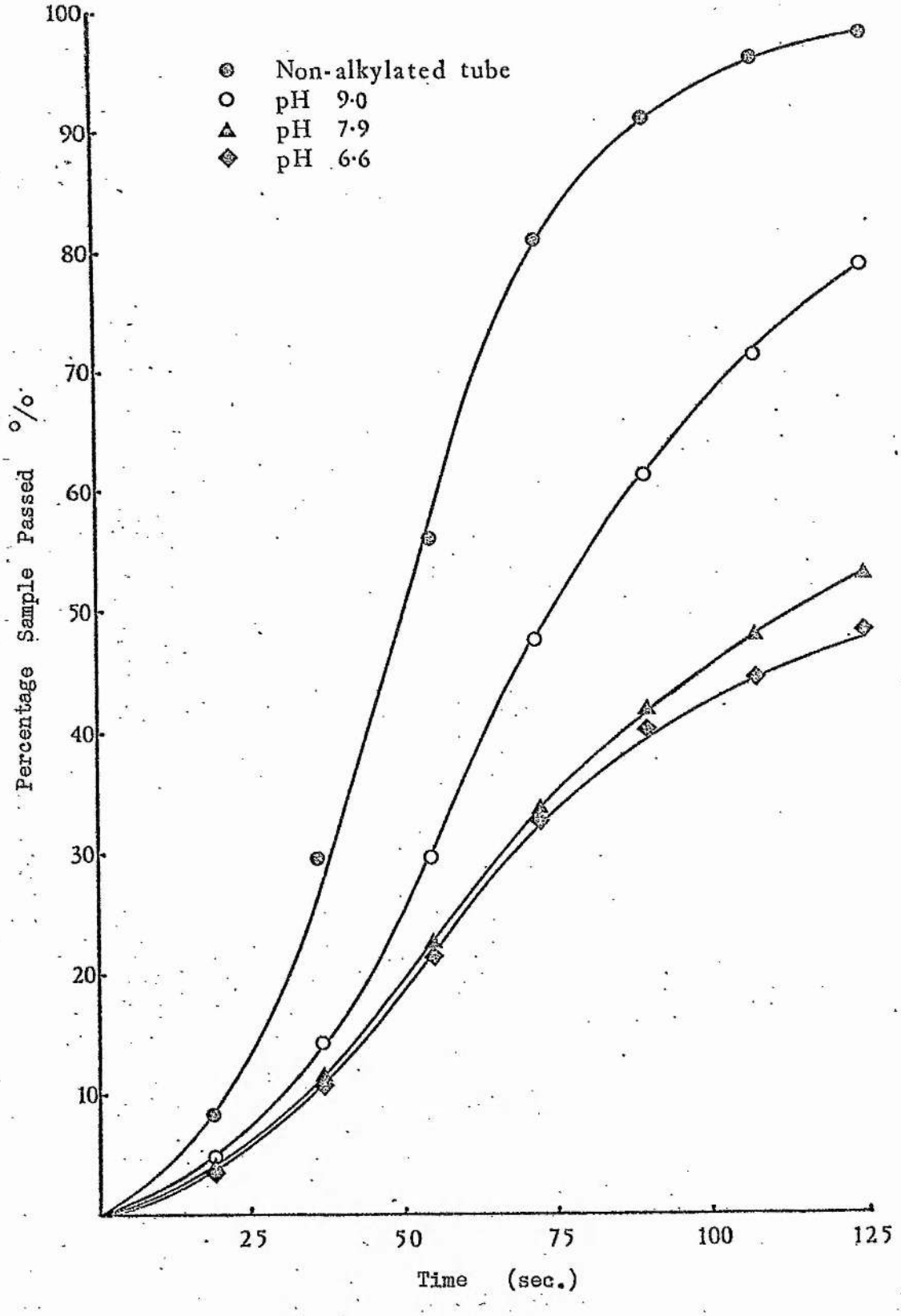


Fig. 38. The effect of pH on the carryover of NADH solution in the continuous flow system when the HMDA-enzyme derivative is used (section 3.4.2d). Carryover is expressed as the percentage sample passed with time (section 3.2).

pH	AH derivative %	HMDA derivative %
9.0	98	79
7.9	96	54
6.6	77	49

Table IX The effect of pH on the carryover of NADH solutions in the continuous flow system when HMDA and AH enzyme derivatives are used (section 3.4.2d). Carryover is expressed as the percentage sample passed after 125 sec. (Time taken for 100% sample passed with non-alkylated tube in the system).

Although this transition has mixed kinetics when enzyme tubes are inserted into continuous flow systems, the concept of half-wash time can still be applied and used as a useful index of carryover. The other method measures the peak distortion or spread by monitoring the percentage sample passed with time. No constant is given with this method but it does provide a simple means of comparing different systems. Both methods are employed to determine the factors which influence sample carryover.

To date, many of the nylon tube immobilized enzymes have been prepared from amine-substituted nylon. When these derivatives are inserted into continuous flow systems pronounced carryover is observed with samples containing negatively charged molecules. The problem is basically one of electrostatic adsorption of samples on the inner surface of the tube. As a consequence of its preparation the support surface of the enzyme tube is positively charged (Fig.39) so that negatively charged species passing through the tube are retarded and neutral or positively charged species pass through unhindered. Fig. 24 shows the formulæ of a series of compounds which have increasing negative charge (riboflavin $NAD^+ < FMN < NADH < NADP^+$). Results confirm that these charges reflect their affinity with the enzyme support (Fig. 25 and Table VI).

The ion-exchange properties of the amine-substituted nylon support can be minimised by the charge screening effect of high ionic strength (Fig. 32, 35, 36 and Tables VII and VIII) and proton removal at high pH (Fig. 37, 38 and Table IX). Such conditions, however, may not always favour the stability and activity of the enzyme. Obviously to prevent the adsorption of charged species a non-charged support is required. In their work on the immobilization of biologically active compounds and affinity chromatography Wilchek and Miron (1974) attempted to eliminate non-specific binding by

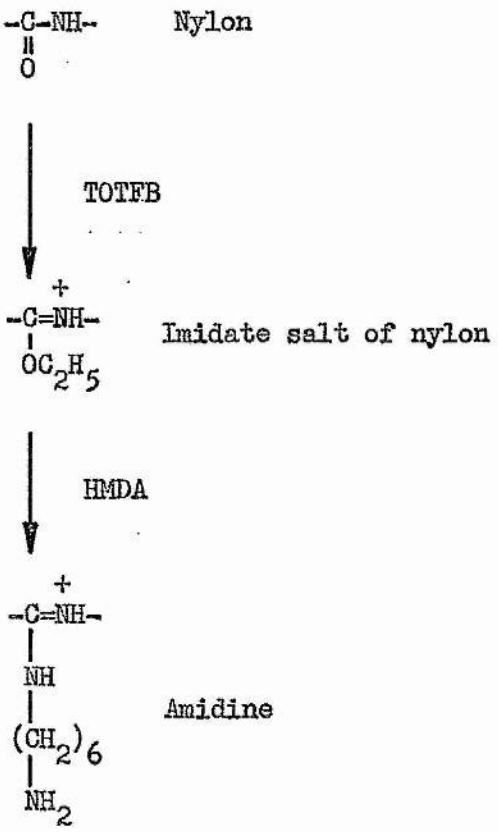


Fig. 39. Preparation of amine-substituted nylon (section 2.4) from O-alkylated nylon (section 2.3)

synthesising a series of agarose supports, one of which was non-charged. When cyanogen bromide activated agarose was treated with a diaminoalkane, a N-substituted imidocarbamate which carries a positively charged nitrogen atom was formed (see Fig. 40). By potentiometric titration of the derivative formed on coupling butylamine to the activated agarose, these workers found that the imidocarbamate group had a pK value of 10.4 Thus the derivative had ion-exchange properties. However, if a dihydrazide was used to replace the diamine, the resultant derivative had a pK of 4.2 and an absence of any ion-exchange properties at neutral and high pH.

Hornby and Norris (unpublished results) demonstrated that similar derivatives could be prepared from caprolactam. Caprolactam, the "monomer" of nylon 6, was O-alkylated with TOTFB to give the caprolactimidate and then reacted with either hexylamine or hexanoic acid hydrazide to give the amine- and hydrazide-substituted caprolactam respectively (Fig. 41). The resulting amidine and amidrazone groups were found to have pK values of 11.2 and 6.5.

The pK values for amidrazones are lower than those of the corresponding amidines because amidrazones have more resonance forms than amidines (see Fig. 42). This makes amidrazones less basic than amidines and therefore less ready to accept protons. Conversely protons are very easily removed from the amidrazones at alkaline pH. Clearly if enzyme tubes are prepared from hydrazine-substituted nylon tube rather than amine-substituted tube then at neutral and high pH conditions the charge on the support is lower and the carryover improved. This reasoning is supported by the evidence obtained in the experiments. Derivatives formed through the amidrazone linkage show less affinity for NADH than do the amidine derivatives at alkaline pH (Table IX) and low ionic strength (Table VIII).

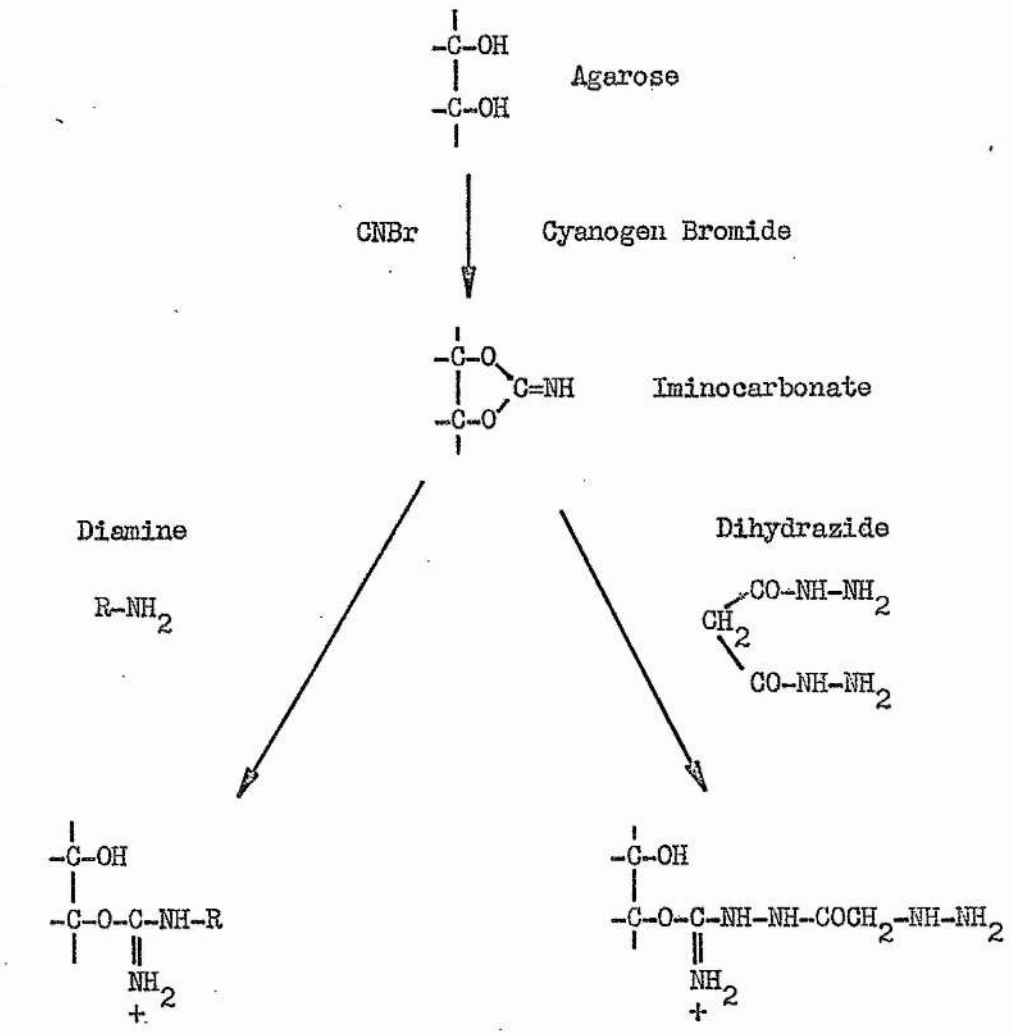


Fig. 40 Preparation of diamine and dihydrazide derivatives of agarose (Wilchek and Miron, 1974).

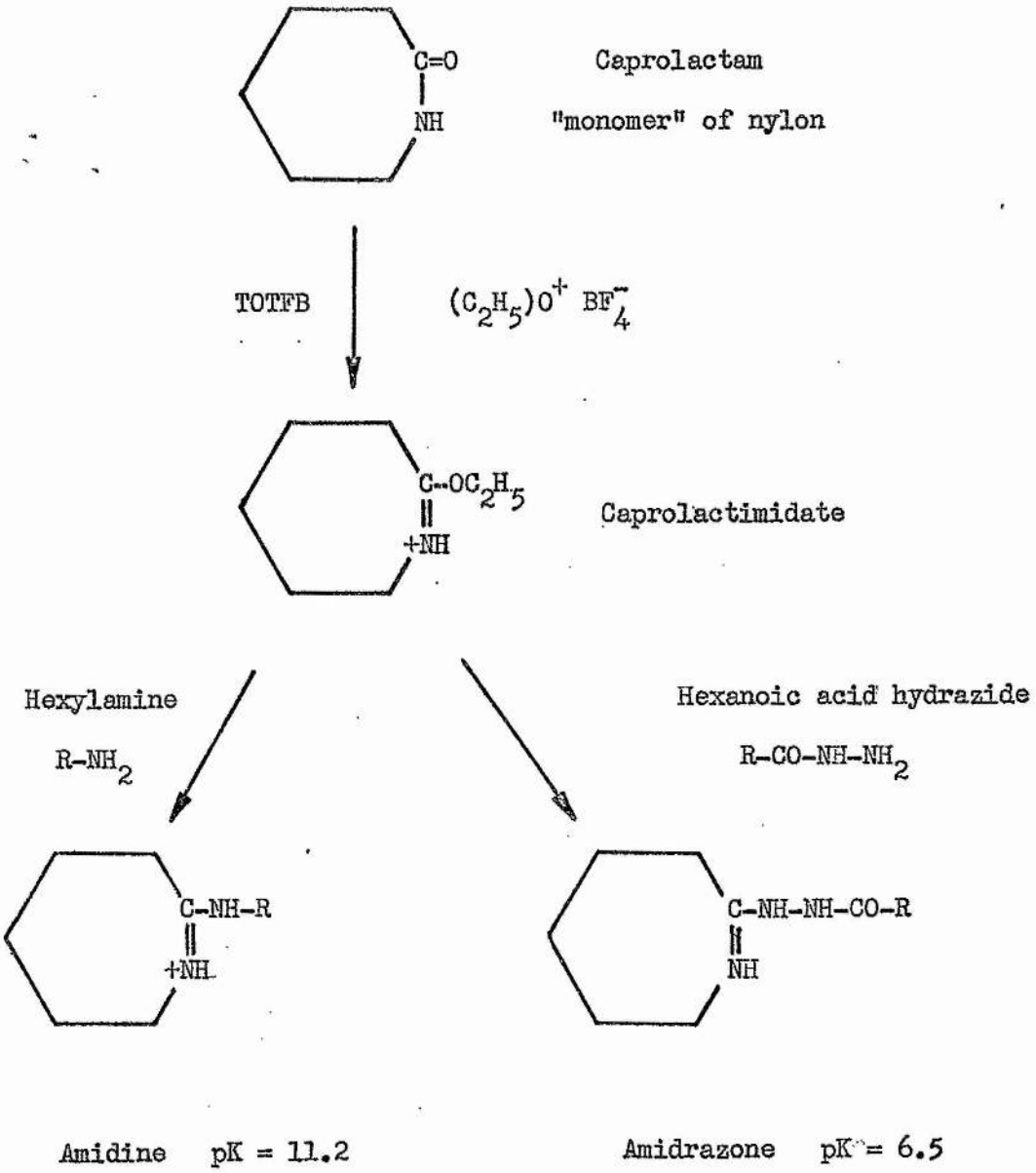
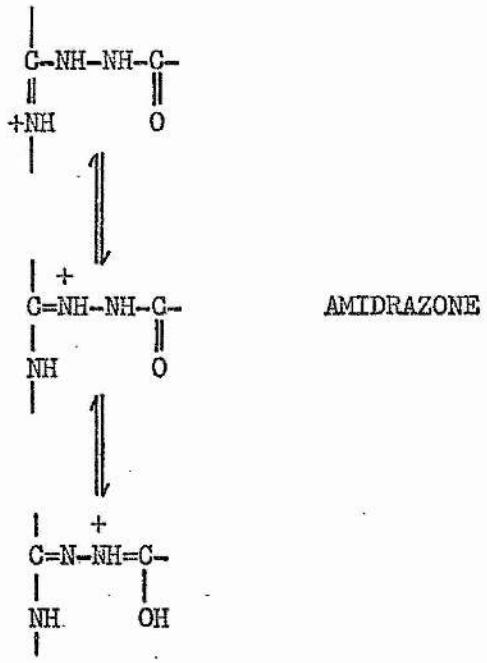


Fig. 41. The preparation of amine- and hydrazide-substituted caprolactam (Hornby and Morris, Unpublished).



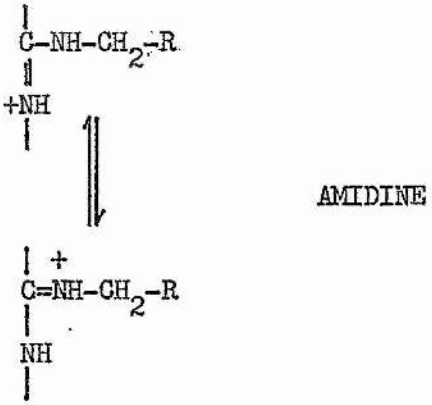


Fig.42. The resonance forms of amidrazones and amides.

Although increased ionic strength will reduce the adsorption of negatively charged species in the case of both the amidine and amidrazone derivatives, inclusion of high concentrations of salt should be avoided where the activity or stability of the enzyme in question can be affected. Instead, enzyme tubes can be tailored to suit the particular assay system by the use of appropriate spacer molecules. As in the case of dehydrogenases, a compromise might have to be made between the activity of the enzyme derivative (Table I, section 2.8b) and their carryover characteristics. Thus at low pH dihydrazide derivatives would be preferred because of their low carryover even though their activity may be poor. Advantage would be taken of diamine derivatives at high pH where the higher activity would compensate for the slight increase in carryover by allowing shorter lengths of tube to be employed.

4. The use and evaluation of immobilized enzymes in continuous flow assay systems.

4.1 Introduction.

The prohibitive cost of some enzymes has meant that, in general, they have been neglected as analytical reagents in continuous flow assay systems. Where large workloads are concerned, analysts prefer to forfeit the specificity of enzyme-based techniques for low cost chemical techniques which often measure groups of related compounds rather than a single compound. In theory, enzyme immobilization should reduce the cost of enzyme-based analysis and provide an attractive alternative to the well-established non-specific chemical methods.

Immobilized enzyme tubes are best suited to the kinetic analysis of metabolites rather than end-point analysis. Unless an enzyme tube has a very high specific activity (U/m) complete substrate-to-product conversion is possible only when long tube lengths are used to supply the assay system with many units of enzyme activity and prolong the incubation period. Such tube lengths will lead to an unacceptable degree of sample interaction where adsorption of reactant occurs and end-point analysis becomes a non-viable proposition for continucus flow systems with high sample turnover. In kinetic analysis, sample interaction can be kept to a minimum by using relatively short enzyme tubes and sensitive monitoring techniques.

The kinetic mode of analysis is used in the methodologies described in the following pages. In each case, the measurement of analyte concentration is based on a dehydrogenase reaction and the production of NADH which is monitored fluorimetrically (Leaback, 1969; Powery and Passenneau, 1972; Rhys-Williams, 1976). Fluorimetry gives

the assay methods another degree of specificity since although many compounds absorb light comparatively few re-emit the absorbed energy as light. Therefore the precision and accuracy of fluorimetric methods are high.

In order to assess the overall performance of new methods, two indices are generally used - precision and accuracy. These allow the new methods to be compared against the standards set by reference methods.

The precision of an analytical method can be defined as its ability to reproduce the same value repeatedly for a single sample. Precision can be determined by aspirating three different samples at a fixed rate in random order and calculating the standard deviation and coefficient of variation from the replicate values obtained for each specimen.

In addition, the carryover between high and low values can be determined by a modified version of the method proposed by Broughton et al. (1969). Three samples containing a high concentration of analyte followed by three samples containing a low concentration of analyte are sampled and the additive effect of the high value on the low value is measured. The third high and low values are assumed to be the true analyte values and the carryover is expressed as a percentage of the true high value.

$$\text{Percentage carryover} = \frac{\text{1st. low value} - \text{3rd. low value}}{\text{3rd. high value}} \times 100\%$$

The accuracy of an analytical method is defined as a measure of its ability to give the "true" analyte concentration. For convenience the estimated values are not compared with the true values but with standard reference values (ie. the values given by

a good method). The accuracy is then expressed in terms of the correlation coefficient and the linear regression plot of paired values.

4.2 Automated fluorimetric analysis of blood ethanol.

In the past the determination of ethanol in body fluids has been carried out by one of three techniques; distillation of ethanol with subsequent reduction of acid dichromate or acid permanganate (Sunshine, 1961), gas chromatography (Leach and Jones, 1971) and enzymatic oxidation (Stiles et al., 1966). Now, as a result of the road traffic safety legislation on "Drink and Driving", blood ethanol assays have become one of the most requested routine analyses in the forensic laboratory. The old methods are not suited to rapid analysis and this has led to the development of new modified procedures to cope with the increased workload.

Speed of analysis, together with convenience of sample handling, comes naturally with a fully automated technique and in this respect the enzymatic oxidation method is more easily adapted to automation than either the distillation or gas chromatographic methods, both of which require some form of pre-treatment of the sample prior to analysis.

Most enzymatic methods for the determination of ethanol derive from the original spectrophotometric technique developed by Bonnichsen and Theorell (1951), in which alcohol dehydrogenase is used to oxidise ethanol to acetaldehyde,

$$C_2H_5OH + NAD^+ \xrightleftharpoons{LADH} CH_3CHO + NADH + H^+$$

ethanol acetaldehyde

This reaction has been followed by a variety of techniques including amperometry (Smith and Olson, 1975), spectrophotometry (Jones et al., 1970) and fluorimetry (Ellis and Hill, 1968).

The method described below uses immobilized alcohol dehydrogenase to catalyse the oxidation of ethanol and the percentage conversion to acetaldehyde is monitored by measuring the fluorescence of the NADH formed in the enzyme catalysed reaction.

Experimental.

Reagents.

1. 0.1M-Pyrophosphate buffer, pH 9.0: 0.1M-$Na_4P_2O_7$ titrated to pH 9.0 with 2M-HCl. 1ml. 10% (v/v Triton-X added per 1.
2. Sample wash: 0.15M-NaCl.
3. Nicotinamide-adenine dinucleotide; 2.5mM-NAD^+ solution in water is freshly prepared. Grade III NAD^+ was obtained from Sigma London Chemical Co.Ltd.
4. Ethanol standards: Prepared by diluting absolute ethanol 1 in 250 with water to give an approximately 69mM solution, which is determined accurately spectrophotometrically. This solution is further diluted to give standards.
5. Enzyme tube: Horse liver alcohol dehydrogenase immobilized on 1mm bore nylon tube. Nylon tube was alkylated (section 2.3), then substituted with DAE (section 2.4), activated with glutaraldehyde (section 2.5a) and coupled with enzyme (section 2.5c). The enzyme was obtained from Sigma London Chemical Co.Ltd. The Schiff's base formed in the coupling step is reduced with borohydride (section 2.5d). A 10cm. length is used in the assay system.

Method.

The flow system used for the determination of blood ethanol is shown in Fig. 43. The apparatus comprises standard Technicon equipment together with the Perkin-Elmer 1000 Fluorescence Spectrophotometer and Kipp-Zonen BD8 recorder.

Aqueous standards and whole blood with added ethanol are sampled at a rate of 60 samples/h. with a 2:1 sample:wash ratio. Each sample is introduced into an air-segmented buffer stream and dialysed against a further stream of buffer. The recipient stream is then supplemented with NAD^+ and passed through a 10cm. length of LADH-tube incubated at 37^oC. After a debubbling step, standards and samples enter the flowcell of the fluorimeter where the NADH formed is monitored (λ_x 366nm: λ_m 465nm.).

The pump tubes on the manifold are arranged in the following manner:

Line	Flow rate ml/min.
1. Sample	0.1
2. Buffer	2.0
3. Air	1.0
4. Buffer	2.0
5. Air	1.0
6. NAD^+	0.6
7. Sample wash	2.0
8. Waste	2.0

Results.

