SOME ANALYTICAL APPLICATIONS OF IMMOBILIZED ENZYMES

A thesis submitted by

MARTIN JOHN TREWHELLA B.Sc. A.R.C.S.

in candidature for the degree

of

Doctor of Philosophy

of the University of London

The Bourne Laboratory
Royal Holloway College
(University of London)
Egham
Surrey

May, 1981

CONTENTS

Page No.

Acknowledgements

I would like to express my gratitude to all the staff at the Bourne Laboratory, (both technical and academic), for their much-appreciated help and cooperation during this work, and my thanks to all of my colleagues on Level 3 for many hours of stimulating discussions and for three very enjoyable years.

In particular, I am most grateful to my supervisor, Dr Arthur Finch for his great interest and help, especially during my first year.

I would like also to thank Dr R. F. Coleman, for his initial involvement with the CASE award with Dr Finch, and the Laboratory of the Government Chemist (and especially Mrs G Alliston), for their support, and interest in this work as well as for kind help in the construction of certain pieces of apparatus.

Thanks are also due to the SRC for the award of a CASE studentship and to Mrs Sylvia Partin for typing this thesis.

ABSTRACT

The use of immobilized enzymes in the analytical determination of enzyme substrates, inhibitors and activators has been examined using, for the majority of the work, heat sensors to monitor the chemical reactions occurring.

The sensitivity of determination of a wide range of enzyme substrates by such means has been substantially improved over similar, previous reported, work by optimization of a number of relevant parameters (particularly in the choice, and dimensions, of the enzyme support material). Reproducibility of the results in most cases appeared excellent, even over relatively long time periods. A brief study has also been conducted using other types of transducers.

Novel techniques for the analytical determination of immobilized enzyme inhibitors and activators have been developed which, although undoubtedly capable of improvement, already show considerable promise. In particular, no reports occur in the literature concerning the use of immobilized enzymes for the quantitative determination of reversible enzyme inhibitors, or enzyme activators.

Using these techniques, the determination of enzyme substrates such as penicillin G, urea, hydrogen peroxide, glucose, sucrose, lactose and uric acid have been accomplished, 0.5 cm^3 of a 2×10^{-5}M solution often being analytically determinable. Enzyme inhibitors (such as Hg^{2+}, Cu^{2+}, caffeine and certain other alkaloids) and activators (such as Cu^{2+} and Zn^{2+}) have also been determined, in certain cases in concentrations as low as 10^{-6} - 10^{-7} mol dm^{-3}.

Additionally, other uses of the combination of immobilized enzymes with thermal detection systems, such as the rapid determination of the overall enthalpies of hydrolysis of urea and penicillinase-sensitive semi-synthetic penicillins have been developed which yield rapid and highly reproducible measurements in an area where little or no data appears in the literature. A correlation has been established between the overall enthalpy of hydrolysis of a semi-synthetic penicillin, and the nature of the side-chain in the 6-amino position of the penicillin.

A strong correlation between the relative inhibitory powers of known anti-cholinesterase compounds on soluble and glass-immobilized cholinesterase enzymes has also been demonstrated, suggesting the possibility of using techniques described in this thesis as a rapid initial screening test for the pharmacological activity of new anti-cholinesterase drugs.

CHAPTER 1 Introduction

Section 1.1. - Enzymes in general

1.1.1. A brief background history and description of enzymes.

Enzymes are biological catalysts, consisting mainly of protein, which participate in many chemical reactions occurring in living organisms. Unlike ordinary chemical catalysts, enzymes characteristically have the ability to catalyze a reaction under very mild conditions, in neutral, or near-neutral aqueous solutions at normal temperatures and pressures, with very high specificity.

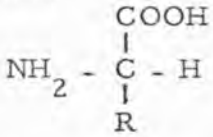
Enzyme technology is truly ancient. Primitive herdsmen discovered in prehistoric times that storing milk in the stomachs of animals resulted in a tasty solid food (cheese). The enzyme, rennin, clots milk by limited hydrolysis which results in the formation of solid cheese. Yeasts have long been utilised by humans in the production of alcoholic drinks, although there was little or no understanding of the means by which such transformations occurred.

What was probably the first clear recognition of an enzyme was made by Payen and Persoz (1) in 1833, when they found that an alcohol precipitate of malt extract contained a thermolabile substance which converted starch into sugar. The word "enzyme" was proposed by Kühne in 1878 (2). and although a number of enzyme reactions were studied in the 19th century, the fruits of this early enzyme research were not harvested until the arrival of the 20th century. Modern enzyme chemistry was heralded by the proposed hypothesis for enzyme

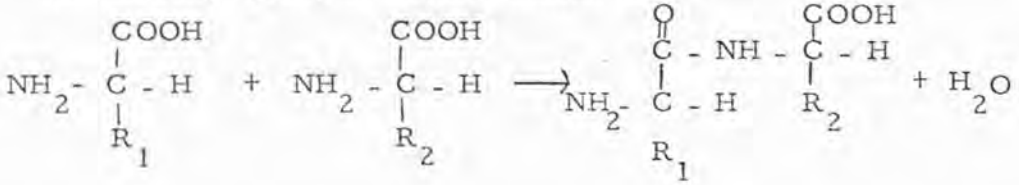
reactions of Michaelis and Menten (3), and the isolation of an enzyme, urease, by Sumner in 1926 (4). Since the introduction of the word "enzyme", there has developed a clearer idea of what it denotes, although an exact definition is not easy. An enzyme is often defined as "a protein, or proteinaceous substance elaborated by a living cell, which catalyses a specific reaction necessary for the maintenance of of life" (5). This definition is satisfactory for an introduction to enzymology, or as a "functional" definition, although it is not complete if one wishes to gain a thorough understanding of an enzyme. To do this, one must comprehend what an enzyme really is, how it exists in its spatial configuration, how it reacts, what factors influence its activity, and why. In an attempt to do this, three words in the above definition must be thoroughly examined:- "protein","catalyze" and "specific".