The calibration curve obtained in this method describes a typical Michaelis-Menten curve ie. rate against substrate concentration (Fig. 44). Therefore the assay method loses a certain

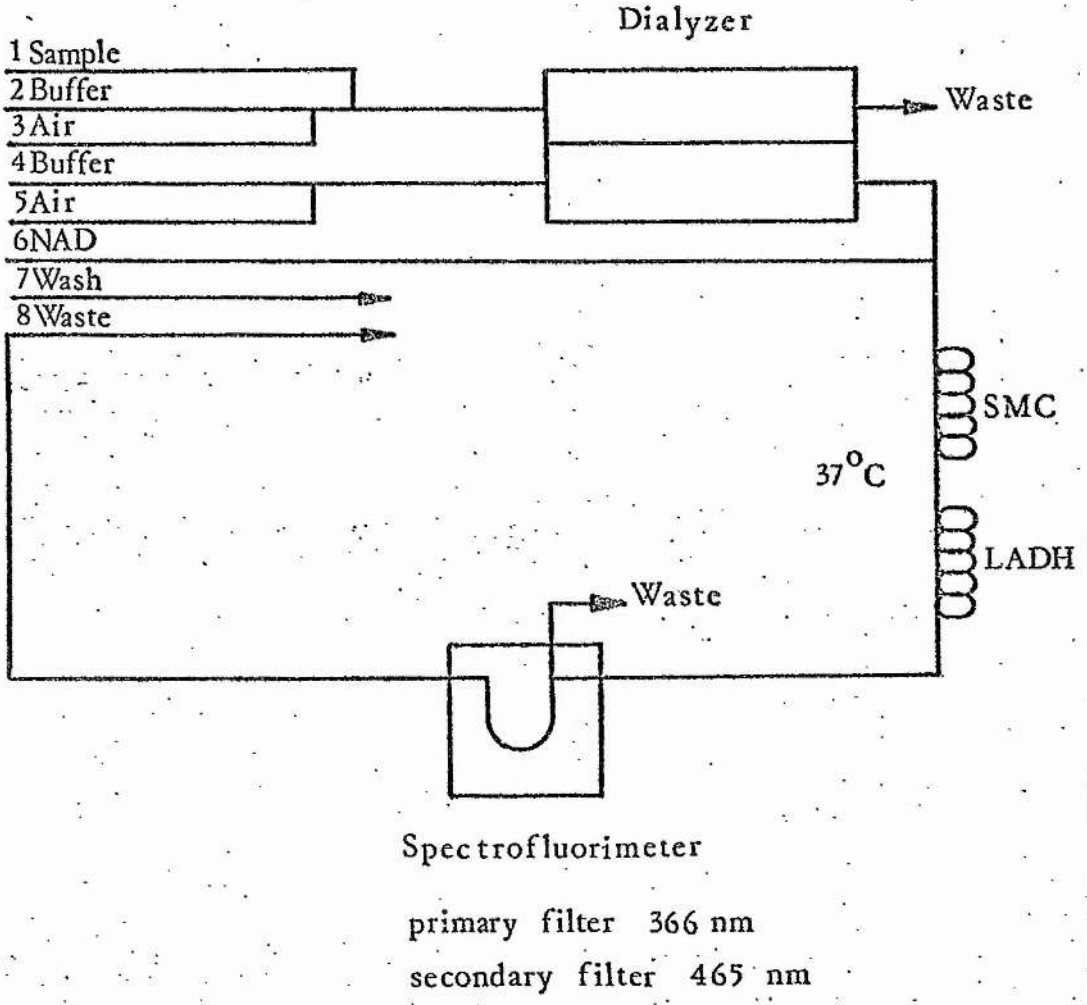


Fig. 43. Flow diagram of the analyser system used to assay blood ethanol concentrations with nylon tube immobilized LADH. (SMC = single mixing coil)

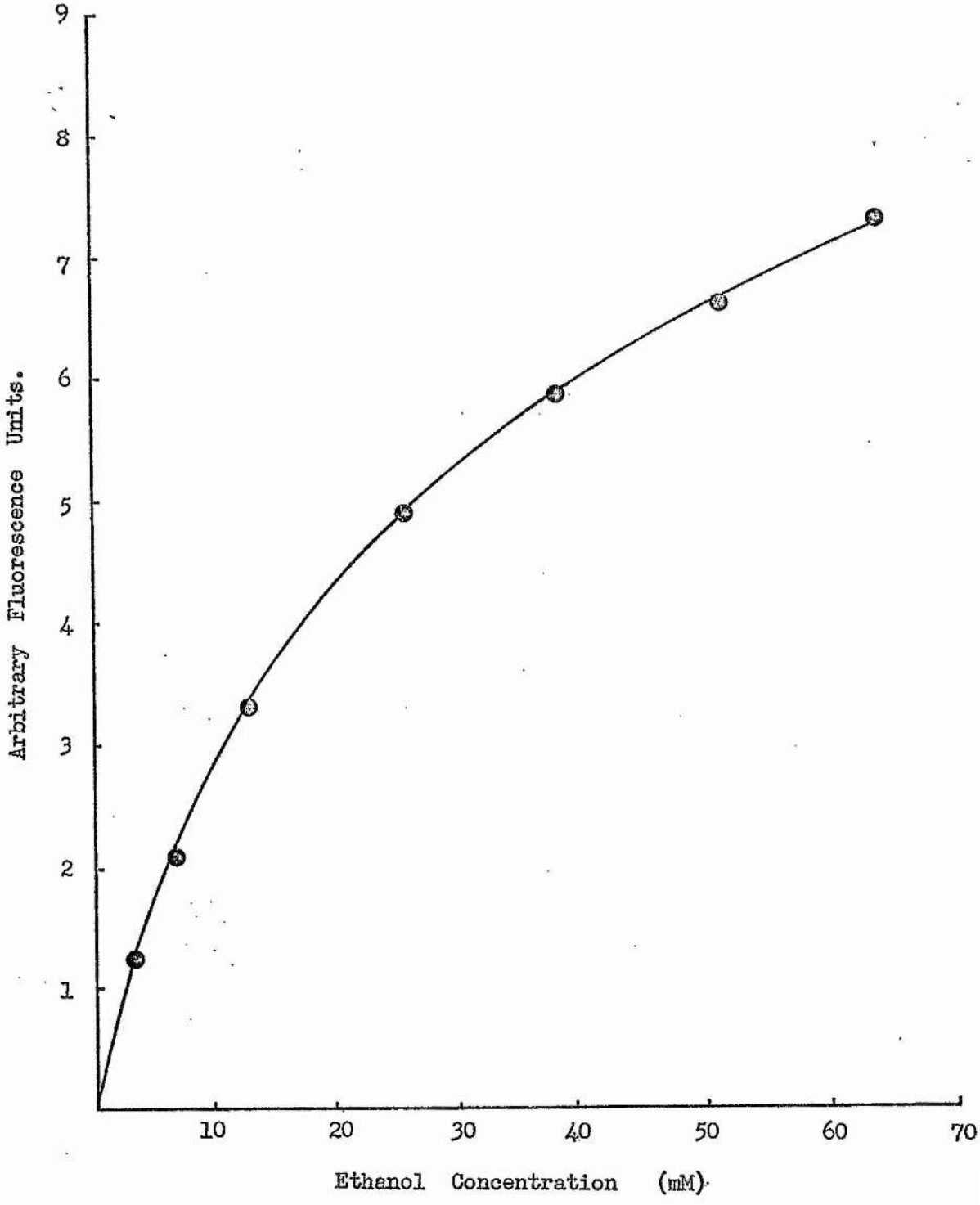


Fig. 44. Typical calibration curve obtained in the automated analysis of blood ethanol using nylon tube immobilized LADH.

amount of sensitivity at the higher end of the curve when the concentration of ethanol presented to the enzyme tube exceeds its K_m (this corresponds to sample concentrations in excess of 30mM ethanol). The overall sensitivity of the method is determined by the NAD^+ concentration and not the ethanol concentration. By sampling a 32mM ethanol standard solution and varying the concentration of NAD^+ in the assay mixture, an increase in sensitivity is observed with increasing NAD^+ concentration (Fig. 45). The most practical NAD^+ concentration to adopt in this method is 0.58mM, above this concentration the rate of increase in sensitivity diminishes rapidly.

Carryover in the system was assessed using the modified version of the method proposed by Broughton et al. (1969). Three high (a= 53mM) and three low (b = 6mM) blood ethanol specimens were sampled at a rate of 60/h. with a 2:1 sample-wash ratio (see Fig.46) and the percentage carryover calculated from the equation,

$$\text{Carryover } c = \frac{b_1 - b_3}{a_3} \times 100\%$$

When a 10cm. length of nylon tube immobilized LADH is used in the flow system c = 0.4% which is not significant.

The precision of the method was determined by sampling 60 randomly distributed blood ethanol specimens and calculating the standard deviation and coefficient of variation from the results obtained. Results are shown in Table X together with comparable results obtained using the manual method of Bernt and Gutmann (1974).

The accuracy of the method was determined by comparison with the method of Bernt and Gutmann. 30 different blood specimens were assayed by each method and the results collated as in Fig. 47.

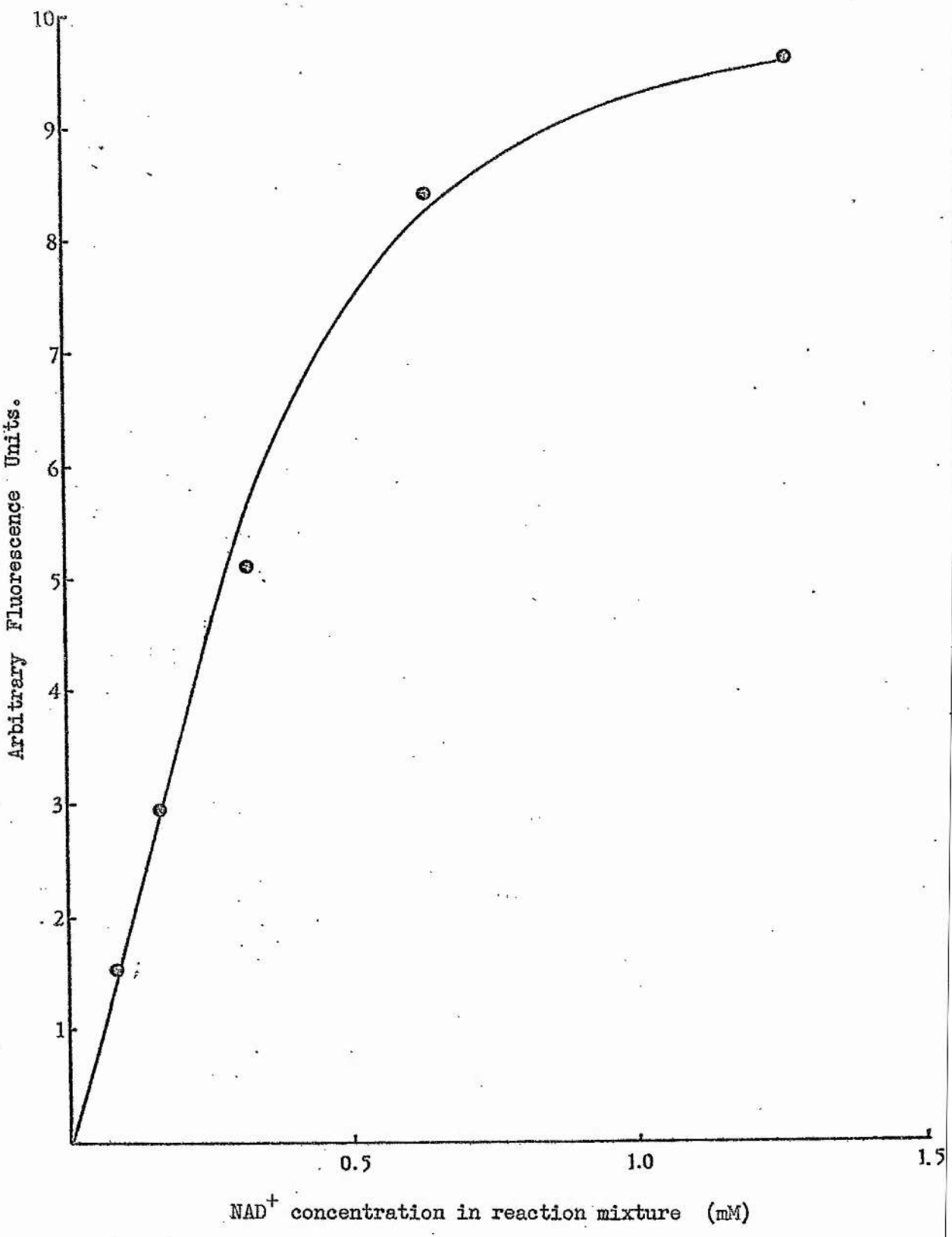


Fig. 45. Variation of sensitivity with NAD^+ concentration in the automated analysis of blood ethanol using nylon tube immobilized LADH. Sensitivity is expressed in terms of the fluorescence given by a 32mM ethanol sample.

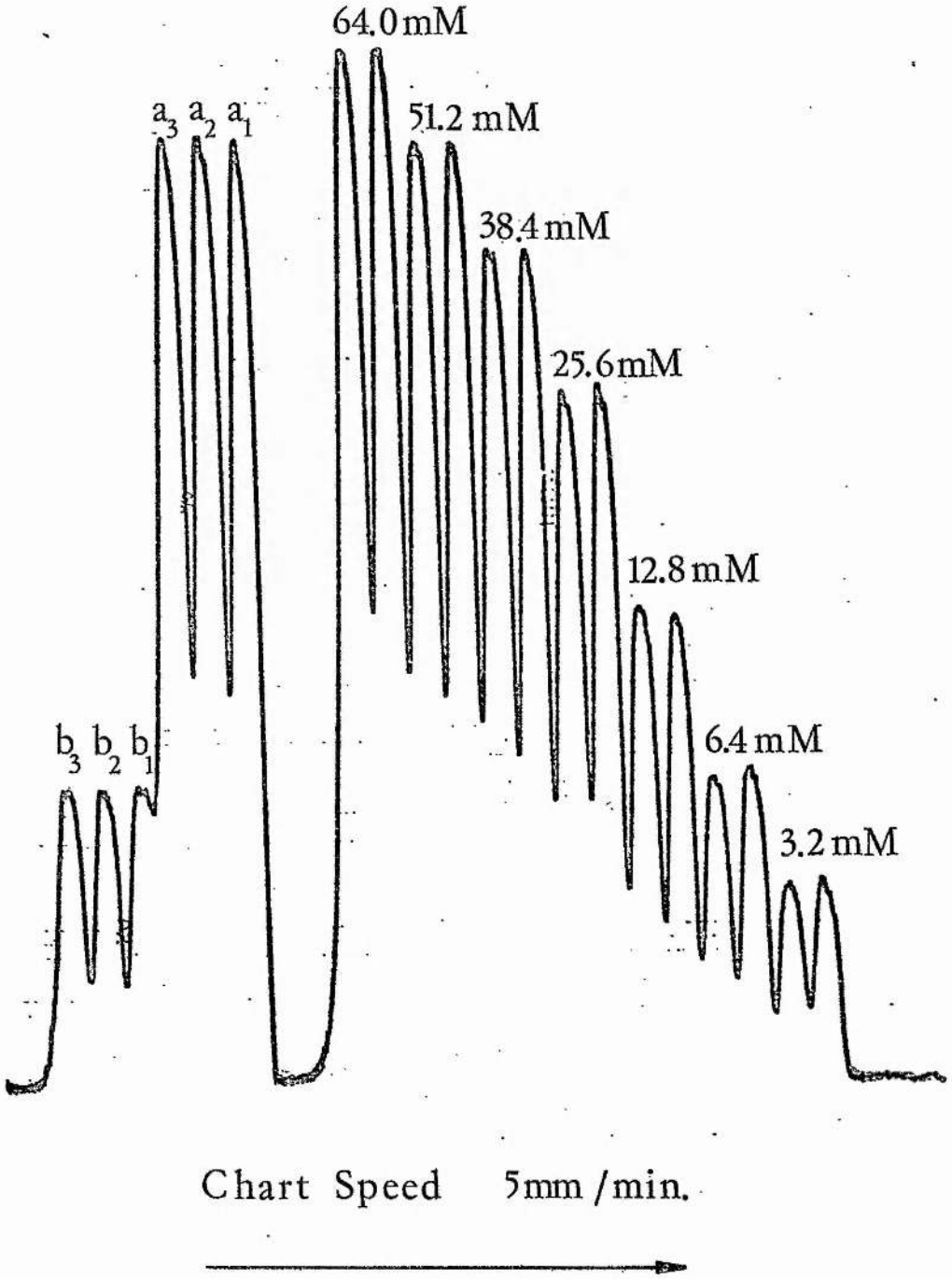


Fig. 46. Typical standard peaks in the ethanol assay system and demonstration of carryover between high and low peaks at 60 samples/h., 2:1 sample:wash.

	Automated Method	Automated Method	Manual Method
Number of assays	30	30	-
Mean	29,5mM	13.0mM	12.0mM
Standard Deviation	0.338mM	0.281mM	0.22mM
Coefficient of Variation	1.46%	2.16%	2 %

Table X. Assessment of precision. Statistical analysis of results obtained by random sampling of two blood ethanol specimens in the immobilized LADH assay system. The precision of the immobilized LADH method is compared with that of the manual method (Bernt and Gutmann, 1974).

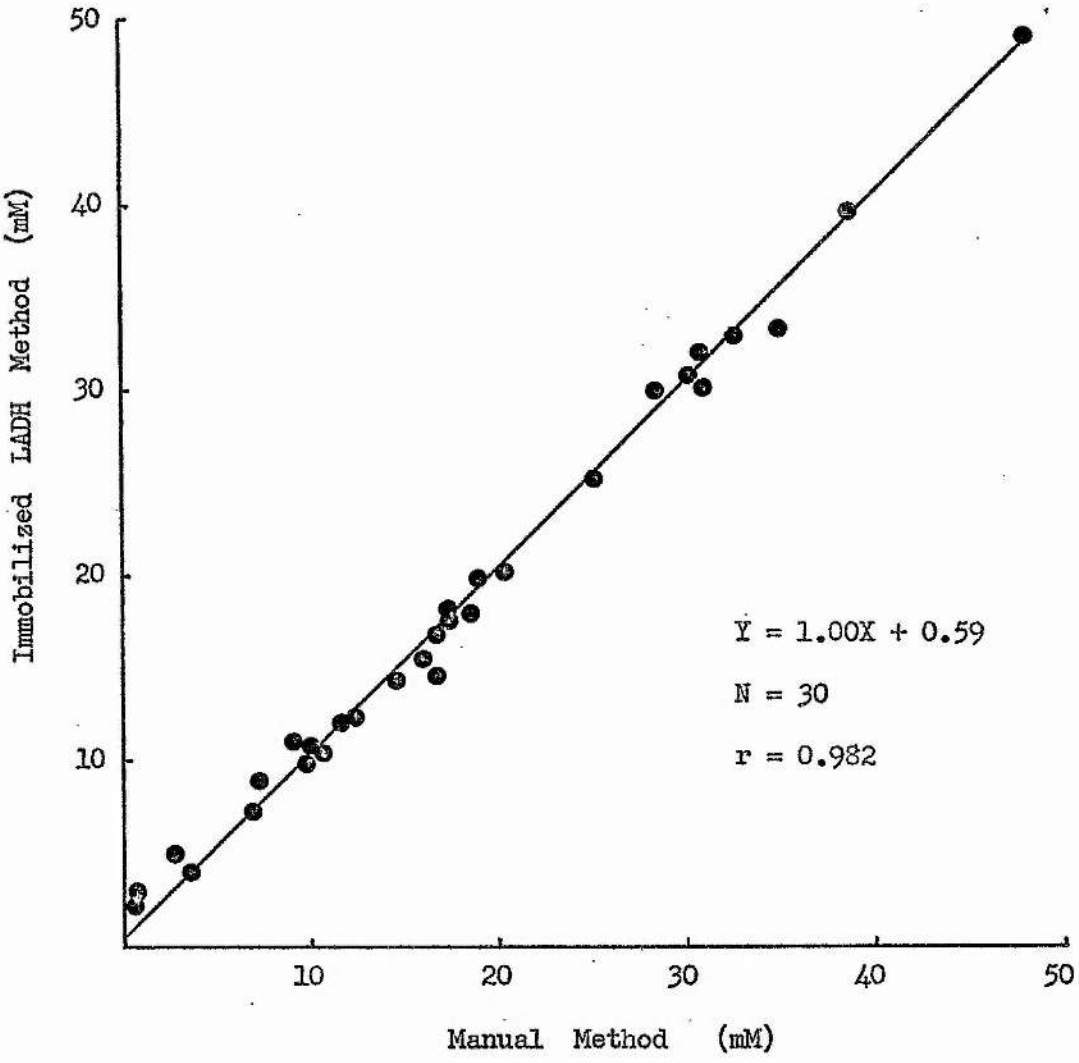


Fig. 47. Comparison of manual (Bernt and Gutmann, 1974) and proposed immobilized LADH methods of determining blood ethanol.

The calculated linear regression ($y = 1.00x + 0.59$) and the correlation coefficient ($r = 0.982$) indicate reasonable agreement between the two methods although the positive intercept shows that there is a general tendency for the immobilized LADH method to produce slightly higher values. The possibility of non-specific fluorescence has been ruled out since no fluorescence is observed when specimens are sampled with no enzyme tube in the flow system.

Comment.

Most manual enzymatic methods for the estimation of ethanol employ yeast ADH rather than liver ADH, mainly because of the high activity and low cost of the yeast enzyme. However, liver ADH has the advantage of being more stable than yeast ADH. Bayne (1974) studied the stability of the immobilized enzymes under a variety of conditions and invariably found the LADH derivative to be more stable than the YADH derivative. When enzymes are used as analytical reagents it is extremely important that they remain stable through the period of analysis. For the enzymatic determination of ethanol, LADH would appear to be the enzyme of choice, however since the high cost of this enzyme prohibits its routine use in the soluble form, the immobilized enzyme must be used.

In this ethanol assay system, a compromise is made between sensitivity, linearity of standard curve and cost of controlling the concentrations of substrates and the length of the enzyme tube used. Sensitivity is increased by reducing the sample dilution and increasing the length of LADH-tube. Linearity of standard curve is approached as sample dilution is increased. Cost is reduced by using short LADH-tubes and optimising the NAD^+ concentration.

The nylon tube immobilized LADH used in this assay system is prepared by covalently linking the enzyme to nylon via a DAE/ glutaraldehyde bridge because the activity is greater when the enzyme is immobilized in this way (Table I). The charge effects and increased carryover found with the DAE spacer are virtually eliminated with a 10cm. length of enzyme tube at the high pH of the reaction. Longer enzyme tubes have little advantage apart from an increase in sensitivity and this is counteracted by a corresponding increase in carryover which detracts from the performance of the system.

The method described is well suited to the rapid analysis of whole blood specimens since no pretreatment of samples or blank channel is required. It is primarily intended as a means of assaying blood ethanol levels for criminal proceedings where the legal limit is 17.4mM (80mg%). This point lies on the steep part of the calibration curve where sensitivity is greatest and the coefficient of variation is about 2%. Overall, the reliability of this enzymatic methods compares favourably with other methods in present use.

4.3 Automated fluorimetric analysis of plasma glucose.

The determination of blood glucose is probably one of the most important routine analyses performed in the clinical chemistry laboratory. This is reflected in the numerous techniques that have been developed in an attempt to achieve more accurate glucose values.

Until recently, reduction methods have been the most widely used form of analysis. These are based on the reduction of either alkaline copper (Folin and Wu, 1920) or alkaline ferricyanide solutions (Folin and Malmross, 1921) by glucose. Early methods estimated the total reducing substances and not only the glucose

present in the blood. These were later modified to exclude or minimise the effect of non-glucose reducing substances. The improved techniques are still used in both manual and automated forms, but now there is a move towards the enzymatic methods with their inherent specificity.

Glucose oxidase is commonly used for the determination of glucose concentrations;

$$\text{Glucose} + H_2O + O_2 \xrightleftharpoons{\text{GOD}} \text{Gluconic acid} + H_2O_2$$

The oxidation reaction can be monitored by measuring either the oxygen uptake using an oxygen electrode (Clark, 1971) or the appearance of hydrogen peroxide with a peroxidase-chromogen system (Trinder, 1969; Gochman and Schmitz, 1972) or polarographic electrode (Life Sciences). Measurement of oxygen uptake would seem to be an ideal method for automation, being practically reagentless when used with immobilized GOD (Kunz and Stastny, 1974; Campbell et al., 1975) and more specific than those involving chromogens which are prone to interference. This is not the case however, the oxygen electrode has a low response time and is therefore more suited to the analysis of single specimens.

Other enzymatic methods use the hexokinase/glucose.6.phosphate dehydrogenase system (HK/G6PDH);

$$\text{Glucose} + \text{ATP} \xrightleftharpoons[Mg^{++}]{\text{HK}} \text{Glucose.6.phosphate} + \text{ADP}$$

$$\text{Glucose.6.phosphate} + NADP^{+} \xrightleftharpoons{\text{G6PDH}} \text{6.phosphogluconolactone} + NADPH + H^{+}$$

The NADPH produced can be measured directly at 340nm. (Peterson and Young, 1968; Neeley, 1972) or indirectly using a chromogen (Coburn and Carroll, 1973; Wright et al., 1971). Again, direct measurement of the NADP makes the method more specific.

Both HK and G6PDH have been immobilized and used in an automated assay system for glucose (Morris et al., 1975) but the high cost of the coenzyme is likely to deter workers from adopting the method in routine analysis. Thus, even with enzyme immobilization the assay of glucose enzymatically is either expensive, subject to interference, or unable to cope with high workloads. The two methods described below are an attempt to overcome these drawbacks.

4.3.1 The glucose oxidase/catalase/aldehyde dehydrogenase method.

In this method glucose oxidase (GOD) and catalase (CAT) are co-immobilized to catalyse the following oxidation reactions in series;

$$\text{Glucose} + H_2O + O_2 \xrightleftharpoons{\text{GOD}} \text{Gluconic acid} + H_2O_2$$

$$H_2O_2 + C_2H_5OH \xrightleftharpoons[\text{peroxidatic}]{\text{CAT}} CH_3CHO + 2H_2O$$

ethanol — acetaldehyde

Co-immobilization ensures that the hydrogen peroxide formed in the first reaction remains at a low level such that deactivation of the CAT will be minimal (Altomare et al., 1974). Also, at these low concentrations of hydrogen peroxide, the enzyme will act predominantly in the peroxidatic rather than the catalatic mode (De Duve and Baudhuin, 1966; Deisseroth and Dounce, 1970);

$$2\ H_2O_2 \xrightarrow[\text{catalatic}]{\text{CAT}} 2\ H_2O + O_2$$

The acetaldehyde formed in the peroxidatic reaction is itself oxidised to acetate by aldehyde dehydrogenase (AlDH) and the percentage conversion is monitored by measuring the fluorescence of the NADH formed in the enzyme catalysed reaction;

$$CH_3CHO + NAD^+ + H_2O \xrightleftharpoons{\text{AlDH}} CH_3COOH + NADH + H^+$$

acetaldehyde — acetate

Experimental.

Reagents.

1. 0.05M-Phosphate buffer, pH 6.5: 0.05M-NaH_2PO_4, 0.15M-NaCl titrated to pH 6.5 with 2M-NaOH. This is used to prepare a 0.17M-ethanol solution containing 1ml. 10% (v/v) Triton-X per l.
2. Sample wash: 0.15M-NaCl.
3. Nicotinamide-adenine dinucleotide: 1.6mM-NAD^+ solution in 1M-tris, 1M-KCl, 5mM-mercaptoethanol is freshly prepared. Grade III NAD^+ was obtained from Sigma London Chemical Co.Ltd.
4. GOD/CAT-tube: GOD and CAT are co-immobilized on 1.5mm. bore nylon tube. Nylon tube was alkylated (section 1.3), then substituted with DAE (section 1.4), activated with glutaraldehyde (section 1.5a) and coupled with the enzymes(section 1.5c). Both enzymes were supplied by Boehringer Mannheim GmbH, fungal GOD as a lyophilised powder and beef heart CAT as a crystalline suspension in thymol saturated water. The pre-coupling solution was prepared in 0.1M-borate buffer pH 8.5 to give an overall protein concentration of 1mg/ml. with GOD and CAT in the ratio 3:1 (w/w) respectively. A 40cm. length of GOD/CAT-tube is used in the assay system.

5. AlDH-tube: AlDH is immobilized on 1.5mm. bore nylon tube. Nylon tube was alkylated (section 1.3), then substituted with DAE (section 1.4), activated with glutaraldehyde (section 1.5a) and coupled with glutathione-free AlDH (section 1.5c). Yeast AlDH was a gift from Boehringer Mannheim GmbH. The enzyme preparation was a lyophilised powder containing 1.0mg. of glutathione per mg. of enzyme. Glutathione-free AlDH was prepared by gel filtration of the enzyme on a column (20 x 1 cm. approx.) of Sephadex G 10 pre-equilibrated with 0.1M-KH_2PO_4, 1M-KCl, 2mM-dithiothreitol pH 6.5. A 50cm. length of AlDH-tube is used in the assay system.
6. Glucose standards: 2.8 - 22.2mM glucose solutions in saturated benzoic acid. The 7.5 standard is used as a drift standard.
7. Control sera: Wellcomtrol One, Two and Three from Wellcome Reagents Ltd.

Method.

The flow system used for the determination of glucose using GOD, CAT and AlDH is shown in Fig. 48. The apparatus comprises standard Technicon equipment together with the Perkin-Elmer 1000 Fluorescence Spectrophotometer and Kipp-Zonen BD8 recorder.

Standards and plasma specimens are sampled at a rate of 60 samples/h. with a 2:1 sample:wash ratio. Each sample is introduced into an air-segmented buffer stream and dialysed against a further buffer stream. The recipient stream then passes through the GOD/CAT tube at 37°C where glucose and ethanol are converted to gluconic acid and acetaldehyde respectively. The effluent stream is subsequently titrated from pH 6.5 to pH 8.5 by the addition of the tris-NAD^+ solution and passed through the AlDH-tube where the acetaldehyde formed is oxidised to acetate. After a debubbling step, standards and samples enter the flowcell of the fluorimeter where the NADH formed is monitored (λ_x 366nm.; λ_m 465nm.).

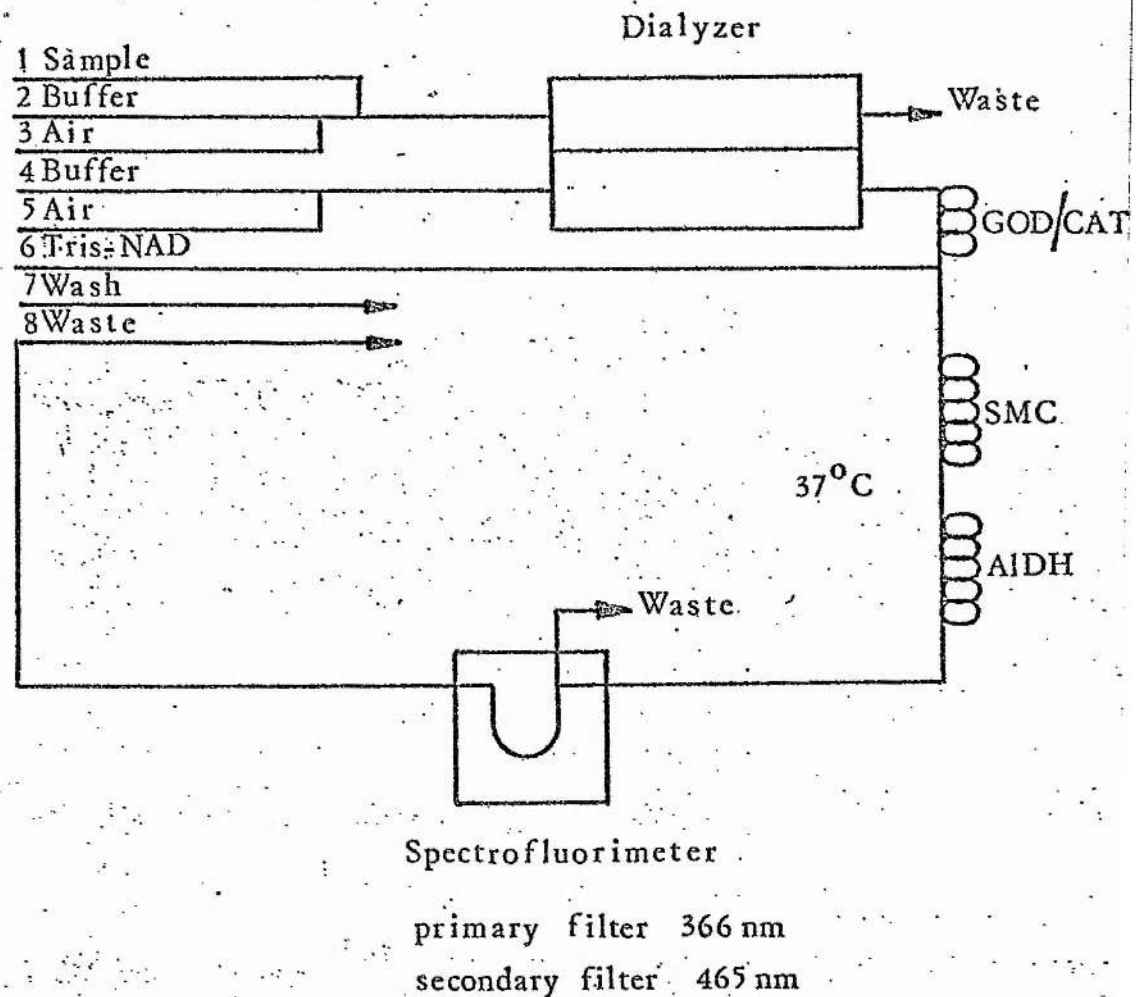


Fig. 48. Flow diagram of the analyser system used to assay plasma glucose with nylon tube immobilized GOD, CAT and AlDH. (SMC = single mixing coil).

The pump tubes on the manifold are arranged in the following manner:

Line.	Flow rate ml/min.
1. Sample	0.23
2. Buffer	2.0
3. Air	0.8
4. Buffer	2.0
5. Air	1.0
6. Tris-NAD^+	0.6
7. Sample wash	2.0
8. Waste	2.0

Results.

The calibration curve obtained in this method is linear over the complete range of standard solutions used ie. 0 - 22.2mM glucose (Fig. 49). The assay system can therefore be calibrated from a single standard. When glucose, hydrogen peroxide, acetaldehyde and NADH standard samples are introduced into the system, in the absence of the dialyser, a series of linear curves are obtained (Fig.50). These illustrate the percentage conversion of glucose, hydrogen peroxide and acetaldehyde in the reactions catalysed by the enzymes GOD (27.9%), CAT (52.9%) and AlDH (3.5%) respectively.

The sensitivity of the method increases with both ethanol and NAD^+ concentrations in the final reaction mixture. This is illustrated in Fig. 51 (ethanol) and Fig. 52 (NAD^+) where sensitivity is expressed in terms of the fluorescence given by the 22.2mM glucose standard. Maximum sensitivity is achieved at 0.17mM-ethanol and 1.3mM-NAD^+ although in the method proposed 0.17 M-ethanol and 0.37mM-NAD^+ are adopted as final reaction mixture concentrations (0.37mM-NAD^+ is the most cost-effective concentration of the coenzyme giving 85% of maximum sensitivity).

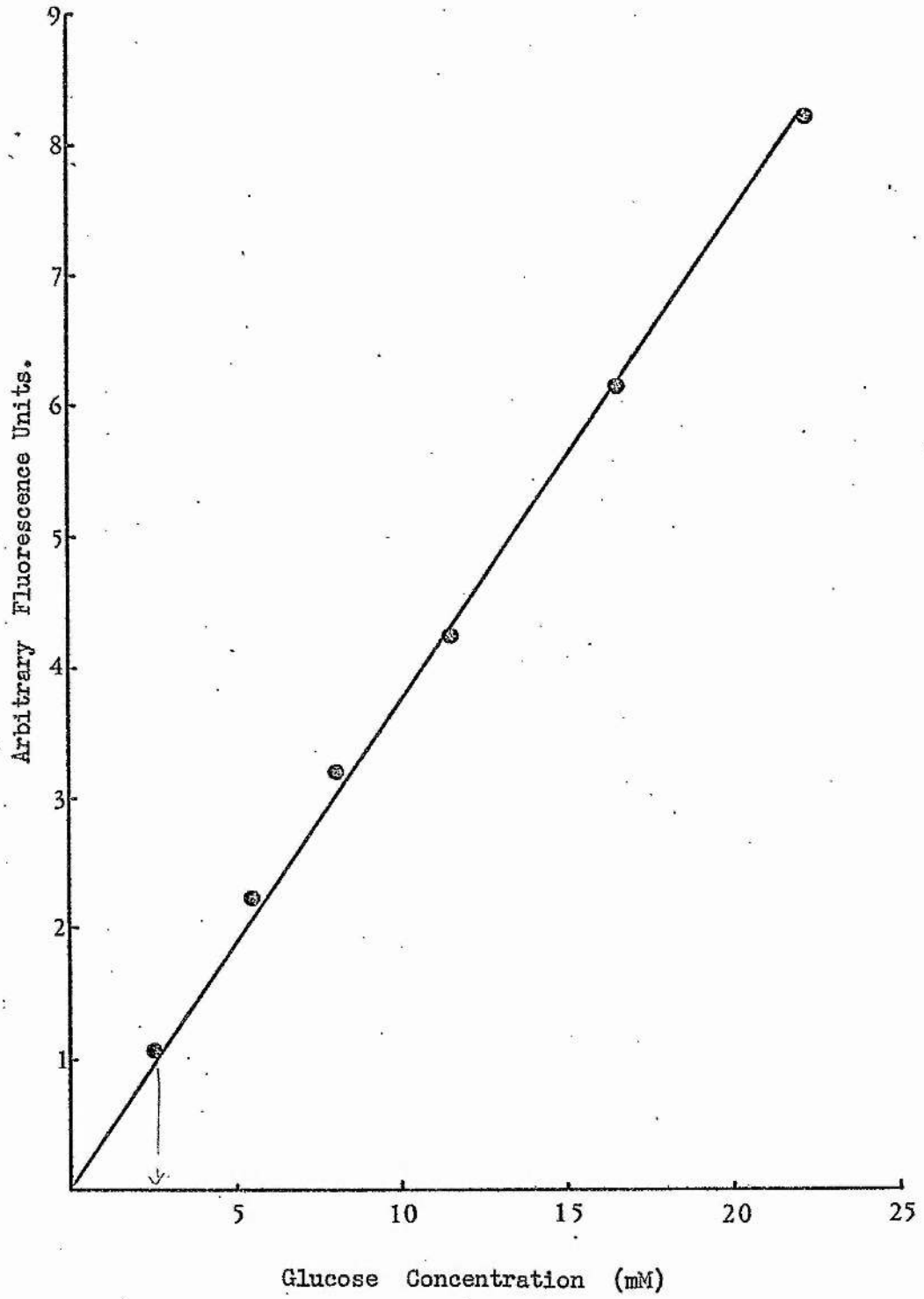


Fig. 49. Typical calibration curve obtained in the automated analysis of plasma glucose using nylon tube immobilized GOD/CAT & AlDH.

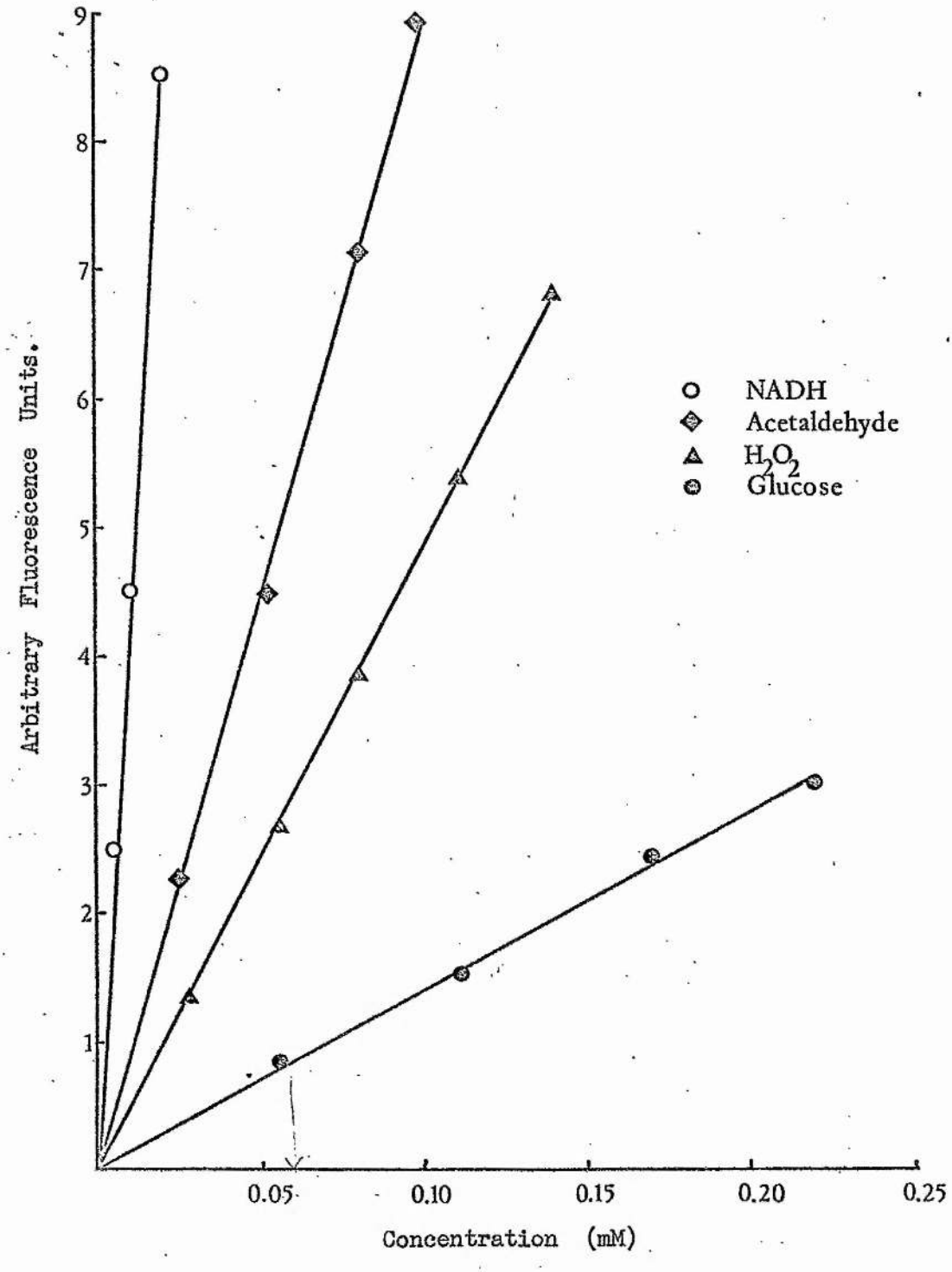


Fig. 50. Comparison of calibration curves of glucose, H_2O_2, acetaldehyde and NADH in the GOD/CAT/AlDH assay system.

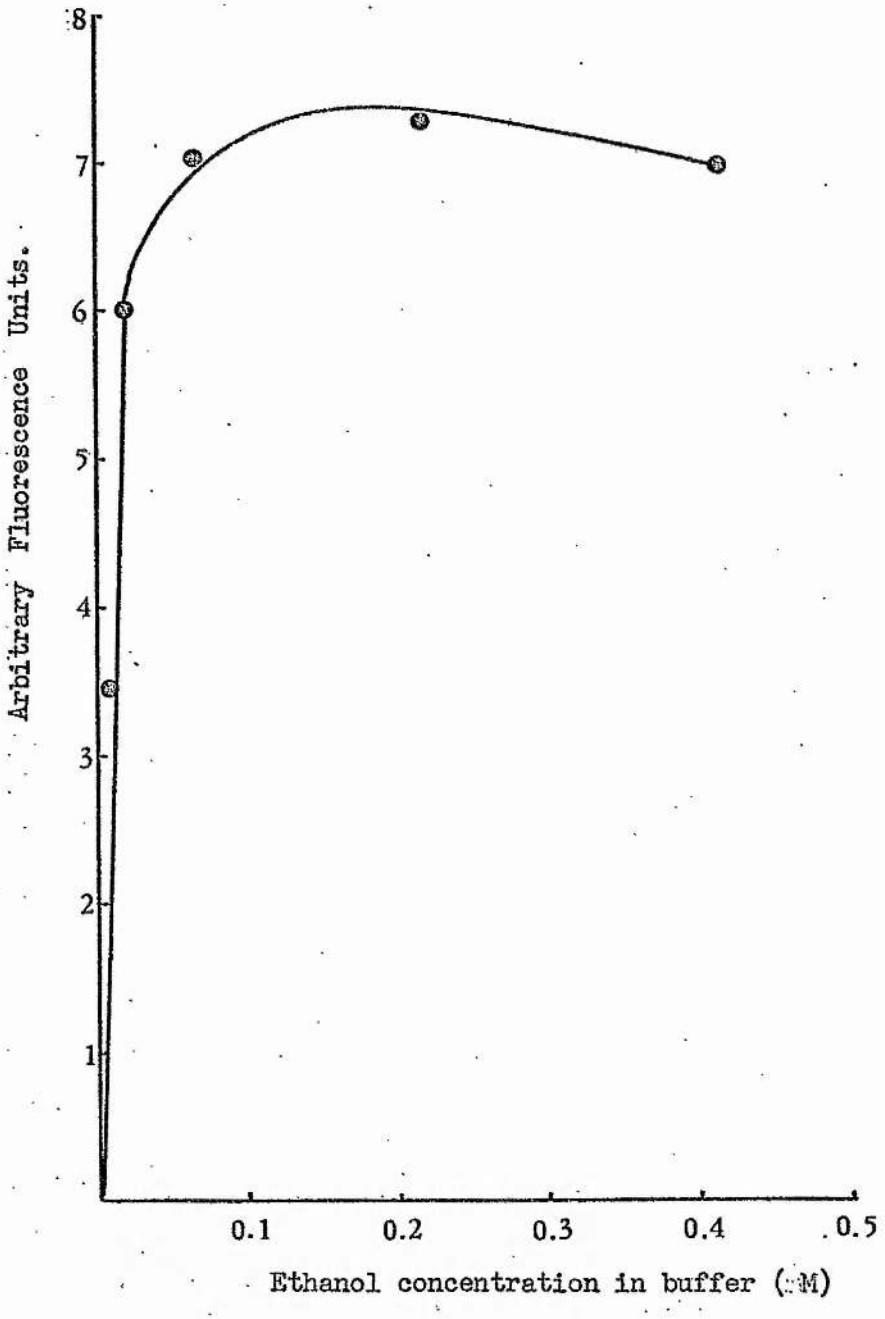


Fig. 51. Variation of sensitivity with ethanol concentration in the determination of glucose by the GOD/CAT/AlDH method. Sensitivity is expressed in terms of the fluorescence given by a 22.2mM glucose sample.

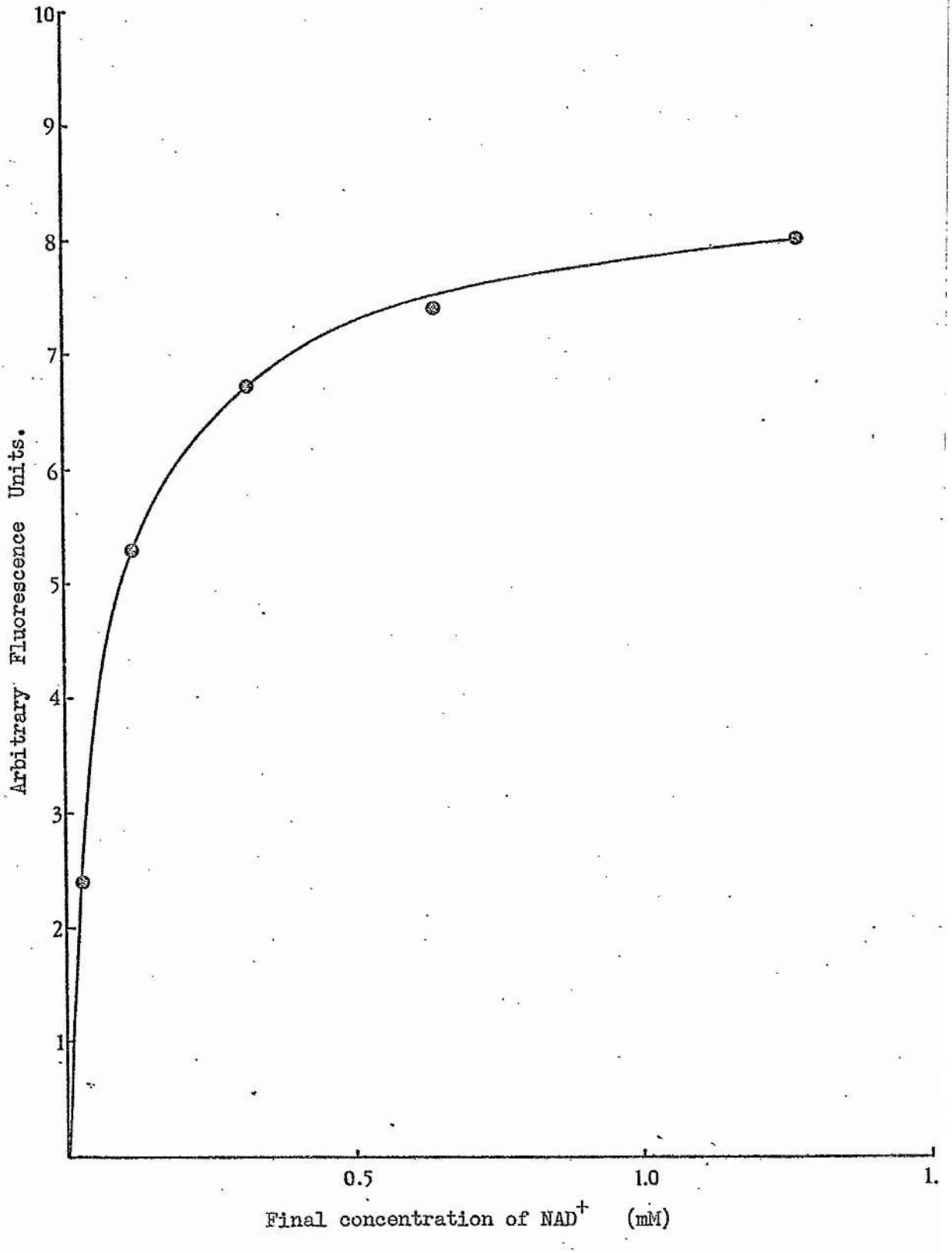


Fig. 52. Variation of sensitivity with NAD^+ concentration in the determination of glucose by the GOD/CAT/AlDH method. Sensitivity is expressed in terms of the fluorescence given by a 22.2 mM glucose sample.

Carryover in the system was assessed using the modified version of the method proposed by Broughton et al., (1969). Three high (a = 17.7mM) and three low (b = 3.0mM) plasma glucose specimens were sampled at a rate of 60/h. with a 2:1 sample:wash ratio (see Fig. 53) and the percentage carryover calculated from the equation:

$$\text{Carryover } c = \frac{b_1 - b_3}{a_3} \times 100\%$$

When a 50cm. length of nylon tube co-immobilized GOD/CAT and a 40cm. length of nylon tube immobilized AlDH are used in the flow system c = 1.6%. Thus the percentage error in the low b_1 value is 6.7% and this lies within the acceptable limits set by the formula of Tonk (1963);

$$\text{Allowable limits of error (maximum } \pm 10\%) = \frac{\frac{1}{4} \text{ of normal range}}{\text{mean of normal range}} \times 100\%$$

The precision of the method was determined by sampling 108 randomly distributed plasma specimens corresponding to glucose levels below, within and above the normal physiological range. The standard deviation and coefficient of variation calculated for each glucose level are shown in Table XI.

The accuracy of the method was determined by assaying a series of samples prepared from commercial quality control sera. Twenty such specimens were assayed in a blind experiment and the values given were compared with the expected values of the commercial sera assayed by an automated colorimetric method (Trinder, 1969) (Fig. 54). The calculated linear regression (y = 0.97x + 0.07) and the correlation coefficient (r = 0.997) indicate a very close agreement between the two methods.

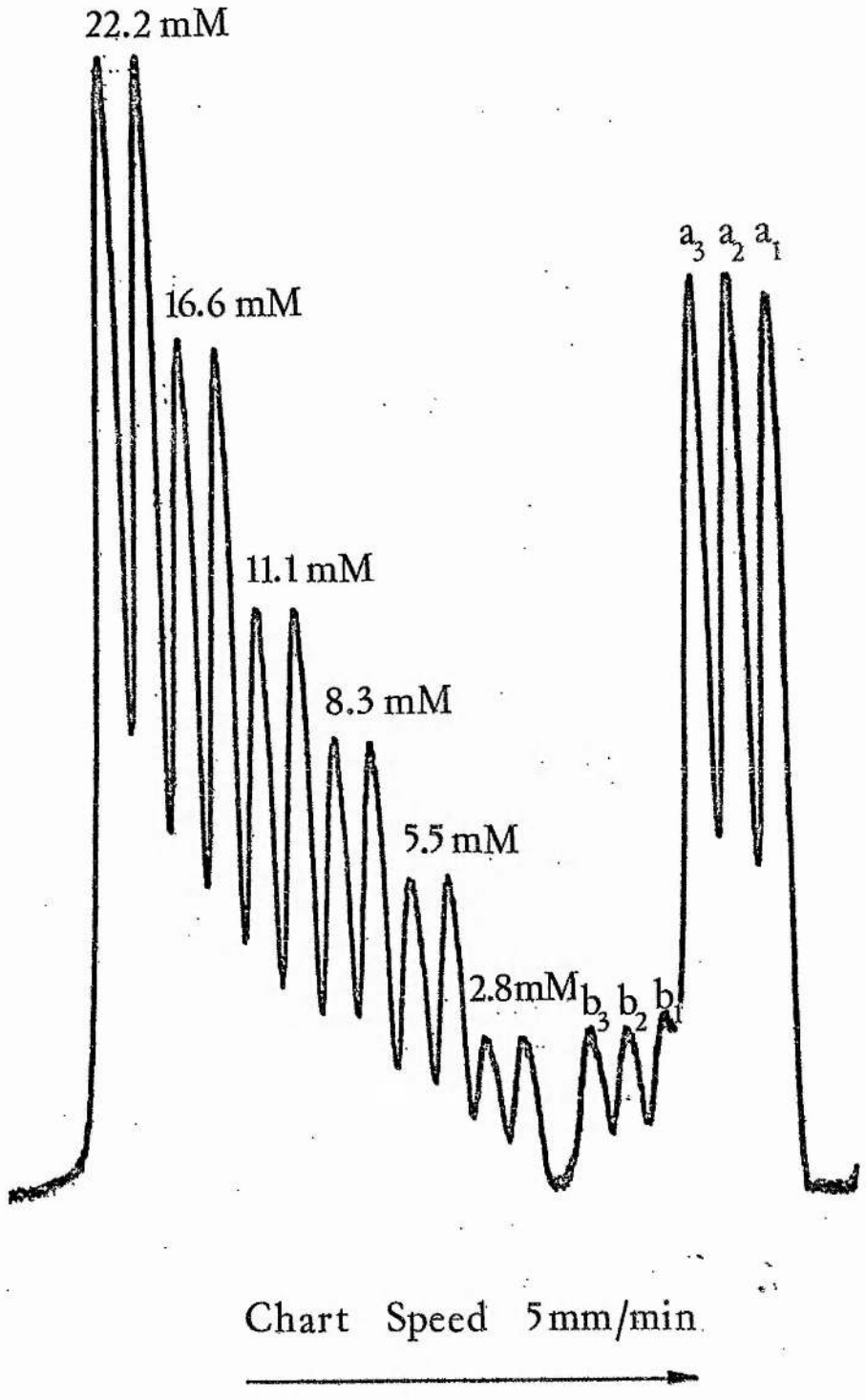


Fig. 53. Typical standard peaks obtained in the determination of glucose using the GOD/CAT/AlDH method and demonstration of carryover between high and low peaks at 60 samples/h., 2:1 sample:wash.

Number of assays	36	36	36
Mean	2.6mM	5.3mM	11.3mM
Standard Deviation	0.071mM	00.122mM	0.201mM
Coefficient of Variation	2.72%	2.36%	1.77%

Table XI Assessment of precision. Statistical analysis of results obtained by random sampling of three plasma glucose specimens in the immobilized GOD/CAT/AlDH assay system.

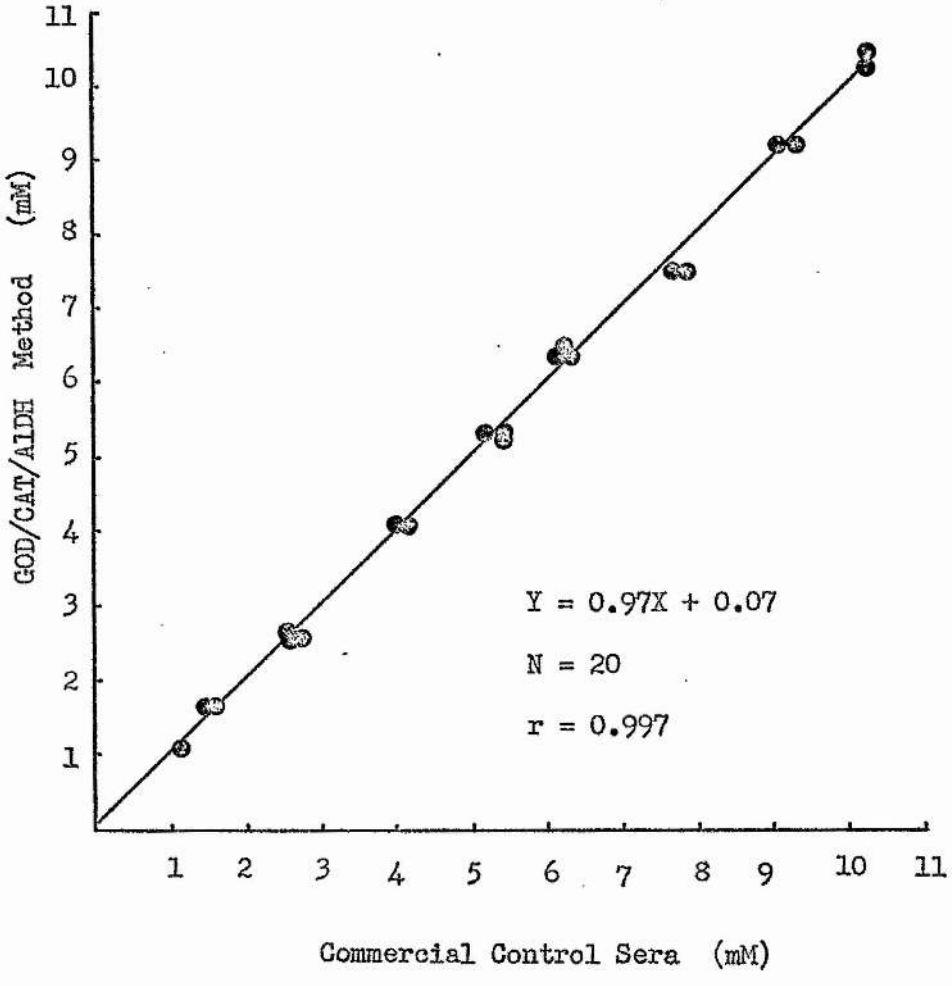


Fig. 54. Comparison of commercial control sera values and those obtained using the GOD/CAT/AlDH method of glucose determination.

4.3.2 The glucose dehydrogenase method.

This method uses nylon tube immobilized glucose dehydrogenase (GDH) to catalyse the oxidation of glucose to gluconolactone;

$$\text{Glucose} + NAD^+ \underset{\text{GDH}}{\rightleftharpoons} \text{Gluconolactone} + NADH + H^+$$

The reaction is monitored by measuring the fluorescence of the NADH formed.

Experimental.

Reagents.

1. 0.1M-Phosphate buffer, pH 7.0: 0.1M-NaH_2PO_4, 0.5M-NaCl titrated to pH 7.0 with 2M-NaOH and containing 1ml. 10% (v/v) Triton-X per 1.
2. Sample wash: 0.15M-NaCl.
3. Nicotinamide-adenine dinucleotide: 2.5mM-NAD^+ solution in water is freshly prepared. Grade III NAD^+ was obtained from Sigma London Chemical Co.Ltd.
4. Enzyme tube: GDH is immobilized on 1mm. bore nylon tube. Nylon tube was alkylated (section 2.3), then substituted with AH (section 2.4), activated with glutaraldehyde (section 2.5a) and coupled with the enzyme (section 2.5c). GDH (Bacillus subtilis) was supplied as a crystalline suspension in ammonium sulphate by Boehringer Mannheim GmbH. A 20cm. length of GDH-tube is used in the assay system.
5. Glucose standards: 2.5 - 25.0 mM glucose solutions in saturated benzoic acid. The 7.5 mM standard is used as a drift standard.

Method.

The flow system used for the determination of glucose using GDH is shown in Fig. 55. The apparatus comprises standard Technicon equipment together with the Perkin-Elmer 1000 Fluorescence

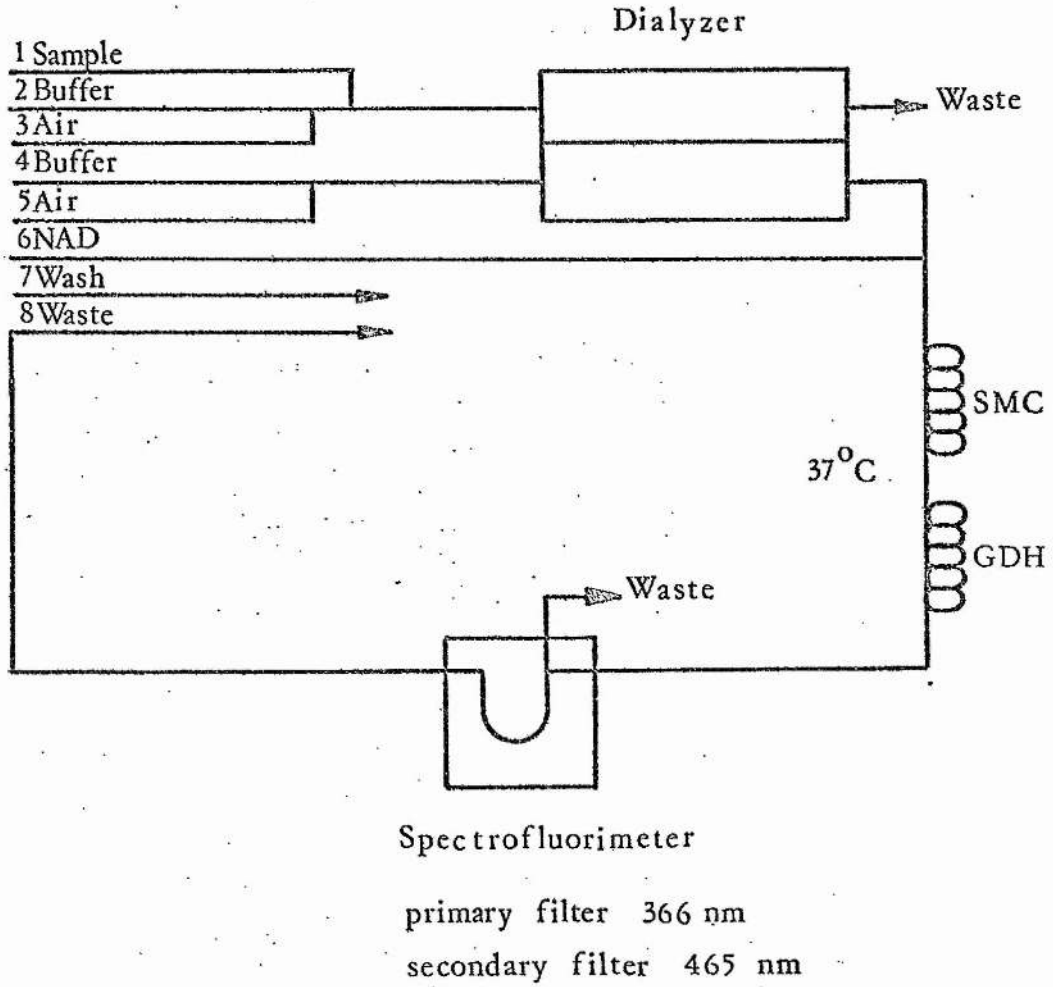


Fig. 55. Flow diagram of the analyser system used to assay plasma glucose concentrations with nylon tube immobilized GDH. (SMC = single mixing coil).

Spectrophotometer and Kipp-Zonen BD8 recorder.

Standards and plasma specimens are sampled at a rate of 60/h. with a 2:1 sample:wash ratio. Each sample is introduced into an air-segmented buffer stream and dialysed against a further buffer stream. The recipient stream is then supplemented with NAD^+ and passed through a 20cm. length of GDH-tube incubated at 37°C. After a debubbling step, standards and samples enter the flowcell of the fluorimeter where the NADH formed is monitored (λ_x 366nm.; λ_m 465nm)

The pump tubes on the manifold are arranged in the following manner;

Line	Flow rate ml/min.
1. Sample	0.23
2. Buffer	2.0
3. Air	0.8
4. Buffer	2.0
5. Air	1.0
6. NAD^+	0.6
7. Sample wash	2.0
8. Waste	2.0

Results.

The calibration curve obtained in this method describes a typical Michaelis-Menten curve (Fig. 56). Since the curve approaches linearity, the sensitivity varies little over the 0 - 25 mM glucose standard range.

Carryover in the system was assessed by the modified version of the method proposed by Broughton et al. (1969). Three high (a = 18.8mM) and three low (b = 2.5mM) plasma glucose specimens were sampled at a rate of 60/h. with a 2:1 sample:wash ratio (see Fig.57) and the percentage carryover calculated from the equation;

$$\text{Carryover } c = \frac{b_1 - b_3}{a_3} \times 100\%$$

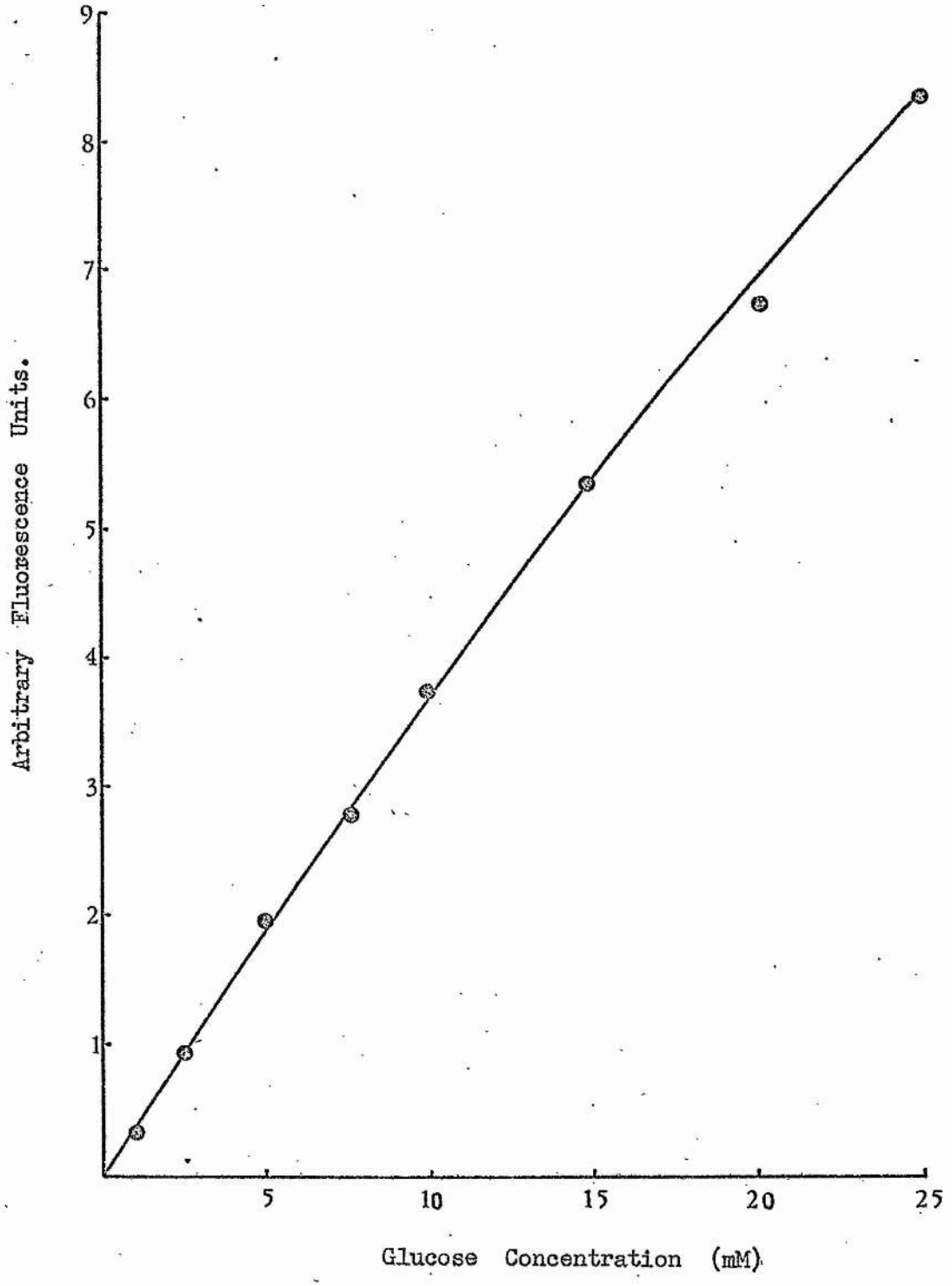


Fig. 56. Typical calibration curve obtained in the automated analysis of plasma glucose using nylon tube immobilized GDH.

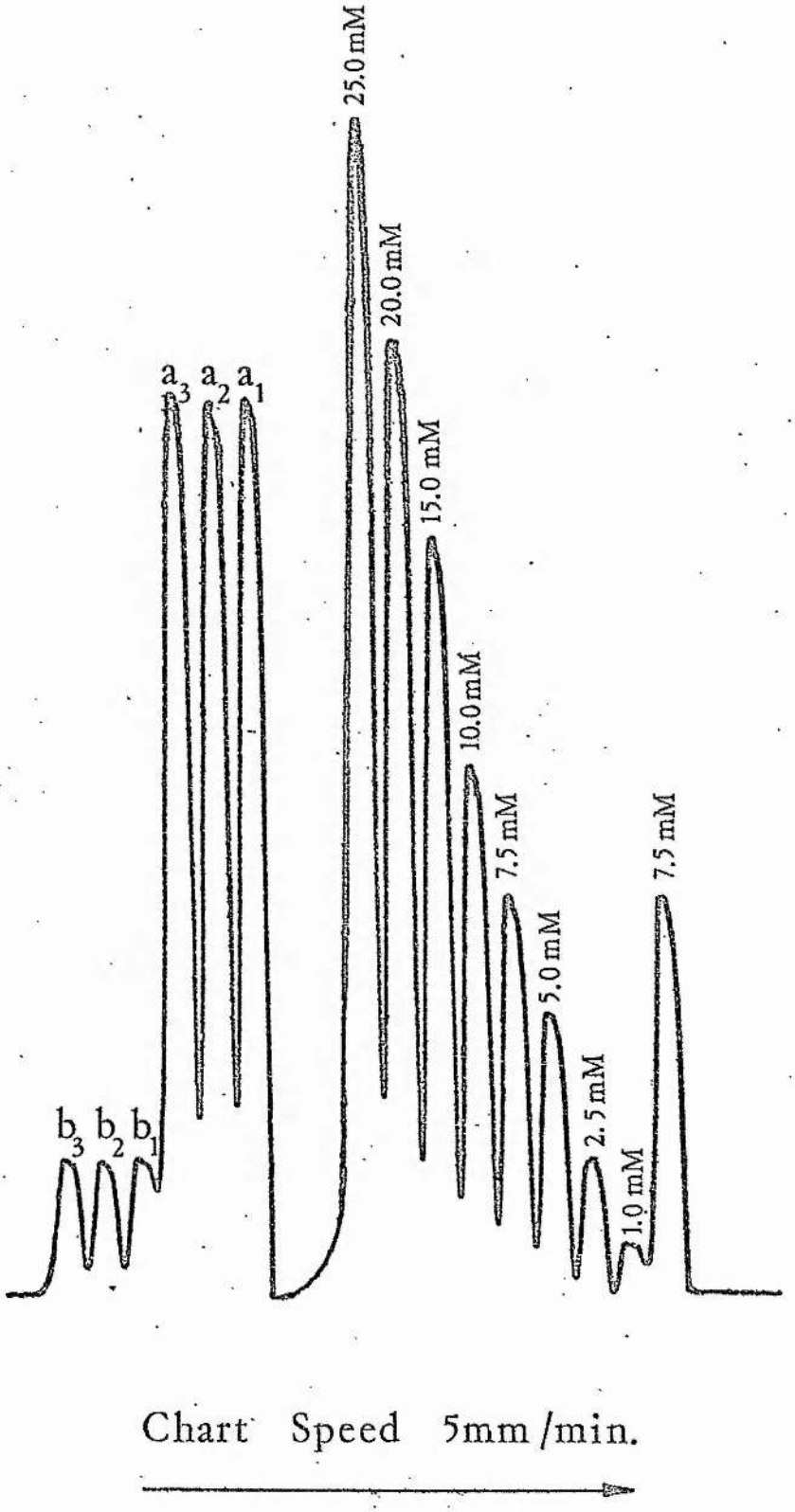


Fig. 57. Typical standard peaks in the immobilized GDH glucose assay system and demonstration of carryover between high and low peaks at 60 samples/h., 2:1 sample:wash.

When a 20cm. length of nylon tube immobilized GDH is used in the flow system c = 0.8%. Thus the percentage error in the low b_1 value is 6.4% and this lies within the acceptable limits set by the formula of Tonk (1963);

$$\text{Allowable limits of error (maximum } \pm 10\%) = \frac{\frac{1}{4} \text{ of normal range}}{\text{mean of normal range}} \times 100\%$$

The precision of the method was determined by sampling 108 randomly distributed plasma specimens corresponding to glucose levels below, within and above the normal physiological range. The standard deviation and coefficient of variation calculated for each glucose level are shown in Table XII.

The accuracy of the method was determined by comparison with the routine method used in the local hospital (Fig.58). Duplicate blood samples were taken from patients at a diabetic clinic and placed into fluoride-oxalate bottles; one set of specimens was analysed by the hospital laboratory using an automated colorimetric method (Trinder, 1969) and the other analysed using the GDH method. The calculated linear regression ($y = 0.98x - 0.09$) and the correlation coefficient ($r = 0.971$) indicate close agreement between the two methods.

Comment.

The two automated glucose methods described above employ enzyme systems which would be prohibitively expensive for routine analysis if the enzymes involved were not immobilized. The reusability of nylon tube immobilized enzymes reduces the cost of these assay methods and makes them attractive alternatives to established methods with which they compare favourably.

Clinically, the importance of glucose assays probably lies in the reliable detection of hypoglycaemic states. Therefore an

Number of assays	36	36	36
Mean	3.4mM	7.6mM	12.5mM
Standard Deviation	0.090mM	0.170mM	0.212mM
Coefficient of Variation	2.61%	2.23%	1.69%

Table XII. Assessment of precision. Statistical analysis of results obtained by random sampling of three plasma glucose specimens in the immobilized GDH assay system.

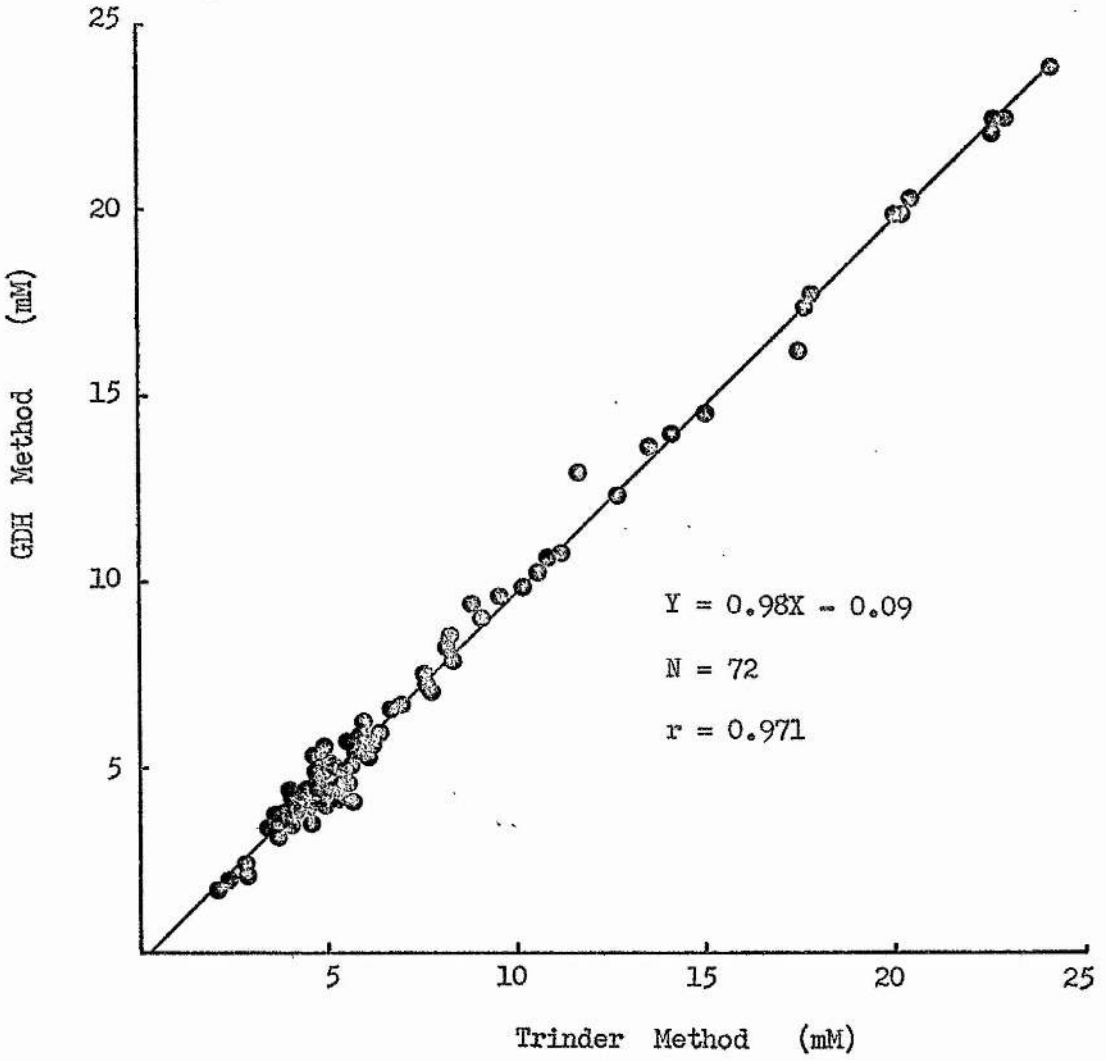


Fig. 58. Comparison of automated Trinder (1969) and proposed immobilized GDH methods of determining plasma glucose concentrations.

assay method which is accurate and sensitive at low glucose levels is required. In the detection of hyperglycaemic states in diabetes mellitus, so long as the same method is used for comparison, it is of little importance which method is adopted. Given the two methods described, the obvious choice for routine glucose analysis is the GDH method which has greater sensitivity, minimal carryover and simplicity. The GOD/CAT/AlDH method is more a demonstration of a hydrogen peroxide measuring system (CAT/AlDH) which, theoretically, can be linked to any system producing hydrogen peroxide. For example, Haeckel (1976) employed a soluble form of this enzyme system to measure uric acid in biological fluids using uricase in place of glucose oxidase.

4.4 Automated fluorimetric analysis of blood lactate.

Most routine methods of determining lactate concentrations in blood are based on the enzymatic oxidation of lactate to pyruvate in reactions catalysed by yeast or muscle lactic dehydrogenase. Yeast lactic dehydrogenase (YLDH) catalyses the transfer of electrons from lactate to ferricyanide;

$$\underset{\text{lactate}}{CH_3CHOHCOOH} + 2\ Fe(CN)_6^{3-} \xrightleftharpoons{YLDH} \underset{\text{pyruvate}}{CH_3COCOOH} + 2\ Fe(CN)_6^{4-} + 2\ H^+ \quad (1)$$

This reaction can be monitored by either amperometry (Durliat et al. 1976) or spectrophotometry (Mann and Shute, 1970). Muscle lactic dehydrogenase (MLDH) on the other hand catalyses the transfer of protons from lactate to NAD^+;

$$\underset{\text{lactate}}{CH_3CHOHCOOH} + NAD^+ \xrightleftharpoons{MLDH} \underset{\text{pyruvate}}{CH_3COCOOH} + NADH + H^+ \quad (2)$$

This reaction can be monitored by either fluorimetry (Cramp, 1968; Boycks et al., 1975) or spectrophotometry (Durliat et al., 1976).

The method described below adopts the MLDH approach to the analysis of lactate using the enzyme immobilized on nylon tube. The equilibrium for this oxidation-reduction reaction (equation 2) lies far to the left at physiological pH but it can be shifted to the right by removing the reaction products. This is accomplished by using a strongly alkaline buffer to remove the hydrogen ions and including hydrazine sulphate in that buffer to combine with the pyruvate to form the hydrazone. The NADH produced in the enzyme catalysed reaction is measured fluorimetrically.

Experimental.

Reagents.

1. 0.5M-Glycine buffer, pH 9.6: 0.5M-glycine, 0.25M-hydrazine sulphate titrated to pH 9.6 with 2M-NaOH. 1ml. 10% (v/v) Triton-X added per 1.
2. Sample wash: 0.15M-NaCl.
3. Nicotinamide-adenine dinucleotide: 2.5mM-NAD^+ solution in water is freshly prepared. Grade III NAD^+ was obtained from Sigma London Chemical Co.Ltd.
4. Enzyme tube: Rabbit muscle LDH immobilized on 1.5mm bore nylon tube. Nylon tube was alkylated (section 2.3), then substituted with HMDA (section 2.4), activated with glutaraldehyde (section 2.5a) and coupled with enzyme (section 2.5c). The enzyme was obtained from Sigma London Chemical Co.Ltd. A 60cm. length is used in the assay system.
5. Lactate standards: 0.06 - 1.14mM lactate prepared from a 3.5mM stock solution which had been assayed spectrophotometrically.
6. Protein precipitant: 0.6M-$HClO_4$

Method.

The flow system used for the determination of lactate using immobilized LDH is shown in Fig. 59. The apparatus comprises standard Technicon equipment together with the Perkin-Elmer 1000 Fluorescence Spectrophotometer and Kipp-Zonen BD8 recorder.

Specimens are deproteinised before they are analysed. 1ml. of whole blood is pipetted into 2ml. of ice-cold $HClO_4$ and the protein precipitate is removed by centrifugation. This treatment serves to prevent glycolysis and subsequent lactate production.

Standards and supernatants are sampled at a rate of 60 samples/h. with a 2:1 sample:wash ratio and introduced into an air-segmented buffer stream. The diluted samples are then supplemented with NAD^+ and passed through a 60cm. length of LDH-tube incubated at 37°C. After a debubbling step, they enter the flowcell of the fluorimeter where the NADH formed is monitored (λ_x 340nm.; λ_m 465nm.).

When calculating the concentration of lactate in whole blood account has to be taken of its specific gravity (1.06) and water content (80%). Since 1ml. of blood and 2ml. of $HClO_4$ are used the dilution factor is calculated from;

$$\text{Dilution } D = \frac{(\text{ml. of blood} \times \text{S.G.} \times \text{water content}) + \text{ml. of } HClO_4}{\text{ml. of blood}}$$

$$= \frac{(1 \times 1.06 \times 0.8) + 2}{1}$$

$$= 2.85$$

The pump tubes on the manifold are arranged in the following manner;

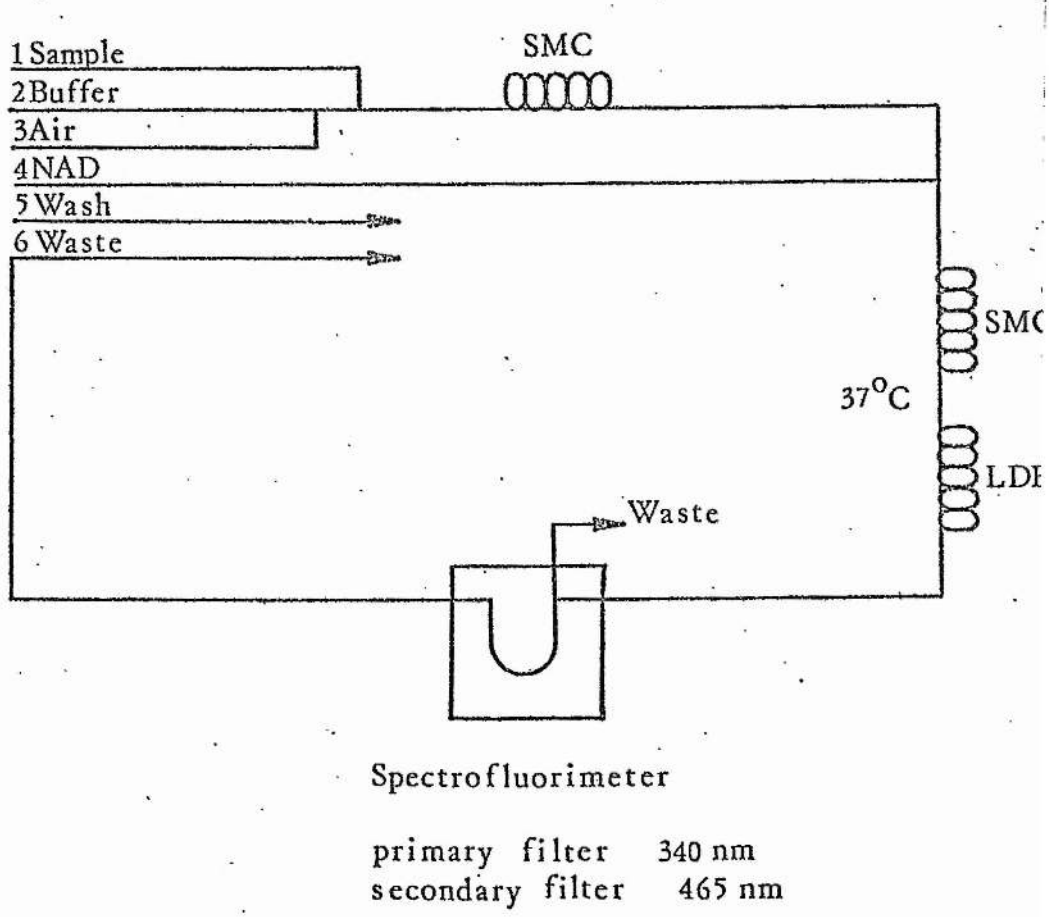


Fig. 59. Flow diagram of the analyser system used to assay blood lactate concentrations with nylon tube immobilized LDH.

Line	Flow rate ml/min.
1. Sample	0.23
2. Buffer	2.0
3. Air	0.8
4. NAD^+	0.6
5. Sample wash	2.0
6. Waste	2.0

Results.

The calibration curve obtained in this method describes a typical Michaelis-Menten curve (Fig. 60). Since the curve approaches linearity, the sensitivity varies little over the 0 - 1.14mM lactate standard range. The overall sensitivity of the method is determined by the NAD^+ concentration in the final reaction mixture and the tube length. The effect of NAD^+ concentration is illustrated in Fig. 61 where sensitivity is expressed in terms of the fluorescence given by the 1.14mM lactate standard. For economy the final NAD^+ concentration used in this assay system is limited to that which gives 85% of the maximum sensitivity (ie. 0.53mM).

Carryover in the system was assessed by the modified version of the method proposed by Broughton et al., (1969). Three high (a = 0.87mM) and three low (b = 0.39mM) lactate solutions were sampled at a rate of 60/h. with a 2:1 sample:wash ratio (see Fig. 62) and the percentage carryover calculated from the equation;

$$\text{Carryover } c = \frac{b_1 - b_3}{a_3} \times 100\%$$

When a 60cm. length of nylon tube immobilized LDH is used in the flow system c = 0.88%. Thus the percentage error in the low a_1 value is only 3% which is well within the 10% limit set by Tonks (1963);

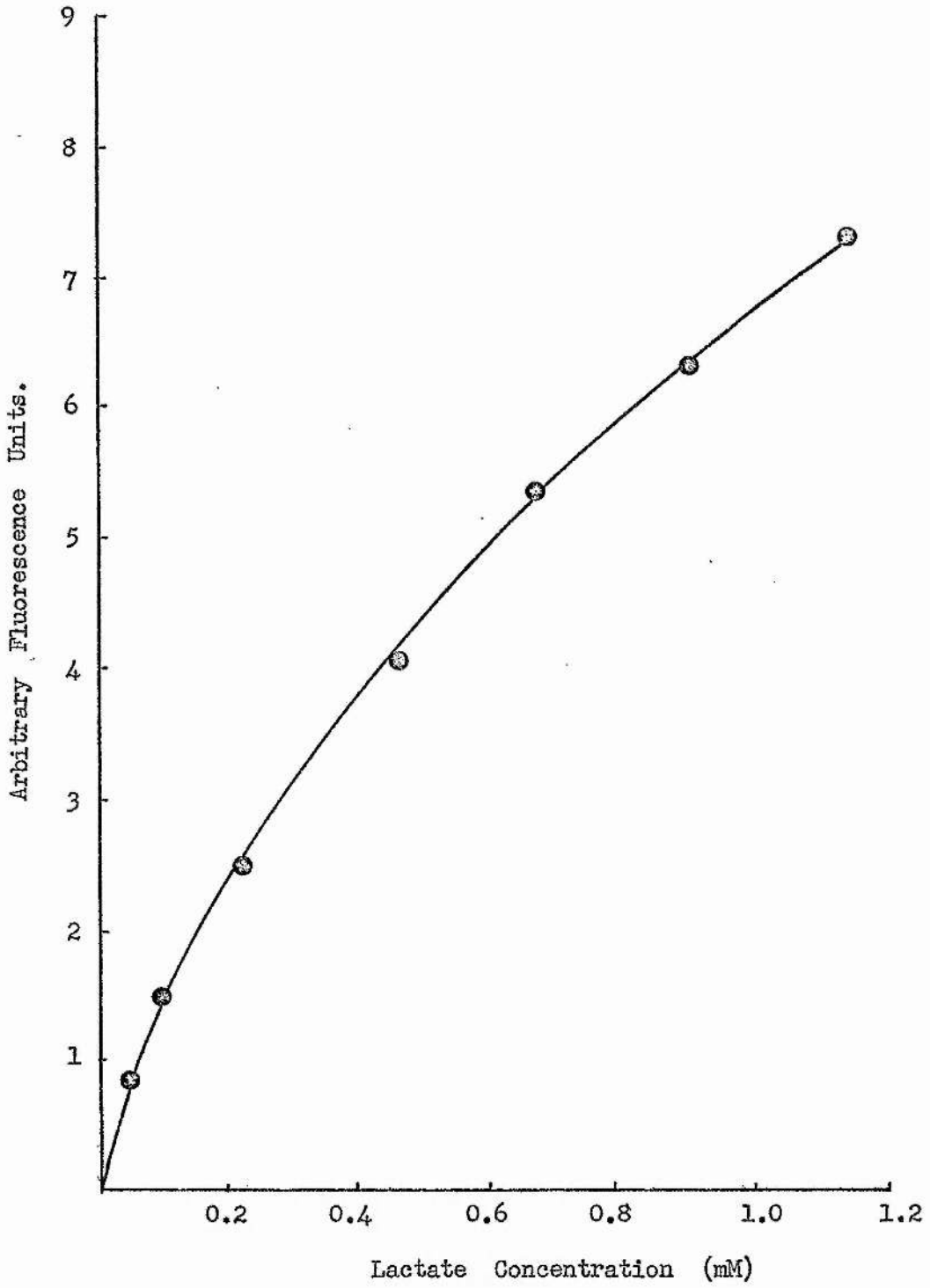


Fig. 60. Typical calibration curve obtained in the automated analysis of blood lactate using nylon tube immobilized LDH.

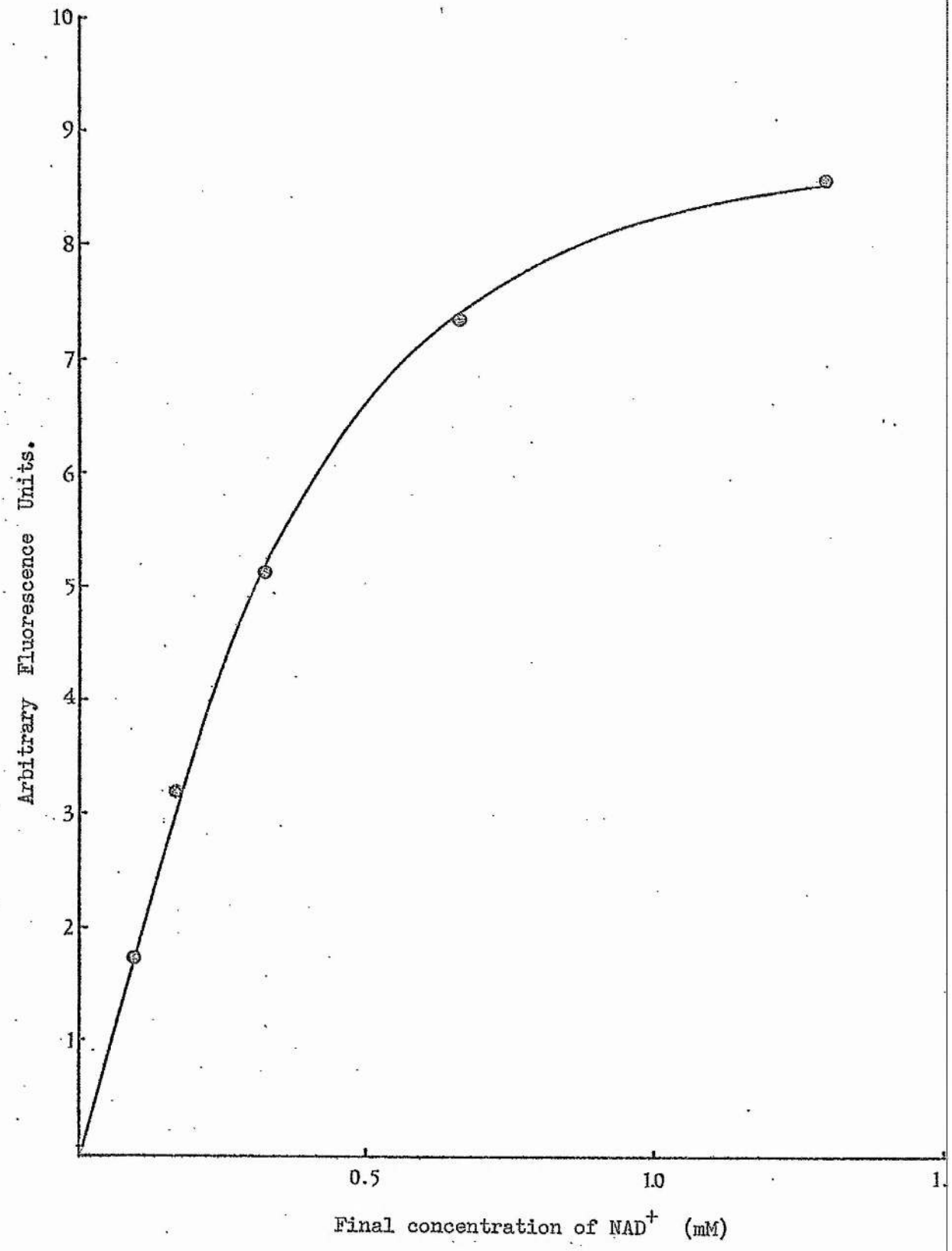


Fig. 61. Variation of sensitivity with NAD^+ concentration in the automated analysis of blood lactate using nylon tube immobilized LDH. Sensitivity is expressed in terms of the fluorescence given by a 1.14 mM lactate standard.

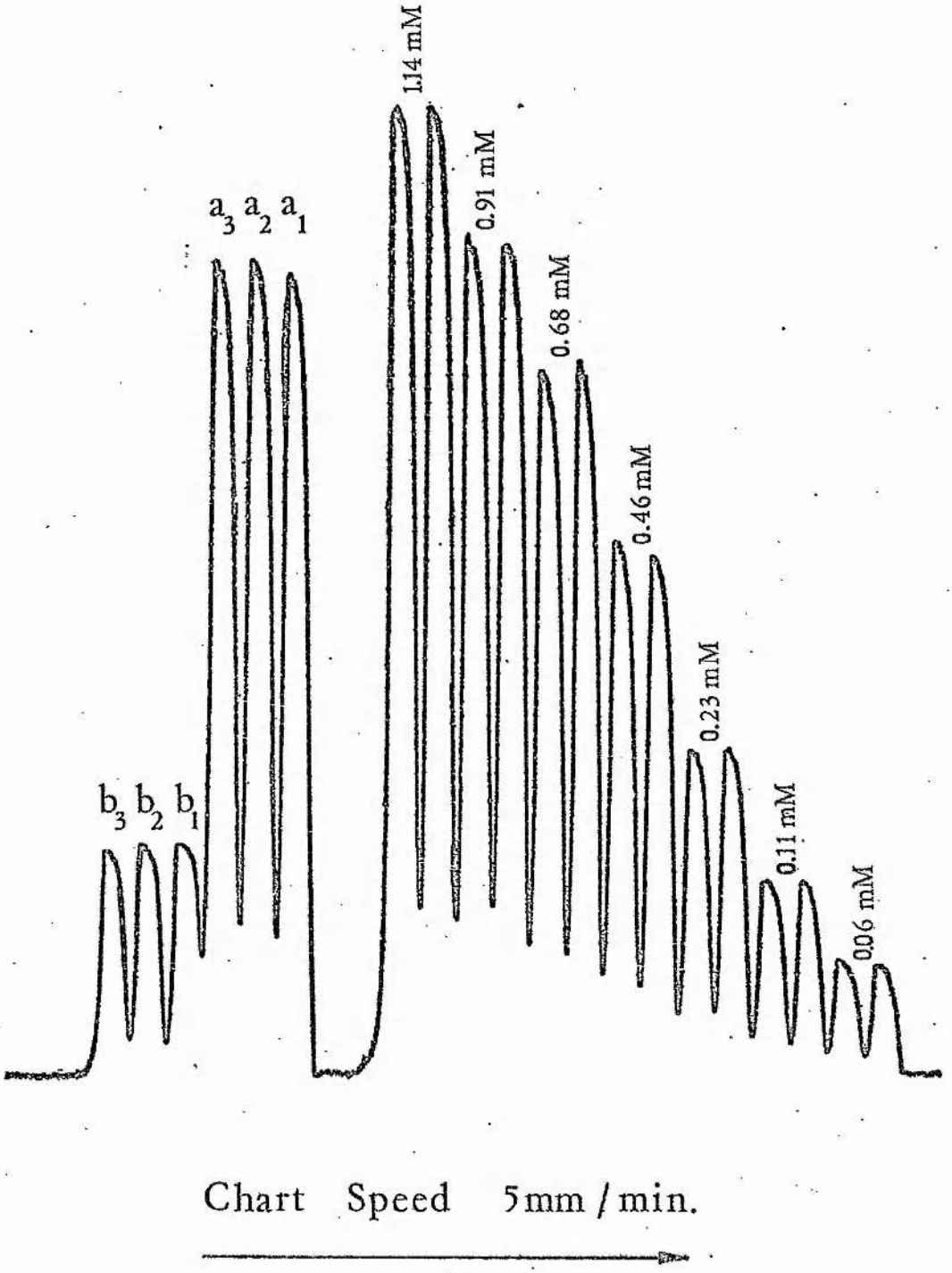


Fig. 62. Typical standard peaks obtained in the immobilized LDH lactate assay system and demonstration of carryover between high and low peaks at 60 samples/h., 2:1 sample:wash.

$$\text{Allowable limits of error} = \frac{\pm \text{ of normal range}}{\text{mean of normal range}} \times 100\%$$

The precision of the method was determined by sampling 98 randomly distributed lactate specimens corresponding to levels below, within and above the normal physiological range. The standard deviation and coefficient of variation calculated for each lactate level are shown in Table XIII.

The accuracy of the method was assessed by assaying a commercial aqueous 3.33mM lactate standard (Sigma London Chemical Co.Ltd.). The standard lactate was diluted with $HClO_4$ and assayed along with the blood supernatants. Exact agreement was observed between the estimated and commercial values.

Comment.

The nylon tube immobilized LDH used in this assay system is prepared by covalently linking the enzyme to nylon via a HMDA-glutaraldehyde bridge because the activity is greater when the enzyme is immobilized in this way (Table I). Although a 60cm. length of this enzyme tube has to be used to achieve the required sensitivity, the expected charge effects and increased carryover with the HMDA spacer are virtually eliminated by the high pH of the reaction. Thus the enzyme tube is working under conditions of optimum performance.

Today great importance is being placed on the blood lactate concentrations of intensive care patients to assess the severity of shock states and circulatory defects. This immobilized enzyme assay for lactate makes an ideal emergency on-call method because of its speed, simplicity and good performance. An added advantage with this system is that it can be kept operational for long periods without either loss of sensitivity through enzyme instability or loss of expensive enzyme discarding to waste.

Number of assays	32	33	33
Mean	0.13mM	0.30mM	0.75mM
Standard Deviation	0.004mM	0.007mM	0.013
Coefficient of Variation	3.5%	2.5%	1.7%

Table XIII. Assessment of precision. Statistical analysis of results obtained by random sampling of three lactate specimens in the immobilized LDH assay system.

5. The stability of enzyme tubes.

5.1 Introduction.

Enhanced stability of an immobilized enzyme is one of the basic requirements for its use in analysis. In order to reduce the cost of enzyme-based analysis, an immobilized enzyme must be able to be gainfully employed over prolonged periods. Therefore, it is necessary to evaluate its overall stability before it can be used routinely. This can be achieved in the following manner;

1. Subject the enzyme derivative to exaggerated conditions of denaturation while monitoring the progressive loss in enzyme activity. The rate of accelerated deactivation of the immobilized enzyme is then compared with that of the soluble enzyme; the method adopted in this work is that of thermal deactivation. Both soluble and immobilized forms of the enzyme are exposed to high temperature and their relative stabilities are measured in terms of the activity half-life value ($t_{\frac{1}{2}}^{t}$), that is, the time required for the enzyme to lose half its initial activity.

2. Determine the stability by incorporating the enzyme derivative into the analyser system for which it is intended and running that system continuously under normal working conditions. This will give an operational stability, an index more useful than thermal stability to the analyst considering the merits of a method. Again, the stability is measured in terms of the activity half-life value ($t_{\frac{1}{2}}^{o}$).

3. Determine the storage stability or shelf-life of the immobilized enzyme which should be assessed for a complete stability characterisation.

5.2 Measurement of thermal stability of nylon tube immobilized enzymes.

a. Alcohol dehydrogenase.

Reagents.

1. 0.1M-Pyrophosphate buffer, pH 9.0: 0.1M-$Na_2P_2O_7$, 0.1M-semicarbazide is titrated to pH 9.0 with 2M-HCl.
2. Ethanol.
3. Nicotinamide-adenine dinucleotide: a) 80mM-NAD^+ solution for soluble enzyme assay; b) 300mM-NAD^+ solution for immobilized enzyme assay. Both solutions are stored at 4°C. Grade III NAD^+ was obtained from Sigma London Chemical Co.Ltd.,
4. Soluble enzyme: 10 U/ml. Horse liver alcohol dehydrogenase (LADH). The enzyme was obtained from Sigma London Chemical Co.Ltd. as a lyophilised powder.
5. Enzyme tube: LADH immobilized on 1mm. bore nylon tube. Nylon tube is alkylated (section 2.3), then substituted with DAE or AH (section 2.4), activated with glutaraldehyde (section 2.5a) or bisimidate (section 2.5b) and finally coupled with the enzyme (section 2.5c). 10 cm. lengths of LADH-tube are used in the assay system.

Method.

Soluble enzyme: 4.9ml. of pyrophosphate buffer without semicarbazide is incubated at 60°C in a water bath and is continuously stirred. At zero time, 0.1ml. of the LADH solution is added to the buffer and 0.1ml. samples are taken out at intervals to be assayed.

The activity of LADH is determined by measuring the rate of increasing absorption at 340nm. due to the production of NADH in the enzyme-catalysed oxidation of ethanol (Bücher and Redetzki, 1951). The acetaldehyde formed in the reaction is effectively removed by condensation with semicarbazide.

$$C_2H_5OH + NAD^+ \xrightleftharpoons{LADH} CH_3CHO + NADH + H^+$$

ethanol — acetaldehyde

All assays are performed at 25°C in reaction mixtures of the following composition:

	Test	Blank
Buffer / semicarbazide	2.5ml.	2.6ml.
Ethanol	0.1ml.	0.1ml.
NAD^+	0.1ml.	0.1ml.
Enzyme	0.1ml.	-

In each case the reaction is initiated by the addition of the enzyme and the change is absorption is measured relative to the blank solution. A zero time reading is obtained by using 0.1ml. of a 1 in 50 dilution of the original enzyme solution.

Immobilized enzyme: 10 cm. lengths of each immobilized enzyme derivative are placed into respective test-tube containing pyrophosphate buffer without semicarbazide and incubated at 60°C in a water bath. At intervals, one piece of each derivative is taken from its test-tube and assayed by the recycling method of Ford et al. (1972) (section 2.7.2). The method is basically the same as that used for the soluble enzyme with concentrations of substrates adjusted for the greater reaction volume. All assays are performed at 25°C in reaction mixtures of the following composition:

Buffer / semicarbazide	9.5ml.
Ethanol	0.4ml.
NAD^+	0.1ml.

In each case the reaction is initiated by the addition of NAD^+ and the increase in absorption at 340nm is measured. A zero time activity is obtained by assaying a 10 cm. length of untreated LADH-tube.

b. Lactic dehydrogenase.

Reagents.

1. 0.1M-Phosphate buffer, pH 7.5: 0.1M-NaH_2PO_4, 0.15mM-EDTA, 5mM-dithiothreitol is titrated to pH 7.5 with 2M-NaOH.
2. Reduced nicotinamide-adenine dinucleotide: a) 6mM-NADH for soluble enzyme assay, b) 10mM-NADH for immobilized enzyme assay. Solutions are prepared in 1mM-tris and stored at 4^oC. Grade III disodium-NADH was obtained from Sigma London Cherical Co.Ltd.
3. Sodium pyruvate: a) 30mM solution for the soluble enzyme assay b) 100mM solution for immobilized enzyme assay. Material was obtained from Sigma London Chemical Co.Ltd.
4. Soluble enzyme: 90 U/ml. Rabbit muscle lactic dehydrogenase (LDH). The enzyme was obtained from Sigma London Chemical Co. Ltd. as a lyophilised powder.
5. Enzyme tube: LDH immobilized on 1mm. bore nylon tube. Nylon tube is alkylated (section 2.3), then substituted with DAE (section 2.4), activated with glutaraldehyde (section 2.5a) and finally coupled with the enzyme (section 2.5c). 10 cm. lengths of LDH-tube are used in the assay system.

Method.

Soluble enzyme: 4.9ml. of phosphate buffer is incubated at 45^oC in a waterbath and is continuously stirred. At zero time, 0.1ml. samples are taken out at intervals to be assayed.

The activity of LDH is determined by measuring the rate of decrease in absorption at 340nm. due to the depletion of NADH in the

enzyme-catalysed reduction of pyruvate (Reeves and Fimognari, 1963).

$$CH_3.\underset{\underset{O}{\|}}{C}.COOH + NADH + H^+ \rightleftharpoons[\text{LDH}] CH_3.\underset{\underset{OH}{|}}{CH}.COOH + NAD^+$$

All assays are performed at 25°C in reaction mixtures of the following composition:

Buffer	2.7ml.
NADH	0.1ml.
Enzyme	0.1ml.
Pyruvate	0.1ml.

In each case the reaction is initiated by the addition of pyruvate. A zero time reading is obtained by using 0.1ml. of a 1 in 50 dilution of the original enzyme solution.

Immobilized enzyme: 10cm. lengths of the immobilized enzyme derivative are placed into a test-tube filled with the phosphate buffer and incubated at 45°C. At intervals, one piece of derivative is taken from the test-tube and assayed by the recycling method of Ford et al. (1972)(section 2.7.2). The method is basically the same as that used for the soluble enzyme with concentrations of substrates adjusted for the greater reaction volume. All assays are performed at 25°C in reaction mixtures of the following composition:

Buffer	9.7ml.
NADH	0.2ml.
Pyruvate	0.1ml.

In each case the reaction is initiated by the addition of pyruvate and the decrease in absorption at 340nm. is measured. A zero time activity is obtained by assaying a 10 cm. length of untreated LDH-tube.

The above procedure is repeated at 55°C with LDH-tubes having different amounts of protein bound to the nylon surface.

5.3 Measurement of operational stability of nylon tube immobilized enzymes.

a. Alcohol dehydrogenase.

Reagents.

As used in the determination of blood ethanol described in methodologies (section 4.2).

Method.

The flow system used is that used in the determination of blood ethanol (section 4.2) and is illustrated in Fig. 63.

Bovine serum containing ethanol is sampled at a rate of 60 samples/h. with a 2:1 sample:wash ratio. The concentration of ethanol used is approximately 65mM and corresponds to the top standard used in the automated determination of blood ethanol. Each sample is introduced into an air-segmented buffer stream and dialysed against a further stream of buffer. The recipient stream is then supplemented with NAD^+ and passed through a 10 cm. length of LADH-tube incubated at 37°C. After a debubbling step, samples enter the flowcell of the fluorimeter where the NADH is monitored (λ_x 366nm; λ_m 465nm.) and recorded as a series of peaks at 1 min. intervals. The system is allowed to return to baseline once every 40 min. to correct for drift.

The pump tubes on the manifold are arranged in the following manner;

Line	Flow rate ml/min.
1. Sample	0.1
2. Buffer	2.0
3. Air	1.0
4. Buffer	2.0

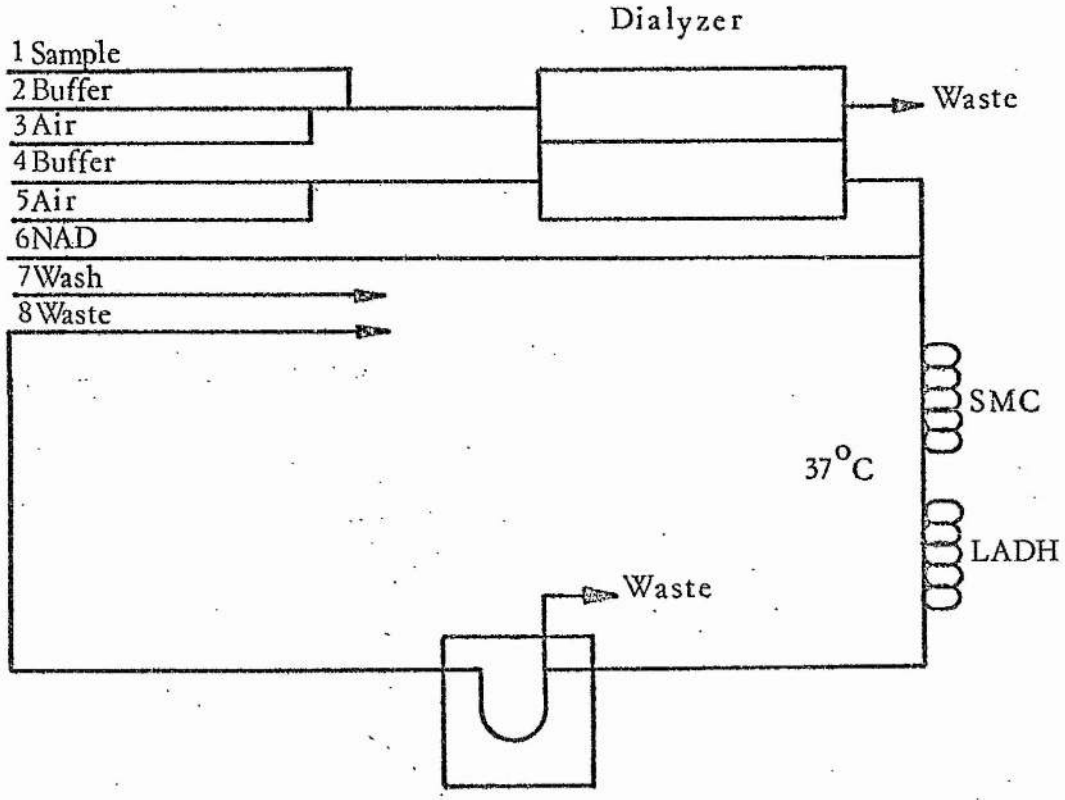


Fig. 63. Flow diagram of the analyser system used to measure the operational stability of nylon tube immobilized LADH. (SMC = single mixing coil).

Line	Flow rate ml/min.
5. Air	1.0
6. NAD^+	0.6
7. Sample wash	2.0
8. Waste	2.0

b. Aldehyde dehydrogenase.

Reagents.

1. 0.1M-Tris buffer, pH 8.5. 0.1M-tris, 1M-KCl titrated to pH 8.5 with 2M-HCl and containing 1ml. 10% (v/v) Triton-X per 1.
2. Sample wash: 0.15M-NaCl.
3. Nicotinamide-adenine dinucleotide: 7.5mM-NAD^+ solution is prepared and stored at 0^oC. AA-grade NAD^+ was obtained from Sigma London Chemical Co.Ltd.
4. Acetaldehyde: 3mM-CH_3CHO, 5mM-mercaptoethanol solution freshly prepared and stored at 0^oC during analysis.
5. Enzyme: Yeast aldehyde dehydrogenase (AlDH) immobilized on 1.5mm. bore nylon tube. Nylon tube was alkylated (section 2.3), then substituted with DAE (section 2.4), activated with glutaraldehyde (section 2.5a) and coupled with enzyme (section 2.5c). A 50 cm. length of AlDH-tube is used in the assay system.

Method.

In the methodology section 4.3.1, it can be seen that AlDH is used together with two other enzymes, GOD and CAT, in the analysis of plasma glucose. Therefore, in order to measure the stability of AlDH only, a flow circuit has to be designed to simulate the operational conditions of AlDH in that proposed method. The flow system used is illustrated in Fig. 64.

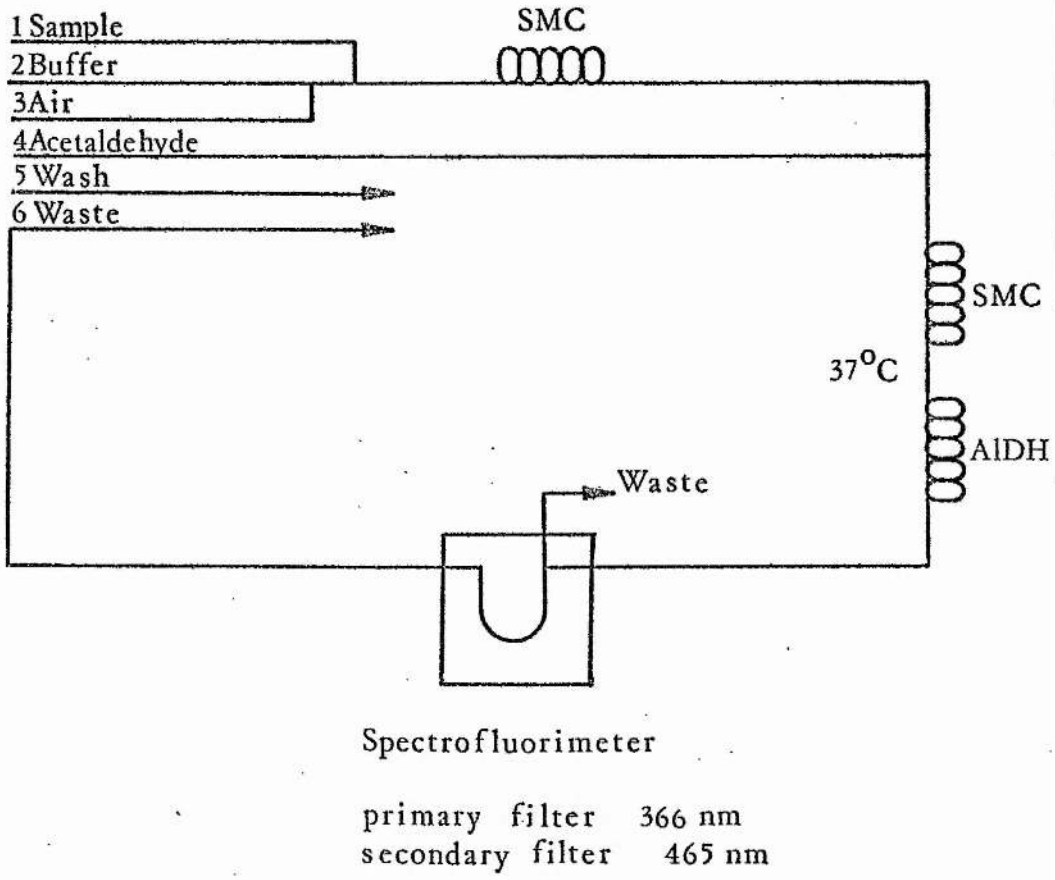


Fig. 64. Flow diagram of the analyser system used to measure the operational stability of nylon tube immobilized AlDH. (SMC = single mixing coil).

The NAD^+ solution is sampled at a rate of 60 samples/h. with a 2:1 sample:wash ratio and introduced into an air-segmented stream of tris buffer. After addition of the acetaldehyde, the reagent stream is incubated at 37°C and passed through the AlDH-tube. The NADH produced in the enzyme-catalysed oxidation of acetaldehyde is measured fluorimetrically (λ_x 366nm.; λ_m 465nm.) and recorded as a series of peaks at 1 min. intervals. The system is allowed to return to baseline once every 40 min. to correct for drift.

The pump tubes on the manifold are arranged in the following manner;

Line.	Flow rate ml/min.
1. Sample	0.1
2. Buffer	2.0
3. Air	1.0
4. Acetaldehyde	0.6
5. Sample wash	2.0
6. Waste	2.0

c. Lactic dehydrogenase.

Reagents.

As used in the determination of blood lactate described in methodologies section 4.4.

Method.

The flow system used is that used in the determination of blood lactate (section 4.4.) and is illustrated in Fig. 65.

The assay sample used to determine the operational stability of immobilized LDH is first deproteinised before analysis. 1 ml. of whole blood (3mM-lactate) is pipetted into 2 ml. of ice-cold $HClO_4$ and the protein precipitate is removed by centrifugation.

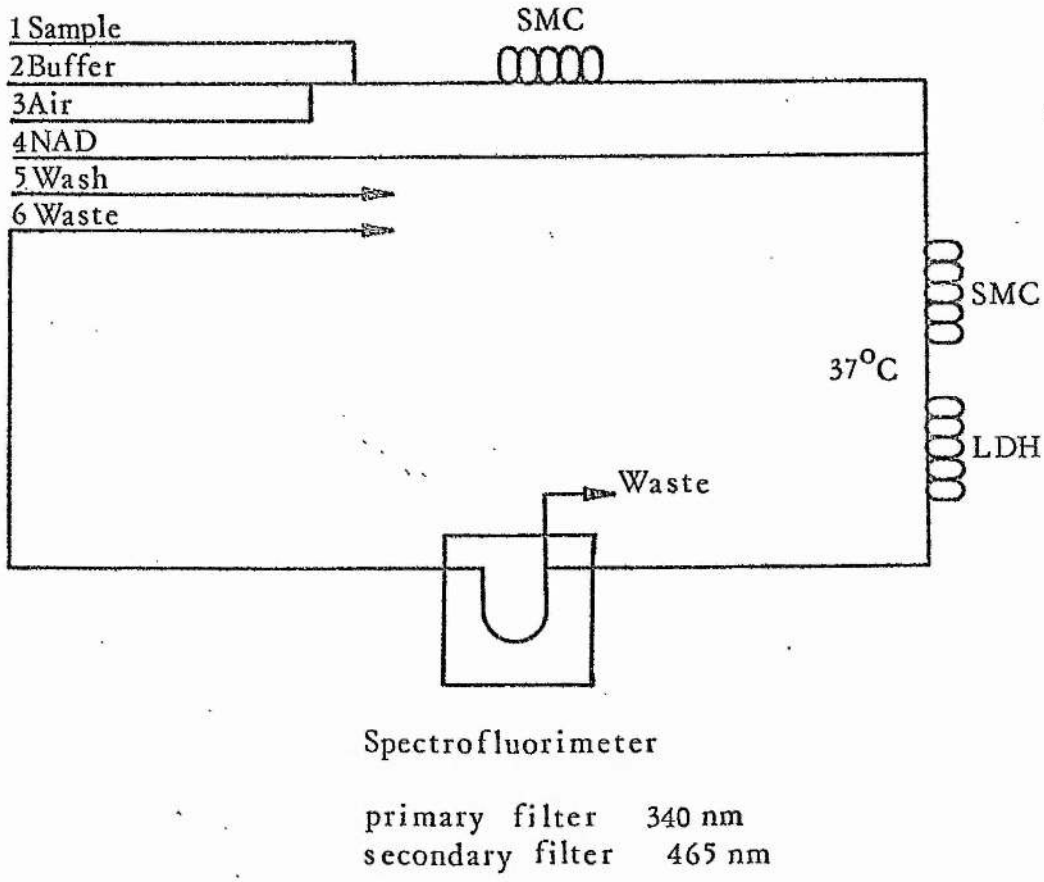


Fig. 65. Flow diagram of the analyser system used to measure the operational stability of nylon tube immobilized LDH. (SMC = single mixing coil).

The supernatant is sampled at a rate of 60 samples/h. with a 2:1 sample:wash ratio and introduced into an air-segmented buffer stream. The diluted samples are then supplemented with NAD^+ and passed through a 60 cm. length of LDH-tube incubated at 37°C. After a debubbling step, they enter the flowcell of the fluorimeter where the NADH formed is monitored (λ_x 340nm.; λ_m 465nm.) and recorded as a series of peaks. The system is allowed to return to baseline once every 40 min. to correct for drift.

The pump tubes on the manifold are arranged in the following manner;

Line	Flow rate ml/min.
1. Sample	0.23
2. Buffer	2.0
3. Air	0.8
4. NAD^+	0.6
5. Sample wash	2.0
6. Waste	2.0

d. Glucose dehydrogenase.

Reagents.

As used in the determination of plasma glucose described in methodologies section 4.3.2.

Method.

The flow system is that used in the determination of plasma glucose (section 4.3.2) and is illustrated in Fig. 66.

An approximation of the operational stability is given by making use of results obtained in the determination of the accuracy and precision of the GDH method for the determination of glucose. The standard calibration curves obtained at intervals during the use of the GDH-tube are compared and the height of the 25mM glucose standard

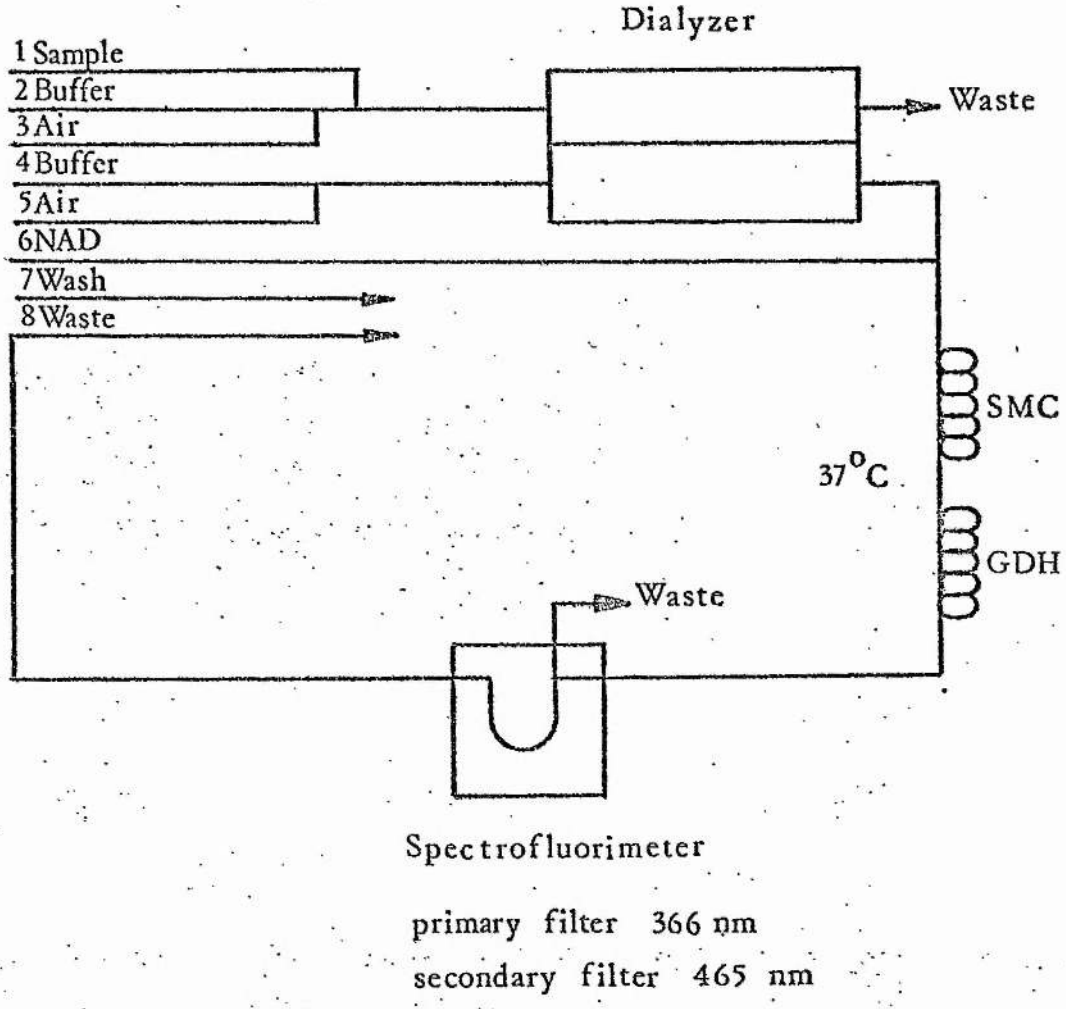


Fig. 66. Flow diagram of the analyser system used to measure the operational stability of nylon tube immobilized GDH. (SMC = single mixing coil).

is used as a measure of the activity.

5.4. Storage stability of nylon tube immobilized enzymes.

Conditions of storage.

1. Glucose dehydrogenase and alcohol dehydrogenase tubes:

These are filled with 0.1M-phosphate buffer, pH 7.5 (0.1M-NaH_2PO_4 titrated to pH 7.5 with 2M-NaOH) and kept at 4°C.

2. Lactic dehydrogenase tubes:

These are filled with 0.1M-phosphate buffer, pH 7.5 (0.1M-NaH_2PO_4, 0.15mM-EDTA, 5mM-dithiothreitol titrated to pH 7.5 with 2M-NaOH) and kept at 4°C.

3. Aldehyde dehydrogenase tubes:

These are filled with 0.1M-phosphate buffer, pH 6.5 (0.1M-NaH_2PO_4, 1M-KCl, 5mM-mercaptoethanol titrated to pH 6.5 with 2M-KOH) and kept at 4°C.

5.5 Results.

a. Thermal stability of nylon tube immobilized enzymes.

The thermal deactivation of both soluble and immobilized enzymes have been studied at temperatures which allow controlled denaturation and convenient monitoring of enzyme activity ie. 60°C for LADH and 45°C for LDH. Each enzyme was immobilized on nylon tube. The nylon tube was first alkylated (section 2.3), then substituted with DAE or AH (section 2.4), activated with glutaraldehyde (section 2.5a) or bisimidate (section 2.5b) and finally coupled with the enzyme (section 2.5c).

The thermal deactivation progress curves of LADH and LDH are illustrated in Fig. 67 and 68 respectively on a semilogarithmic scale.

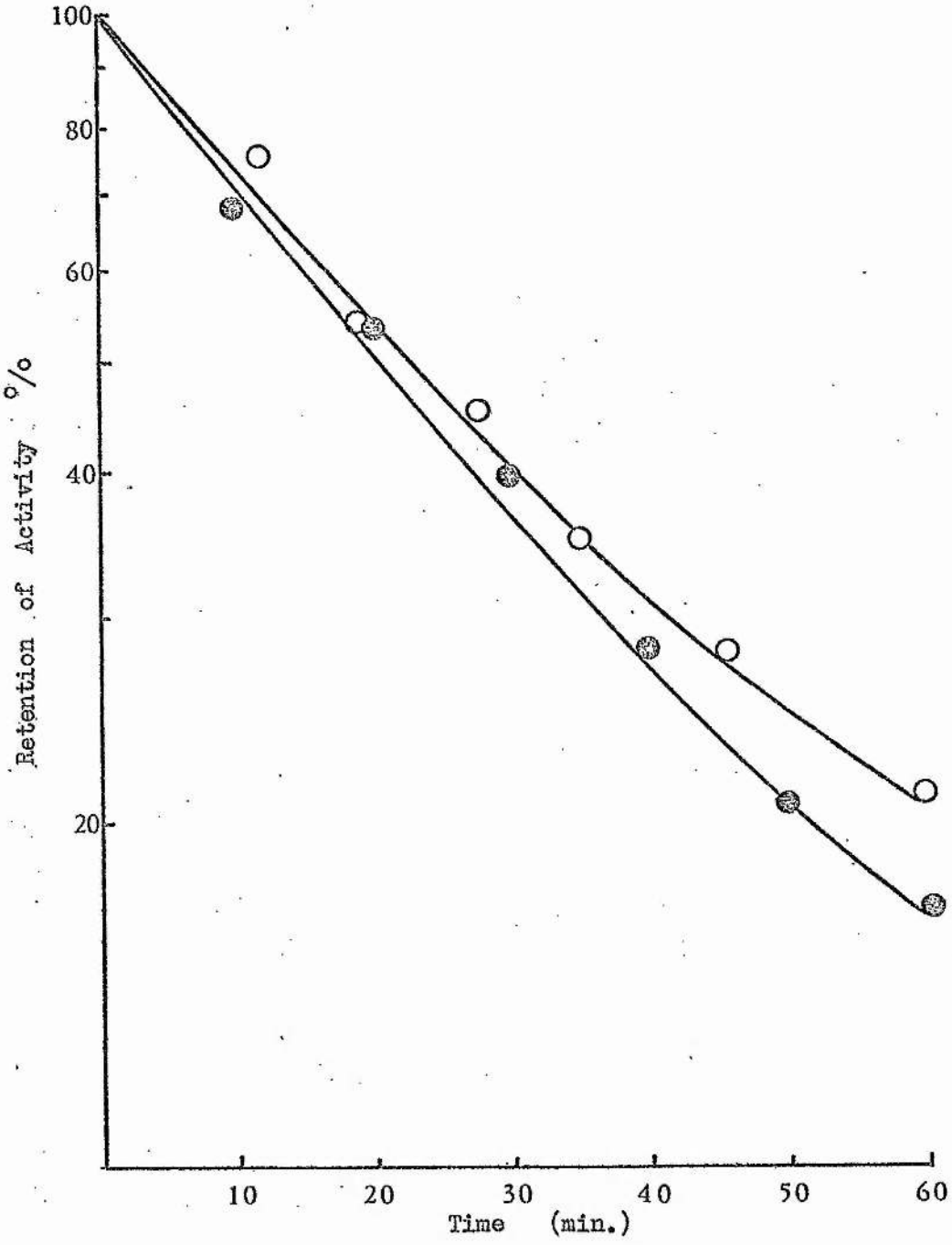


Fig. 67. The thermal deactivation progress curves of LADH at 60°C. Enzyme activities are expressed as a percentage of the zero time activity. (○ soluble LADH; ◍ immobilized LADH represented by the nylon-DAE-glutaraldehyde- LADH derivative.)

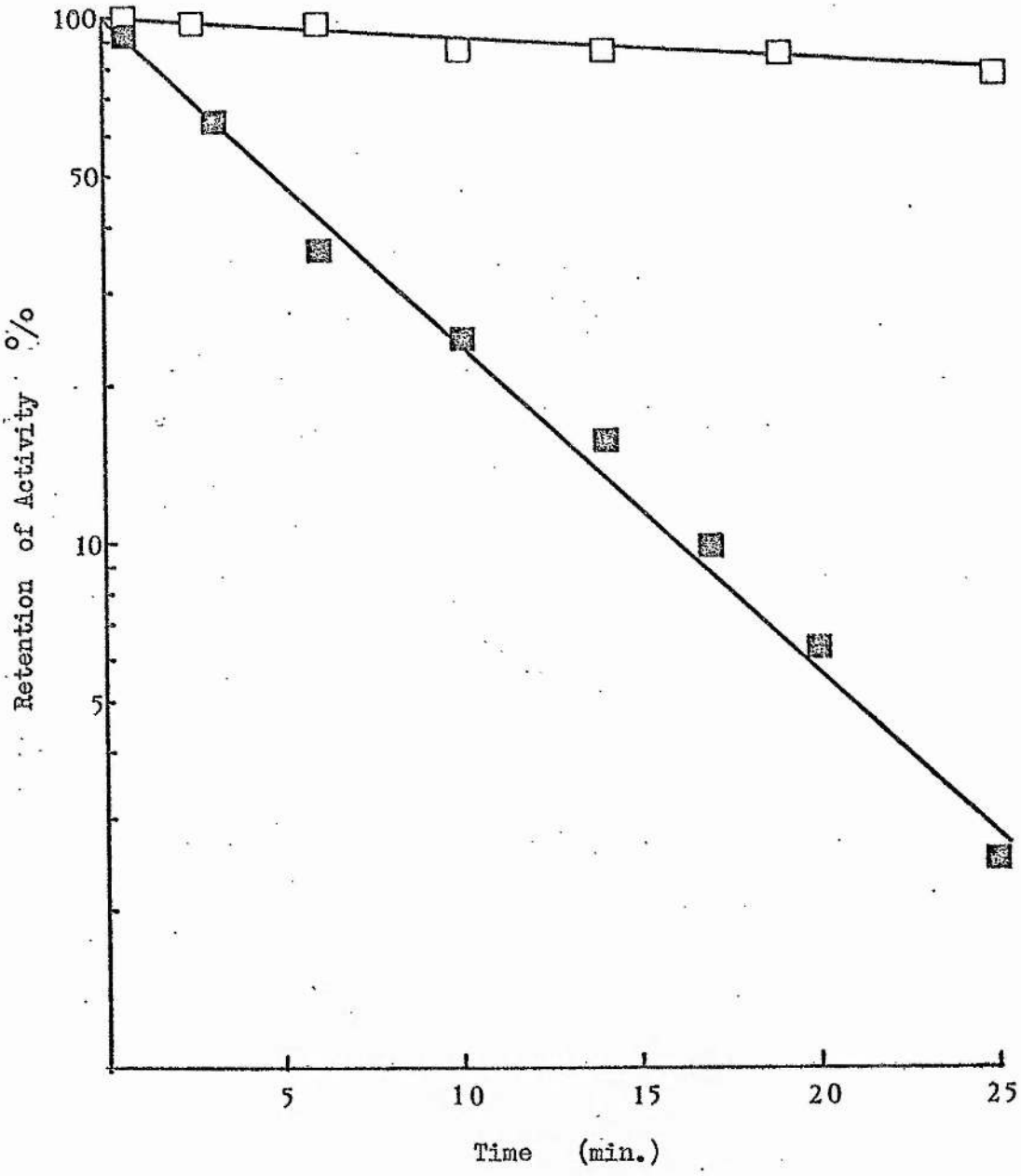


Fig. 68. The thermal deactivation progress curves of LDH at 45°C. Enzyme activities are expressed as a percentage of the zero time activity. (▩ soluble LDH; □ immobilized LDH in the form of the nylon-DAE-glutaraldehyde-LDH derivative.)

In these figures, the activities of sequential enzyme assays are expressed as a percentage of the zero time activity to enable easy comparison of the relative stabilities of soluble and immobilized enzymes.

In the case of nylon tube immobilized LADH, all derivatives exhibit the same thermal stability. Therefore the deactivation progress curve of the nylon-DAE-glutaraldehyde-LADH derivative is plotted as representative of all LADH-derivatives studied. At 60°C, a slightly lower thermal stability is demonstrated by LADH on immobilization ($t_{\frac{1}{2}}^{t}$ = 20.5 min. for immobilized LADH derivatives; $t_{\frac{1}{2}}^{t}$ = 23.5 min. for soluble LADH).

Immobilized LDH does have enhanced thermal stability (Fig. 68). At 45°C, the half-life of the immobilized enzyme is approximately 13 times that of the soluble enzyme ($t_{\frac{1}{2}}^{t}$ = 62.5 min. for immobilized LDH; $t_{\frac{1}{2}}^{t}$ = 4.9 min. for soluble LDH). The amount of protein bound to the nylon support has no major effect upon the overall thermal stability of the immobilized enzyme although a slightly enhanced stability is observed with increasing bound protein. Fig. 69 illustrates the thermal deactivation progress curves of 4 immobilized LDH derivatives (nylon-DAE-glutaraldehyde-LDH derivatives with increasing amounts of bound LDH) which have been incubated at 55°C. These curves are non-linear and therefore unsuitable for half-life calculations.

b. Operational stability of nylon tube immobilized enzymes.

The operational stability of nylon tube immobilized enzymes has been studied under assay conditions essentially the same as those used in the routine analytical methods described in section 4. Nylon tube was first alkylated (section 2.3), then substituted with DAE (LADH, LDH, and AlDH) or AH (GDH)(section 2.4), activated with glutaraldehyde (section 2.5a) and finally coupled with enzyme (section 2.5c). These

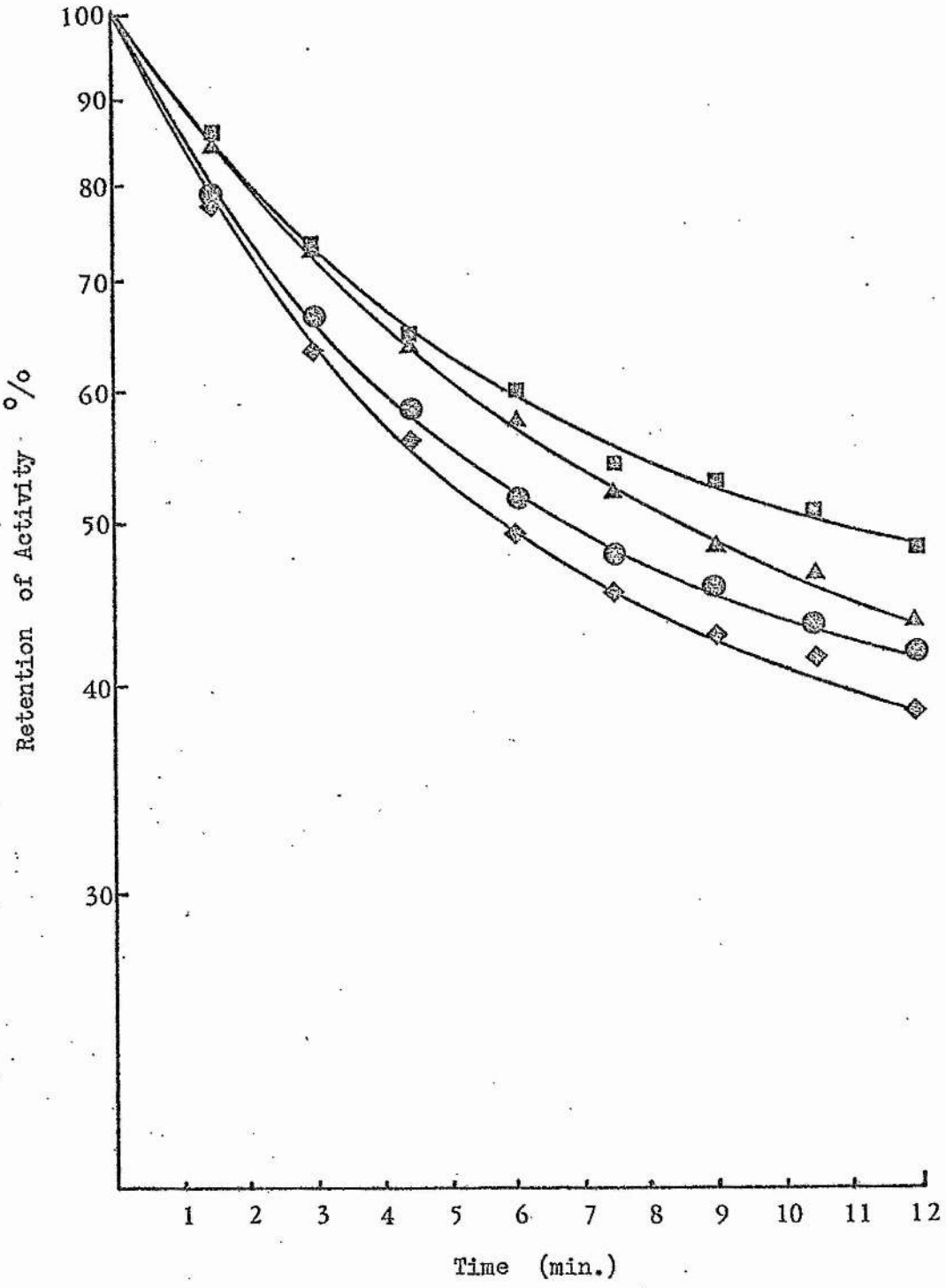


Fig. 69. The thermal deactivation progress curves of immobilized LDH at 55°C. Different amounts of enzyme are bound to a nylon-DAE-glutaraldehyde support. (▣ 0.58 mg/m.; △ 0.36 mg/m.; ◉ 0.25mg/m.; ◈ 0.13mg/m.)

immobilized enzyme tubes were then used in their respective assay systems for prolonged periods.

The loss of activity by all immobilized enzyme tubes (LADH, AlDH, LDH and GDH) is found to be less than 5% during a 2h. run of 120 specimens. This trend appears to continue for the long term use of LADH, LDH and AlDH. The operational deactivation progress curves of these three immobilized enzymes are illustrated in Fig. 70 where the logarithm of the activity (relative to zero time activity) is plotted against the number of hours of analytical use. From these curves, the initial deactivation is observed to be exponential such that the operational half-life of the enzyme can be calculated. Each enzyme is found to have an operational half-life of the same order of magnitude ($t^{o}_{\frac{1}{2}}$ = 30.5 h. for immobilized LADH; $t^{o}_{\frac{1}{2}}$ = 27.5 h. for immobilized AlDH; $t^{o}_{\frac{1}{2}}$ = 23.0 h. for immobilized LDH)

c. Storage stability of nylon tube immobilized enzymes.

All immobilized enzyme derivatives used in the proposed analytical methods described in section 4 have been stored under conditions most favourable to the particular enzyme (see section 5.4) and not under the adverse operational conditions. Long term storage of the enzyme tubes has not yet been fully evaluated although over a 2 week period no significant loss of activity has been observed with any of the enzyme derivatives. The longest recorded storage time of an enzyme tube is 3 months for immobilized LDH (nylon-DAE-glutaraldehyde-LDH). The derivative retained 69% of its original activity after this period. In this particular case, the Schiff's base formed during the glutaraldehyde coupling step (section 2.1, Fig.1) was stabilized by reducing the carbon-nitrogen double bond with borohydride (section 2.5d).

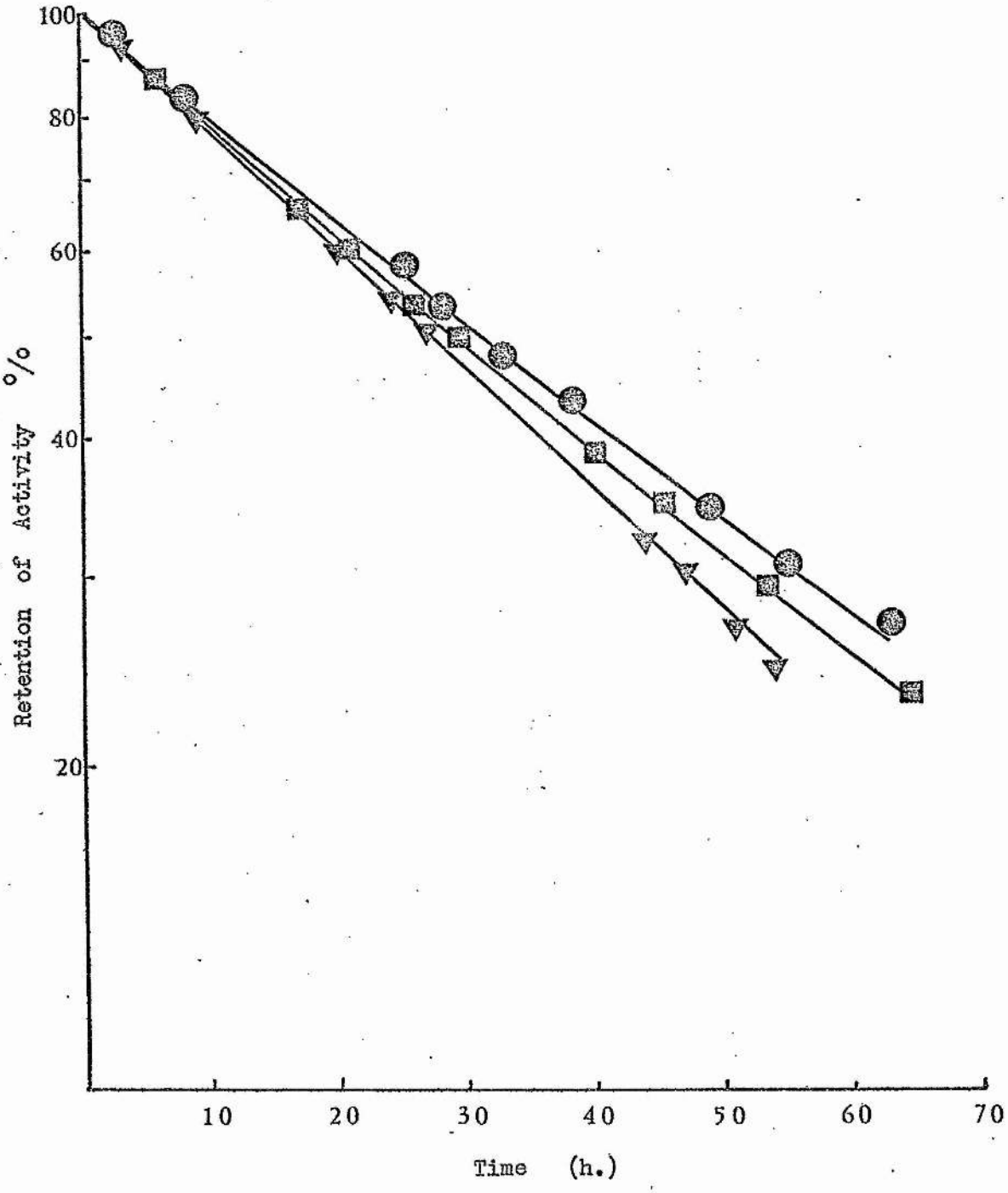


Fig. 70. The operational deactivation progress curves of immobilized LDH, LADH and AlDH at 37°C. The nylon-DAE-glutaraldehyde derivative of each enzyme was used. (▣ immobilized LDH; ◉ immobilized LADH; ▼ immobilized AlDH.)

5.6 Discussion.

Enzyme immobilization does not necessarily confer enhanced stability upon the enzyme. Stability is dependent upon the support material, nature of attachment and the enzyme immobilized. In this work, the stability of nylon tube immobilized enzymes has been assessed at high temperatures and under operational and storage conditions. Thermal stabilities are a useful and rapid means of comparing different enzyme derivatives whereas operational and storage stabilities are important when assessing the reliability and maximum working life of a particular enzyme derivative in analysis.

In the study of the thermal deactivation of LADH and LDH, only LDH demonstrates an enhanced stability on immobilization. This is probably a reflection of the relative stabilities of the two enzymes. Since soluble LDH is far more susceptible to deactivation than LADH, as illustrated by the respective thermal deactivation temperatures and percentage retention of activity on immobilization (section 2, Table I), LDH might be expected to benefit relatively more from immobilization. Thus, multipoint attachment of the enzyme may effectively prevent thermal disruption of enzyme conformation and in doing so prevent subsequent loss of activity. The amount of protein bound and the chemistry of immobilization have little or no effect on the rate of thermal deactivation of immobilized LDH. Its stability would appear to be largely due to the actual binding of the enzyme to the nylon support and the number of points of attachment.

From the thermal stability profiles (Fig. 68 and 69) it can be seen that the shape of the deactivation curve of LDH changes from being apparently linear at 45°C to definitely non-linear at 55°C. A possible explanation for this is the presence of both weakly and strongly bound enzyme giving rise to an immobilized enzyme derivative

with varying degrees of stability, For example, the weakly bound enzyme might behave like the soluble enzyme having little resistance to thermal deactivation and subunit dissociation whereas the strongly bound enzyme, because of more points of attachment for instance, might retain its conformation and be less readily denatured. The resulting non-linearity of the thermal stability profile at the higher temperature is possibly a combination of decay curves from both weakly and strongly bound enzyme, these two curves are not resolved at the lower temperature.

The operational stability of nylon tube immobilized enzymes is entirely dependent upon the assay system into which the tubes are incorporated because conditions will vary from one system to another. In end-point analysis an excess of immobilized enzyme is used in the assay system, thus loss of activity will be apparent only when the level of enzyme activity falls below that required for complete analyte conversion. Initially therefore, a false impression of immobilized enzyme stability is given and operational stability profiles show only the deactivation of the final fraction of enzyme molecules. In kinetic analysis, on the other hand, loss of enzyme activity results in an immediate loss of sensitivity because the percentage conversion of analyte is small and proportional to enzyme activity. Thus operational stability profiles trace the deactivation of all immobilized enzyme molecules.

The percentage conversion of analyte in each of the systems described in this work is never greater than 5% and therefore they all operate in the kinetic mode. Operational stability profiles of these systems (Fig. 70) thus demonstrate the apparent exponential deactivation of the immobilized enzyme derivatives with no initial lag phase. In fact the profiles of LADH, LDH and AlDH are remarkably

similar and operational half-life values are of the same order, probably because once immobilized and in a more stable conformation, these enzyme derivatives are susceptible to similar conditions of deactivation.

The operational stabilities of immobilized LADH, LDH and AlDH, although relatively low, are sufficiently favourable to merit their use in routine automated analysis. No system is going to be run continuously for very long periods. Instead, it is more likely that a maximum of a few hundred analyses are performed each day. All of the enzyme tubes considered above are quite capable of handling such workloads with very little loss of activity and this is reflected in the precision results obtained (section 4). However, although the inclusion of drift standards can be used to correct values to a certain extent, it is though advisable to recalibrate at two-hourly intervals when running systems for long periods.

The enzyme tubes have been stored for short periods only at present and, although proving to be economical in the short term on a small scale, have yet to prove themselves on the long term commercial scale where there is a need for a shelf-life at least equivalent to that already found with the unmodified enzyme.

6. The effect of pH on the activity of nylon tube immobilized enzymes.

6.1 Introduction.

The activity of an enzyme is greatly dependent upon the pH of the assay system used. Changes in pH affect not only the ionic state of the enzyme molecule but often that of the substrate and product as well. These effects generally restrict enzyme activity to a relatively narrow pH range. On either side of this range the activity decreases considerably such that the overall pH-activity profile frequently describes a characteristic bell-shaped curve (see Fig. 76). Activity is greatest when amino acid residues involved with catalysis at the active site are in the correct ionisation state for substrate binding. Small changes in pH result in reversible removal or attachment of protons to these residues and subsequent alteration of substrate and product binding affinities. Obviously, pH charge effects on substrate and product will also influence these binding affinities. Generally, for small changes in pH the loss of enzyme activity can be restored by titration back to the optimum pH. At extremely high or low pH values, however, enzyme denaturation occurs and activity is lost irreversibly.

The pH-activity plots of immobilized enzymes have been shown to differ on occasion from those of the unmodified enzyme both in the shape of the curve (Line et al., 1971; Axen and Ernback, 1971; Silman and Karlin, 1967) and the pH optimum (Goldstein et al., 1964; Goldstein and Katchalski, 1968). Distortion and shift of pH-activity profiles is attributed to chemical modification of the enzyme on immobilization, charge on the support and/or the diffusion barrier which exists at the enzyme surface. These factors affect enzyme activity by setting up concentration gradients of reactants and buffer ions between enzyme and bulk solution.

Both charge and diffusion effects will be influenced by the method of assaying the immobilized enzyme activity. Since the purpose of this work is to evaluate the use of nylon tube immobilized enzymes in automated continuous flow systems, all pH-activity profile determinations described in the following sections assay the enzyme tubes under conditions equivalent to those used in the corresponding automated assay systems.

6.2 Buffers used in the determination of pH-activity profiles.

All buffers used in the determination of pH-activity profiles are prepared from information given by Datta and Grzybowski (1961). Three different buffering systems are used - phosphate, tris and carbonate, each prepared with an ionic strength of 0.1. The pH of the buffer solutions are checked using a pH-meter which has been previously calibrated with three standard buffer solutions (Beckman Instruments) covering the pH range of the buffers used in the experiments (pH 6.0 - 10.2). To ensure continuity and detect buffer ion effects, the ranges of the different buffering systems overlap.

Phosphate buffer (Na_2HPO_4 / NaH_2PO_4)	pH 6.0 - 7.6
Tris buffer (tris / HCl)	pH 7.2 - 9.0
Carbonate buffer (Na_2CO_3 / $NaHCO_3$)	pH 9.0 - 10.2

6.3 Determination of the pH-activity profile of alcohol dehydrogenase.

Reagents.

1. Buffers: pH 6.2 - 10.2 (see section 6.2).
2. Sample wash: 0.15M-NaCl.
3. Nicotinamide-adenine dinucleotide: 2.5mM-NAD^+, 3mM-ethanol solution is freshly prepared. Grade III NAD^+ was obtained from Sigma London Chemical Co.Ltd.

4. Enzyme tube: Horse liver alcohol dehydrogenase (LADH) immobilized on 1mm. bore nylon tube. Nylon tube was alkylated (section 2.3), then substituted with DAE (section 2.4), activated with glutaraldehyde (section 2.5a) and coupled with enzyme (section 2.5c). The enzyme was obtained from Sigma London Chemical Co.Ltd. A 10 cm. length is used in the assay system.
5. Soluble enzyme: 0.1 U/ml. solution of LADH.
6. Reduced nicotinamide-adenine dinucleotide: 1.5mM-NADH solutions in phosphate buffer, pH 7.5 a) with 0.1 U/ml. LADH, b) without LADH.
7. Acetaldehyde: 10mM-CH_3CHO solution is prepared from freshly distilled CH_3CHO and stored at $0^{\circ}C$.

Method.

The flow system used for the determination of the pH-activity profiles of LADH is shown in Fig. 74. The apparatus comprises standard Technicon equipment together with the Perkin-Elmer 1000 Fluorescence Spectrophotometer and Kipp-Zonen BD8 recorder.

a. Oxidation of ethanol.

$$C_2H_5OH + NAD^+ \rightleftharpoons^{LADH} CH_3CHO + NADH + H^+$$

The analyser circuit is essentially the same as that used for the determination of blood ethanol apart from the absence of a dialyser unit. Also, to facilitate the inclusion and exclusion of the enzyme tube, two 3-way taps (A and B) are incorporated into the system.

In order to measure the activity of the soluble enzyme the system (excluding enzyme tube) is first equilibrated with the appropriate buffer until a steady baseline is observed on the recorder. Once this has been achieved, the enzyme solution is

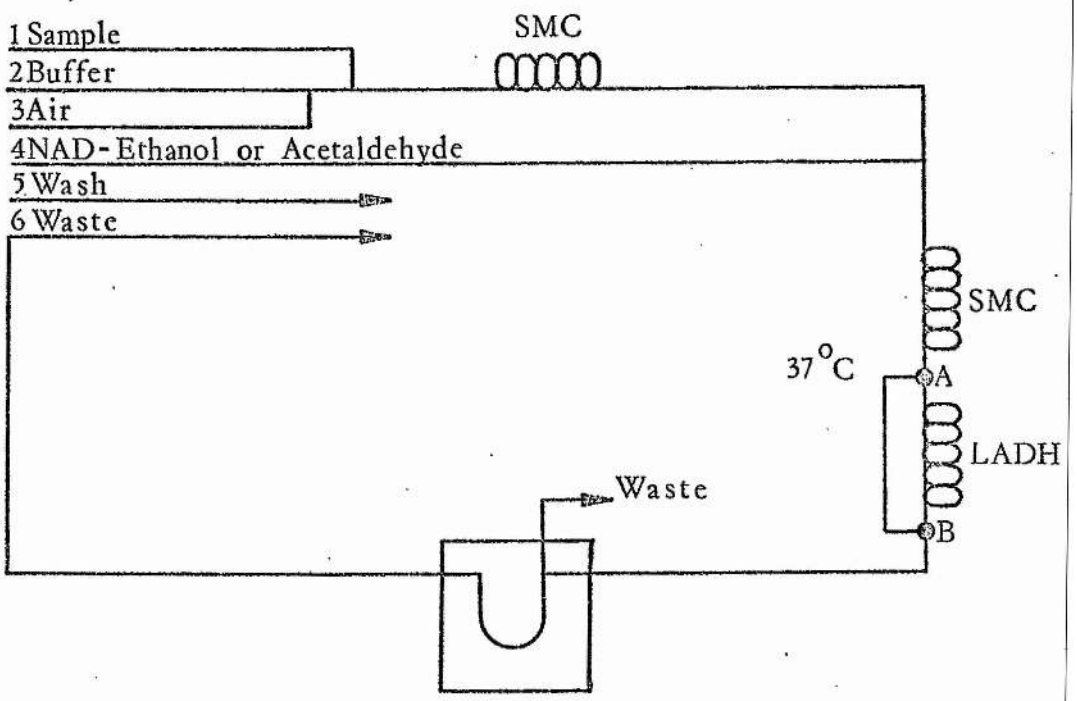


Fig. 71 Flow diagram of the analyser system used to determine the pH-activity profiles of LADH. (SMC = single mixing coil; A & B = 3-way taps.)

sampled automatically, diluted by the buffer and incubated at 37°C with NAD^+ and ethanol. The NADH produced in the enzyme catalysed oxidation of ethanol is measured fluorimetrically (λ_x 366nm.; λ_m 465nm.). This procedure is subsequently repeated with each buffer in turn, the peaks produced being a measure of the enzyme activity.

When measuring the activity of the nylon tube immobilized LADH, the system is equilibrated with the appropriate buffer via the shunt. On attainment of a steady baseline the tape (A and B) are used to include the enzyme tube and the NADH formed is monitored until a steady state value is achieved. The increase in fluorescence between baseline and steady state value is used as a measure of the enzyme activity.

Using the above method, both immobilized and soluble enzymes can be assayed under identical conditions and a valid comparison of pH-activity profiles can be made.

The pump tubes on the manifold are arranged in the following manner;

Line.	Flow rate ml/min.
1. Sample	0.1
2. Buffer	2.0
3. Air	1.0
4. NAD^+ / ethanol	0.6
5. Sample wash	2.0
6. Waste	2.0

b. Reduction of acetaldehyde.

$$C_2H_5OH + NAD^+ \underset{\text{LADH}}{\rightleftharpoons} CH_3CHO + NADH + H^+$$

The reverse reaction pH-activity profiles are derived in a manner similar to that above using the same analyser circuit.

In order to measure the activity of the soluble enzyme the system (excluding enzyme tube) is first equilibrated with the appropriate buffer. The NADH / LADH solution is then sampled and incubated with the buffer at 37°C to give a series of reproducible reference peaks. These peaks represent the maximum level of fluorescence (λ_x 366nm; λ_m 465nm.) given when there is zero enzyme activity and no substrate conversion. Once the system has been calibrated, acetaldehyde is introduced into the circuit via pump line 4 and the NADH / LADH solution is sampled again to give assay peaks. The height difference between reference and assay peaks is used as a measure of the enzyme activity.

When measuring the activity of the nylon tube immobilized LADH the system is equilibrated with the appropriate buffer and the acetaldehyde via the shunt. The NADH solution is then aspirated until a steady state value is recorded. The taps (A and B) are now used to include the enzyme tube and NADH monitoring continues until a second steady state value is obtained. The decrease in fluorescence between the two steady state values is used as a measure of the enzyme activity.

The pump tubes on the manifold are arranged in the following manner;

Line	Flow rate ml/min.
1. Sample	0.1
2. Buffer	2.0
3. Air	1.0
4. Acetaldehyde	0.6
5. Sample wash	2.0
6. Waste	2.0

6.4 Determination of the pH-activity profile of aldehyde dehydrogenase.

Reagents.

1. Buffers: pH 7.2 - 10.2 (see section 6.2).
2. Sample wash: 0.15M-NaCl.
3. Substrate solution: 3mM-CH_3CHO, 1.25mM-NAD^+, 5mM-mercaptoethanol, 1M-KCl solution freshly prepared and stored at 0^oC.
4. Enzyme tube: Yeast aldehyde dehydrogenase (AlDH) immobilized on 1.5mm. bore nylon tube. Nylon tube was alkylated (section 2.3), then substituted with DAE (section 2.4), activated with glutaraldehyde (section 2.5a) and coupled with enzyme (section 2.5c). A 40cm. length is used in the assay system.
5. Soluble enzyme: 0.6 U/ml. AlDH in 1M-KCl. The enzyme was a gift from Boehringer Mannheim GmbH.

Method.

The flow system used for the determination of the pH-activity profile of AlDH is shown in Fig. 72. The apparatus comprises standard Technicon equipment together with the Perkin-Elmer 1000 Fluorescence Spectrophotometer and Kipp-Zonen BD8 recorder.

$$CH_3CHO + NAD^+ + H_2O \xrightleftharpoons{AlDH} CH_3COOH + NADH + H^+$$

The pH-activity profile is studied in the forward reaction with AlDH catalysing the oxidation of acetaldehyde. The system used is basically the same as that used in the determination of the operational stability of the AlDH with the addition of two 3-way taps (A and B) so that the enzyme tube can be removed from the circuit.

In order to determine the soluble pH-activity profile the system (excluding enzyme tube) is first equilibrated with the appropriate buffer until a steady baseline is obtained. The soluble enzyme is then sampled automatically, diluted by the buffer and

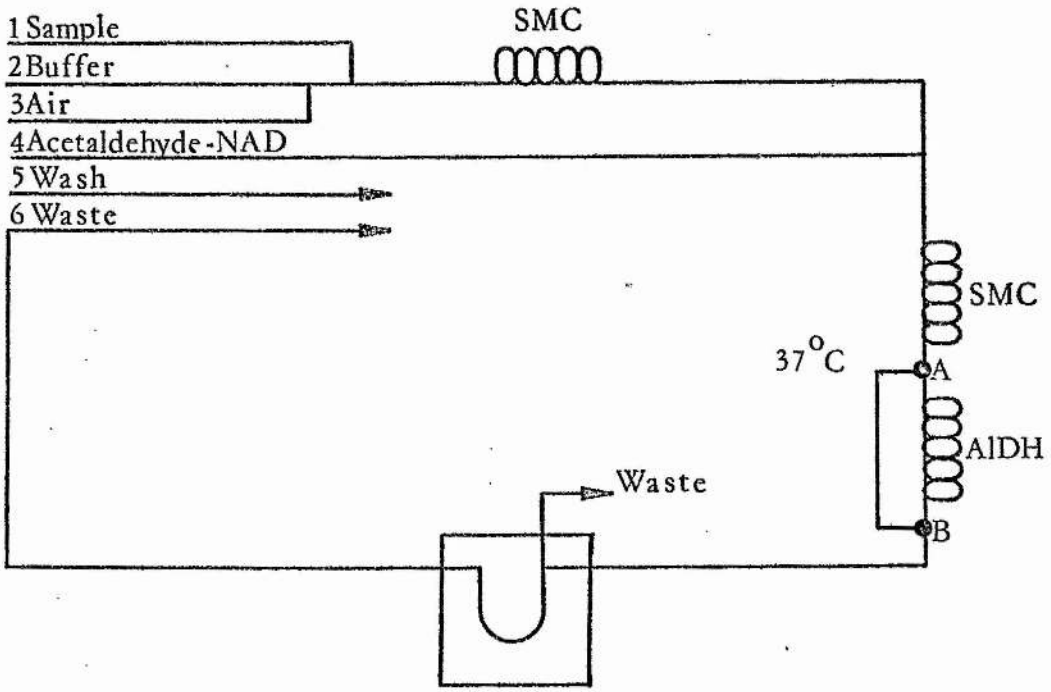


Fig. 72 Flow diagram of the analyser system used to determine the pH-activity profile of AlDH. (SMC = single mixing coil; A & B = 3-way taps).

incubated at 37°C with its substrates. The NADH produced in the enzyme catalysed oxidation of acetaldehyde is measured fluorimetrically (λ_x 366nm.; λ_m 465nm.). This procedure is repeated for each of the buffers and the peaks used as a measure of the activity.

The immobilized enzyme pH-activity profile is determined by initially equilibrating the system with the appropriate buffer via the shunt. On attainment of a steady baseline, the taps (A and B) are used to include the enzyme tube and the NADH formed is monitored until a steady state value is achieved. The increase in fluorescence between baseline and steady state value is used as a measure of the enzyme activity.

The pump tubes on the manifold are arranged in the following manner;

Line	Flow rate ml/min.
1. Sample	0.1
2. Buffer	2.0
3. Air	1.0
4. Acetaldehyde / NAD^+	0.6
5. Sample wash	2.0
6. Waste	2.0

6.5 Determination of the pH-activity profile of lactic dehydrogenase.

Reagents.

1. Buffers: pH 7.2 - 10.2.
2. Sample wash: 0.15-NaCl.
3. Substrate solution: 10mM-$CH_3CHOHCOOH$, 2.5mM-NAD^+ solution freshly prepared.

4. Enzyme tube: Rabbit muscle lactic dehydrogenase (LDH) immobilized on 1.5nn. bore nylon tube. Nylon tube was alkylated (section 2.3), then substituted with DAE (section 2.4), activated with glutaraldehyde (section 2.5a) and coupled with enzyme (section 2.5c). A 20 cm. length is used in the assay system.
5. Soluble enzyme: 4 U/ml. LDH in 1% (w/v) albumin. The enzyme was supplied as a lyophilised powder by Sigma London Chemical Co.Ltd.

Method.

The flow system used for the determination of the pH-activity profile of LDH is shown in Fig. 73. The apparatus comprises standard Technicon equipment together with the Perkin-Elmer 1000 Fluorescence Spectrophotometer and Kipp-Zonen BD8 recorder.

$$CH_3CHOHCOOH + NAD^+ \underset{\leftarrow}{\xrightarrow{LDH}} CH_3CO.COOH + NADH^+ + H^+$$

The pH-activity profile is studied in the forward reaction with LDH catalysing the oxidation of lactate. The system used is basically the same as that used in the determination of blood lactate with the addition of two 3-way tap (A and B) so that the enzyme tube can be removed from the circuit.

In order to determine the soluble pH-activity profile the system (excluding enzyme tube) is first equilibrated with the appropriate buffer until a steady baseline is obtained. The soluble enzyme is then sampled automatically, diluted by the buffer and incubated at 37°C with its substates. The NADH produced in the enzyme catalysed oxidation of lactate is measured fluorimetrically (λ_x 340nm.; λ_m 465nm). This procedure is repeated for each of the buffers and the peaks used as a measure of the activity.

The immobilized enzyme pH-activity profile is determined by initially equilibrating the system with the appropriate buffer via

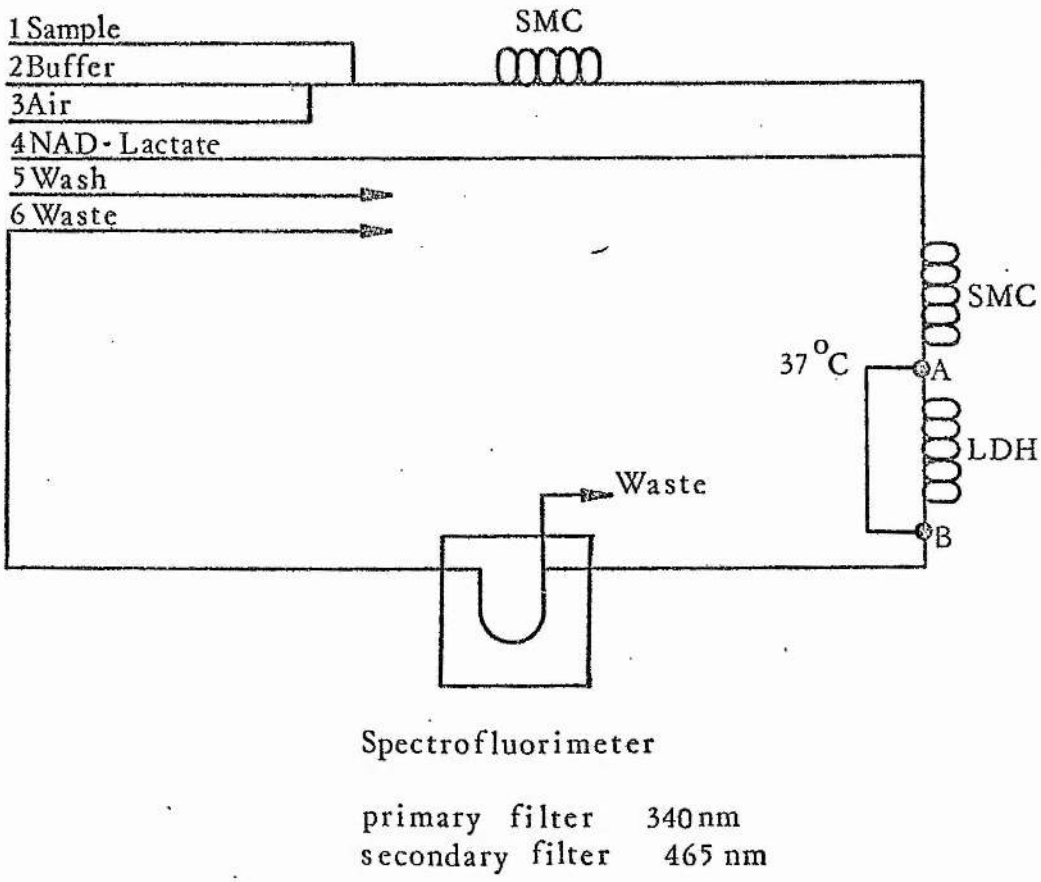


Fig. 73 Flow diagram of the analyser system used to determine the pH-activity profile of LDH. (SMC = single mixing coil; A & B = 3-way taps.)

the shunt. On attainment of a steady baseline, the tape (A and B) are used to include the enzyme tube and the NADH formed is monitored until a steady state value is achieved. The increase in fluorescence between baseline and steady state value is used as a measure of enzyme activity.

The pump tubes on the manifold are arranged in the following manner;

Line.	Flow rate ml/min.
1. Sample	0.23
2. Buffer	2.0
3. Air	0.8
4. Lactate / NAD^+	0.6
5. Sample wash	2.0
6. Waste	2.0

6.6 Results.

The enzyme activities are calculated in terms of arbitrary fluorescence units. In order to facilitate easy comparison of the pH-activity profiles of soluble and immobilized enzymes, these activities are expressed as a percentage of the optimum pH activity. The effect of pH on the activity of soluble and immobilized enzymes is illustrated in Fig. 74, 75 and 76 for the enzymes LADH, AlDH and LDH respectively. The pH activity profile of each immobilized enzyme is seen to be slightly broader than that of the corresponding soluble enzyme. In addition, shifts in pH optima of between 0.6 and 1.4 pH units (see Table XIV) are demonstrated by the immobilized derivatives of LADH and LDH. In the case of LADH a shift is also found in the reverse reaction (Fig. 77).

The effect of increased ionic strength (ie. 0.6M-NaCl in the assay solution) on the pH-activity profiles of soluble and immobilized LADH is not apparent apart from a possible decrease in maximum

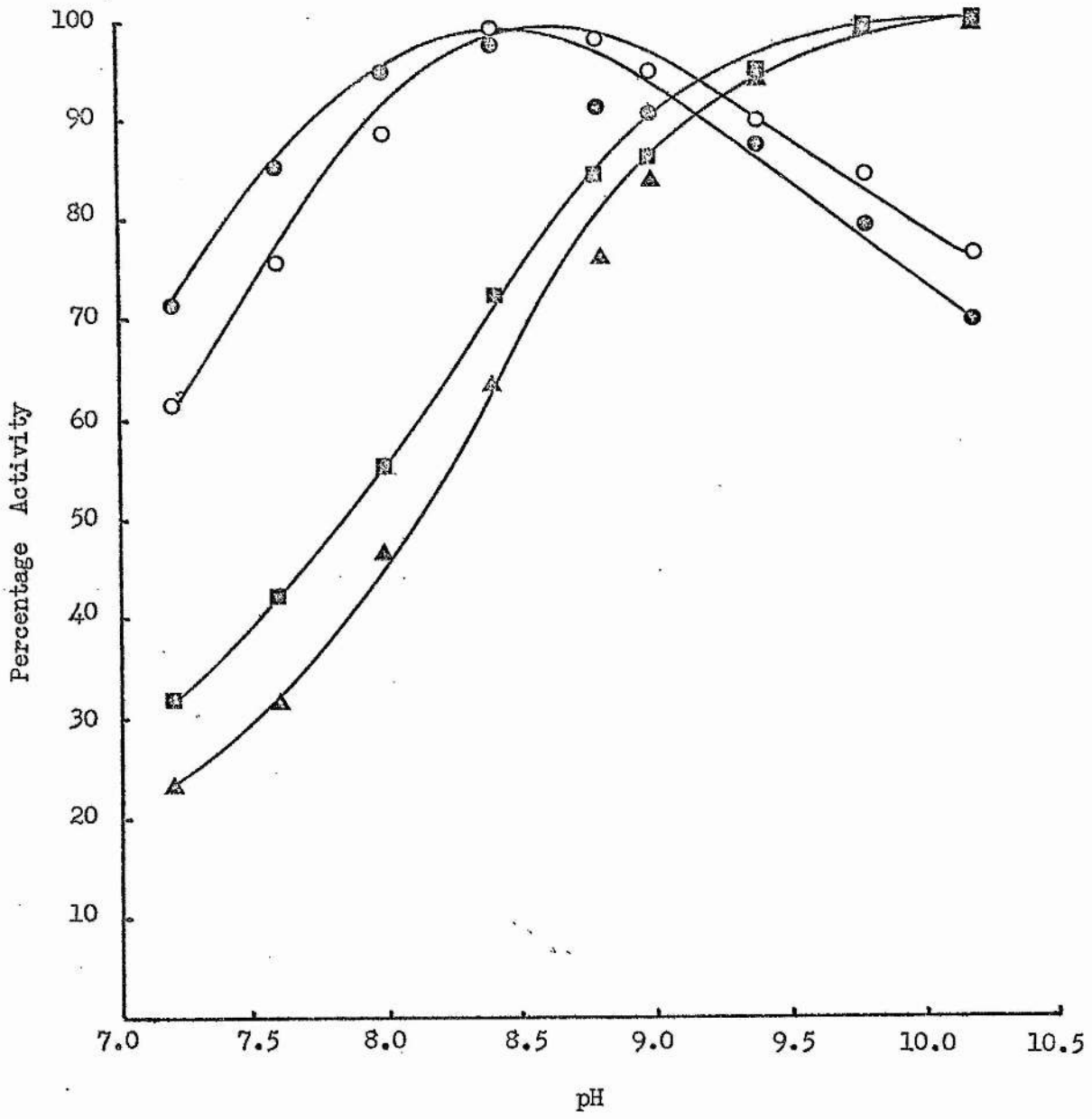


Fig. 74 The pH-activity profiles of soluble and immobilized LADH in the forward reaction. Enzyme activity was determined as described in section 6.3 over the pH range 7.2 - 10.2 and expressed as a percentage of the maximum activity. (◉ soluble enzyme with no NaCl; ○ soluble enzyme with 0.6M-NaCl; ■ immobilized enzyme with no NaCl; ▲ immobilized enzyme with 0.6M-NaCl.)

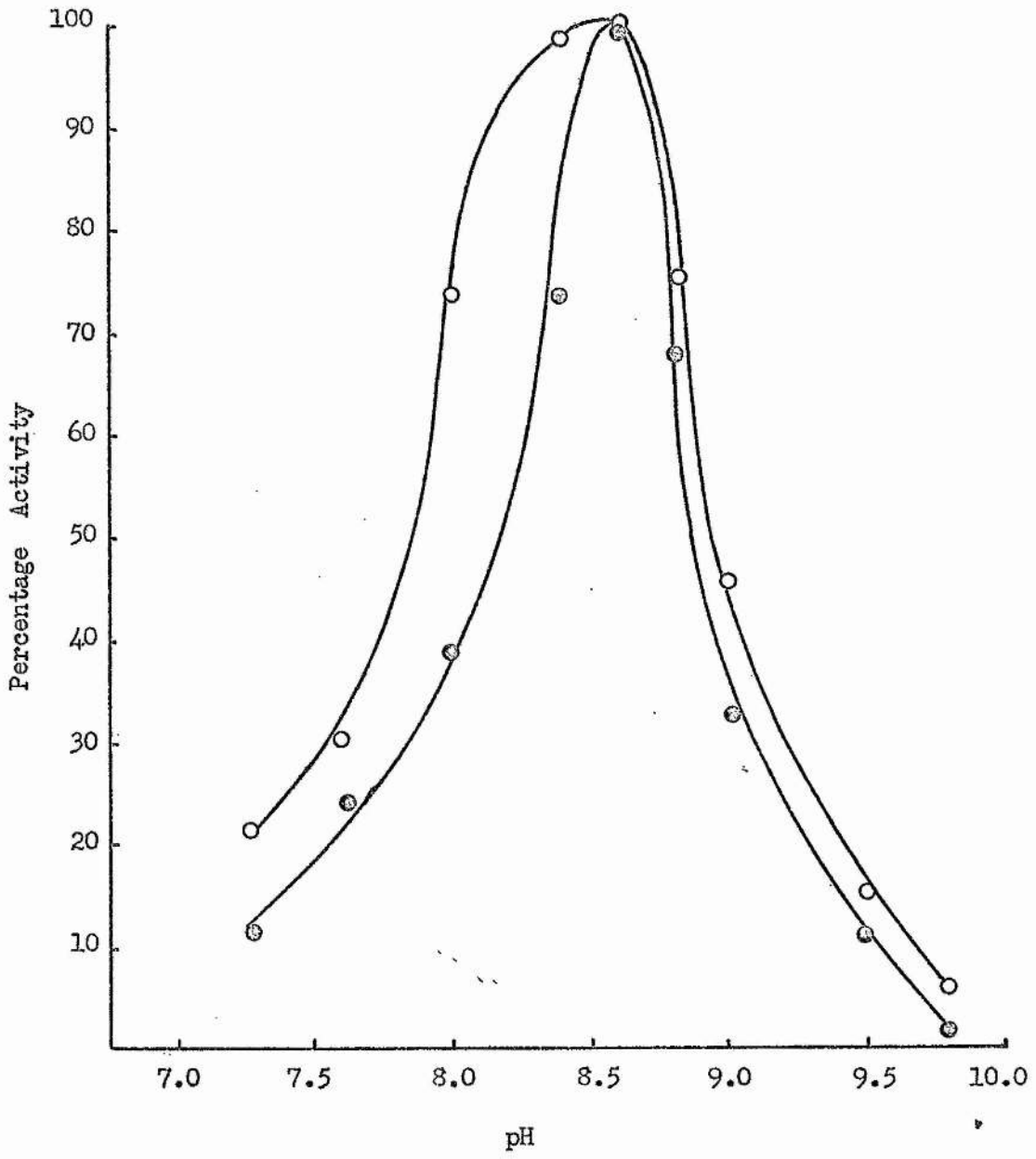


Fig. 75 The pH-activity profiles of soluble and immobilized AlDH. Enzyme activity was determined as described in section 6.4 over the pH range 7.2 - 10.2 and expressed as a percentage of the maximum activity. (● soluble enzyme; O immobilized enzyme.)

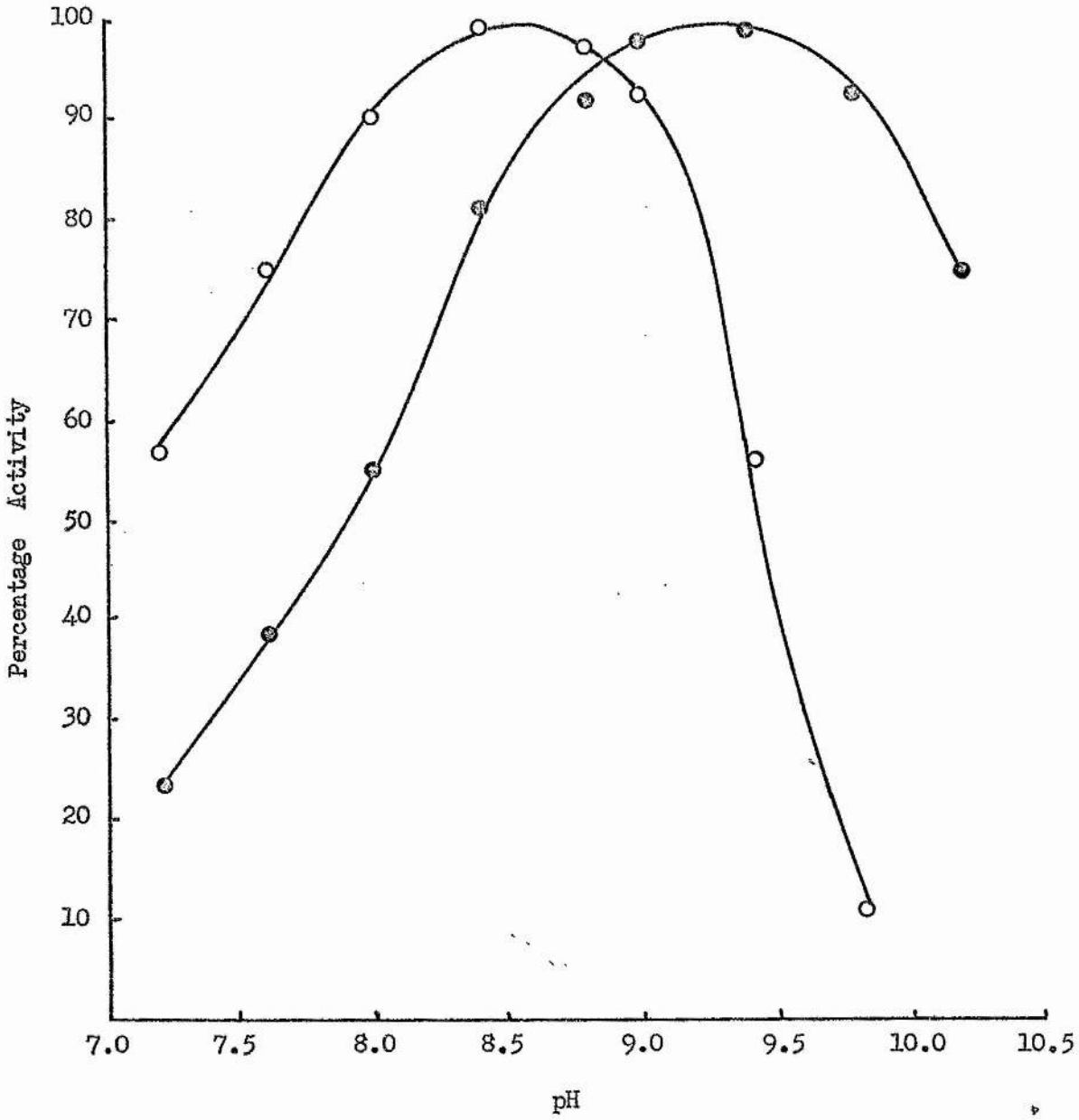


Fig. 76 The pH-activity profiles of soluble and immobilized LDH. Enzyme activity was determined as described in section 6.5 over the pH range 7.2 - 10.2 and expressed as a percentage of the maximum activity. (O soluble enzyme; ● immobilized enzyme.)

Enzyme	Direction of Reaction	pH optimum
LADH soluble	Acetaldehyde to ethanol	7.4
LADH immobilized	Acetaldehyde to ethanol	6.8
LADH soluble	Ethanol to acetaldehyde	8.4
LADH immobilized	Ethanol to acetaldehyde	9.8 and above
LADH soluble	Ethanol to acetaldehyde (high salt)	8.4
LADH immobilized	Ethanol to acetaldehyde (high salt)	9.8 and above
LDH soluble	Lactate to pyruvate	8.6
LDH immobilized	Lactate to pyruvate	9.3
AlDH soluble	Acetaldehyde to acetate	8.7
AlDH immobilized	Acetaldehyde to acetate	8.6 - 8.8

Table XlV. The pH optima of soluble and immobilized dehydrogenases. Values have been taken from the pH-activity profiles illustrated in Fig. 74, 75, 76 and 77.

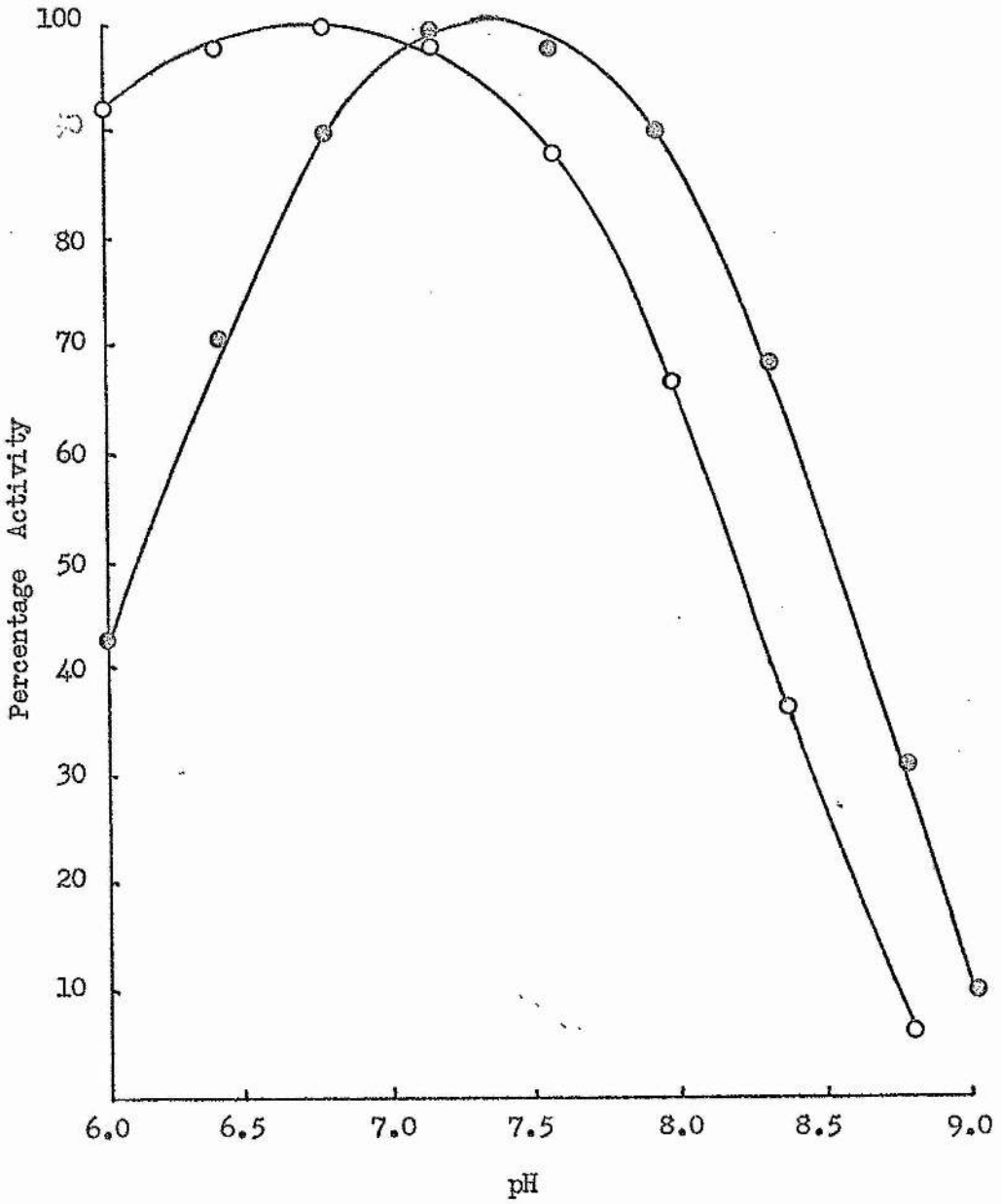


Fig. 77 The pH-activity profiles of soluble and immobilized LADH in the reverse reaction. Enzyme activity was determined as described in section 6.3 over the pH range 6.0 - 9.0 and expressed as a percentage of the maximum activity. (● soluble enzyme; ○ immobilized enzyme.)

activity and there is no further displacement of the pH optimum of the immobilized enzyme towards that of the soluble enzyme.

6.7 Discussion.

Why are there changes in the pH optimum on immobilization? Goldstein et al. (1964) observed that similar shifts, towards either lower or higher pH values, can be brought about by charged support systems. These workers argued that the displacement is caused by an unequal distribution of hydrogen ions. Thus, positively charged supports repel hydrogen ions and negatively charged supports attract hydrogen ions such that, in the vicinity of the bound enzyme, the pH is higher or lower respectively than that of the buffer solution being used. Charged supports will therefore displace pH optima towards more alkaline pH values (polyanionic supports) or more acidic pH values (polycationic supports).

The enzymes described in this work are attached to amine-substituted nylon which is positively charged. However, the pH-optimum shifts observed are not those expected for a cationic support. For example, the pH optimum of the immobilized LADH derivative is displaced towards the higher pH values in the forward reaction (ethanol tc acetaldehyde) and towards the lower pH values in the reverse reaction. In addition, high ionic strength has no effect on the position of the pH optimum. Therefore, although the effect observed is definitely not electrostatic in nature, it is probably still associated with an unequal distribution of hydrogen ions, possibly brought about by a diffusional effect.

It has been demonstrated (Hornby et al., 1968) that an enzyme attached to a solid support may be considered as being removed from the bulk of the assay solution by a diffusion layer in which there is no fluid motion. Thus the nylon tube derivative will have its

inner enzyme surface completely covered by a diffusion layer, a barrier to both substrates and products. The substrates in the bulk solution diffuse across this barrier, the enzyme catalyses the reaction and the products subsequently diffuse out. This results in the production of two concentration gradients, one in which the concentrations of substrates in the bulk solution are greater than those at the enzyme surface and the other in which the concentrations of products in the bulk solution are less than those at the enzyme surface. The magnitude of these gradients depends upon the thickness of the diffusion layer, enzyme activity and the rate of transport of substrates and products.

The thickness of the diffusion layer in a tube is related to the rate of flow of fluid (Bunting and Laidler, 1974; Royer, 1975). Diffusion effects are predominant at low flow rates where there is little turbulence or agitation and flow is laminar. Low flow rate conditions are present in all the immobilized enzyme analysis systems described and subsequently the diffusion layer at each enzyme surface will be thick. In view of this, diffusion might well be expected to influence the pH-activity profiles as observed.

In each pH-activity assay system, diffusion of the substrates can be neglected because concentrations at the enzyme surface, although lower than concentrations in the bulk solution, are still high enough to give maximum reaction rates. Diffusion of the products, on the other hand, cannot be ignored because the hydrogen ions released (in the forward reaction) or taken up (in the reverse reaction) could result in significant localised changes in the pH.

$$XH_2 + NAD^+ \rightleftharpoons X + NADH + H^+$$

where X = acetaldehyde, acetate or pyruvate.

If the rate of production of hydrogen ions in the forward reaction is greater than the rate of diffusion then the pH of the microenvironment about the immobilized enzyme will be lower than the pH of the bulk solution because of the concentration gradient produced. This will lead to a displacement of the pH optimum to a more alkaline "apparent" optimum pH, the pH at the surface of the enzyme being at, or near, the pH optimum of the soluble enzyme. The reverse reaction, conversely, will result in a deficit of hydrogen ions at the enzyme surface and an increase in pH. Thus the displacement of the pH optimum will be towards the acidic pH values.

This argument may explain why there are variations in the effect of diffusion on the different immobilized dehydrogenases. LADH and LDH, being the more active derivatives, are diffusion controlled at their respective pH optima and so a pH optimum shift is observed. The extent of the shift is dependent upon the activity of the enzyme and the rate of accumulation of hydrogen ions at the enzyme surface. Thus LADH has the largest shift, then LDH and finally AlDH has no shift. AlDH apparently never reaches a state at which it is diffusion controlled and the rate of hydrogen ion production is greater than the diffusion rate.

The pH optimum of the immobilized enzyme presumably tends towards that of the soluble enzyme as the diffusion layer tends towards zero thickness and/or diffusional control no longer operates, allowing for any deviation brought about by modification of the actual enzyme structure. This pH value can be classed as the true pH optimum of the immobilized enzyme. However, it would be pointless to determine the pH optimum in a system with minimal diffusion effects when considering systems where diffusion plays a significant role.

Thus the "apparent" pH optimum, obtained in the manner described in the previous pages, can be considered of greater value than the true pH optimum because it is related to the conditions found in continuous flow analyser systems.

7. General Discussion.

Amine- and hydrazide-substituted nylon tubes have been prepared from nylon-6 tubing by alkylation of the inner surface with triethyloxonium tetrafluoroborate and subsequent saturation with various spacer molecules (diamines and dihydrazides). These tubes have then been reactivated with either glutaraldehyde or bisimidate in order to covalently link dehydrogenases. The conditions necessary to perform these separate procedures, once elucidated and controlled, result in the synthesis of reproducible nylon tube immobilized enzyme derivatives. In general, the dehydrogenases favour covalent linkage through the diamine-glutaraldehyde chemistry. These glutaraldehyde derivatives have activities either greater than, or equal to, those of the bisimidate chemistry although the amount of protein bound varies considerably with each enzyme. This is contrary to observations made by Campbell (1974) who, on immobilization of glucose oxidase, achieved more active tubes using bisimidate as the coupling reagent.

Differences in the retention of activity on immobilization appear to depend largely on the structure of the enzyme. It has been shown (Richards and Knowles, 1968) that the glutaraldehyde reaction involves not only amine groups but various other reactive groups whereas bisimidate is specific for the amine group (Hunter and Ludwig, 1972). Therefore use of the glutaraldehyde chemistry in the immobilization of an enzyme may bestow upon its derivative the advantage of a greater percentage retention of activity when that enzyme has amine groups essential for catalytic activity.

Immobilization by covalent bonding of the enzyme can lead to loss of activity in various ways. These include linkage through an essential group, the masking of the active site (ie. orientation of

enzyme) and multiple point binding through several groups resulting in conformational stress with concomitant loss of the active configuration. Conformation stress could also lead to dissociation of sub-unit enzymes and further activity loss.

Rather than measure the activity of the enzyme tube derivatives by a slug-flow method, in which the substrate conversion across the tube is measured, a recirculating system devised by Ford et al. (1972) is employed where, because of high flow rates, diffusional effects are minimal. This allows for a more acceptable comparison of tube activities for the different enzymes. With the slug-flow method, flow rates are necessarily low and the calculated enzyme activities would vary with the flow rate and the diffusional coefficients of the reactants. It is therefore suitable only for comparison of different derivatives of the same enzyme under identical conditions and not of different enzyme derivatives.

Although the recycling method is found to be an excellent tool for comparative studies, its use in kinetics experiments is found wanting. This is due to the instability of tube derivatives to the high-flow mechanical stress incurred at the nylon-enzyme surface. The rate of flow in the recirculating system is 40 ml/min. which means that fluid velocities of up to approximately 50 m/min. along the enzyme tube are realised. The stress effect is more apparent on the wider bore tubes where the sloughing off of the activated nylon surface with its enzyme is greater and actually visible. Use of lower flow rates cannot be entertained as diffusional effects (Bunting et al., 1974) are pronounced and Lineweaver-Burk (1934) plots become non-linear (concave downwards). Such results have been observed by Lilly and Sharp (1968).

The activated nylon tube, as prepared by the method of Hornby and Morris (1974b), is a polycationic support and the nylon itself is hydrophobic. Thus, when an enzyme is attached to the support, partitioning effects of electrostatic and hydrophobic origin might well be expected. Substrates carrying an overall charge will be either attracted to or repelled from the enzyme tube surface leading to unequal distribution of the substrate between enzyme and bulk solution. Similarly, the substrate concentration at the enzyme site will deviate from that of the bulk solution as a result of being either hydrophilic or hydrophobic. Hydrophilic substrate molecules will not be as soluble at the support surface as in the bulk solution and conversely, the hydrophobic molecules will be very soluble.

These properties, while excellent in affinity chromatographic techniques, are not always welcomed in continuous flow analyser systems. The benefits sought from automated methods are the elimination of operator error, the increase of precision and the increase of sample turnover. This cannot be achieved when there is significant adsorption of substrates and/or products because the speed of analysis, or sampling rate, in continuous flow is directly dependent upon the interaction between the trailing edge of one sample and the leading edge of the following sample. Adsorption leads to increased overlap of these edges causing greater interaction. The carryover in the proposed assay systems is reduced by the use of increased ionic strength buffers and spacer molecules which have low pK values (ie. dihydrazides). Together, these factors ensure that there are minimal charge effects at the pH values used in the assays.

The carryover can be further reduced by decreasing the length of the enzyme tube and making use of the scale expansion facilities of the fluorimeter. It follows from this that, rather than use

the enzyme tube to the extent of its working life, it might be wiser to use short lengths of enzyme tube for single runs and discard. Great savings in the amount of enzyme used can still be made, but in addition, the sensitivity and reliability of the method can be maintained at a constant level from one run to the next. Longer lengths of enzyme tube can be used in continuous monitoring systems where carryover is of less importance and the stability will show an apparent improvement because of the greater percentage conversion of the substrate by the enzyme. Often in soluble enzyme analysis, much care has to be taken to stabilise the enzyme solution being used but on immobilization of course the stability is generally enhanced. If enzyme tubes are used in the manner described above, very little loss of activity is observed during normal assay runs and precision is high.

The dependence of enzyme tube activity on pH follows a slightly broader profile than that of the soluble enzyme often with shifts of optimum pH. This can be put to advantage in certain two-enzyme systems where enzymes having two different pH optima can be co-immobilized. The broader profiles mean that an acceptable compromise can be made and the two enzymes run at some pH value between the two optima. In continuous flow, this would confine the system to a simple configuration, eliminating the necessity of titrating buffers to another pH within the flow system. Any loss of overall activity on immobilization is frequently compensated for by increased activity due to the proximity of the two enzymes (Mosbach, 1976). Diffusion of substrate and products through the diffusional barrier occurs only at the beginning of the first reaction and the end of the second reaction. The product of the first enzyme becomes the substrate of the second enzyme and is therefore not required to pass through the diffusional barrier. This results in an effectively increased substrate

concentration for the second enzyme and hence increased rate of reaction.

The limiting factor when using immobilized enzyme to measure metabolites in continuous flow systems is the normal level of metabolite concentration found in the blood. Concentrations, after dilution, should be high enough to give detectable amounts of product when using short lengths of enzyme tube and minimum sample. (This latter consideration is necessary in view of the number of assays performed on patients and the need to keep the volume of blood taken to a minimum.) This is especially important when considering the use of dehydrogenases in the reverse reaction. For example, the normal blood pyruvate levels are very low (0.05 - 0.11 mM) so that a high NADH concentration is required to give detectable amount of NAD^+ in the LDH catalysed reduction reaction. This leads not only to raised noise levels on the recorder but also to effective dilution of the NADH by the sample and a fall in baseline. Soluble assays overcome this problem by using small samples, lower concentrations of the coenzyme, increased amounts of enzyme and longer incubation times. This solution is not applicable to the immobilized enzyme tube where incubation of substrate and the amount of enzyme is limited to the tube length.

All the automated systems described in this work have proved economical for routine use and compare favourably with the standard methods performed in the analytical laboratory at present. Moreover, a new method has been introduced which becomes financially practical only on immobilization of the enzyme. This is the glucose dehydrogenase method for the determination of blood glucose, the enzyme being so expensive as to rule out its use in soluble assays. Doubtless many more new enzymatic methods can be brought from research into routine use by employing techniques of immobilization since with careful choice of immobilization chemistry, immobilized enzyme tubes

can be tailored to suit the particular assay procedure.

The use of open-ended tubular enzyme reactors in continuous flow systems is still in its infancy. Although a number of methods have appeared in the various journals over the years, few have found their way into the routine analytical laboratory. This may in part be due to the necessary developmental effort required to produce suitable enzyme tube derivatives. Now that industry has taken a commercial interest in the immobilized enzyme tube, their future use in automated assay systems would appear to be affirmed and proposed methods should conform strictly to the criteria set by the analyst.

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