A. Enzymes as proteins

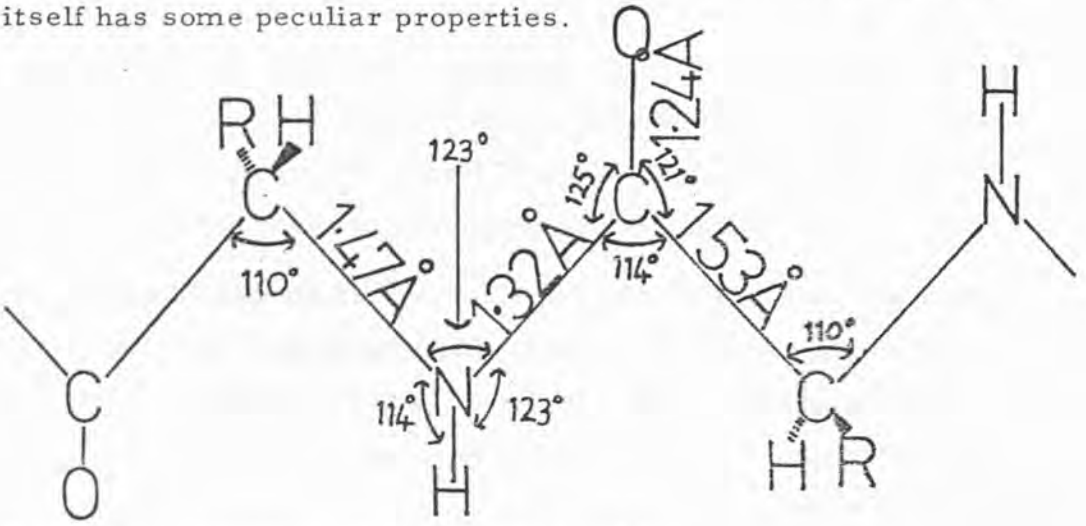
Proteins are macromolecules composed of amino acids, which have the general formula



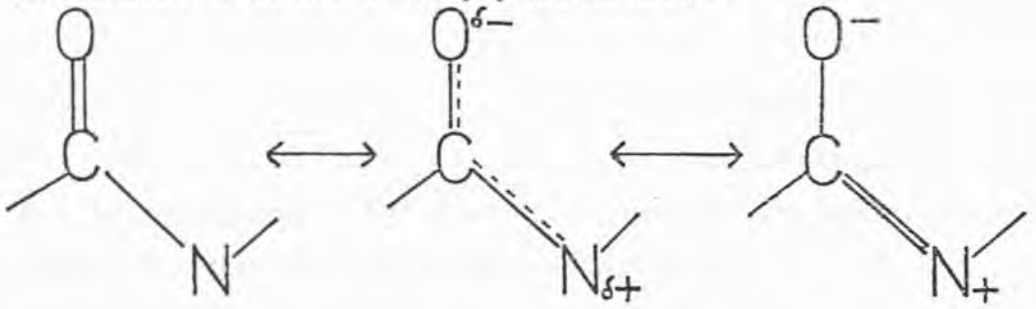
From this formula it is obvious that there are two active groups on the amino acid which participate in protein formation, since the amino group of one amino acid can form an amide bond with the carboxyl group of another amino acid:-



This bond is called a peptide bond. Considering that all amino acids of importance to protein chemistry (with rare exceptions) are in the L-configuration about the alpha carbon atom, this gives some order to the repeating chain. In addition, the bond itself has some peculiar properties.



Resonance structure of the peptide bond is:-



Looking at just one peptide bond, and visualizing extensions in both directions, one can see that, since only L-amino acids are involved, a pattern of "up and down" R-groups tends to emerge. Thus, some degree of repeatability is introduced into the chain. In addition, it can be noticed that the C-N bond between the carbonyl and amino groups is shorter (1.32Å), than the alpha C-N bond (1.47Å). This results from the resonance structure

of the peptide bond, which imparts two important characteristics to this group.

(1) The bond has partial double bond character, which restricts free rotation about this bond, and

(2) the atoms in the bond (the O and NH) can participate in hydrogen bonding with other amino acids or other molecules.

This peptide bond is the "basic" bond in protein structure because it forms the backbone to the entire protein chain.

Molecular biologists have clearly shown that proteins are biosynthesized in one long polypeptide chain. These chains my consist of several hundred amino acids which would be thermodynamically unstable if they remained in an extended form. In a review by Wetlaufer and Ristow (6) on the acquisition of the three-dimensional structure of proteins, they state "one of the first functions of every protein is its own self-assembly".

This is accomplished by a folding process in which various different types of bonds or stabilizing forces, are important:-

(1) Hydrogen bonds - the sharing of a proton from one group with a pair of electrons from another group, e.g.

$$C = O:^{\delta-} \quad H^{\delta+} - N$$

(2) Ionic bonds - the interaction of two oppositely-charged groups, e.g.

$$\begin{array}{c} O \\ \| \\ -C-O^{-} \end{array} \quad H_3^{+}N-$$

(3) Disulphide linkages - a disulphide bond may be formed by the oxidation of two -SH groups (of cysteine amino acid residues)

e.g. $2(-CH_2SH) \xrightarrow{[O]} -CH_2-S-S-CH_2 + H_2O$

(4) Electrostatic interactions - the attraction between two polarized species. Van der Waals' forces and dipole-dipole interactions are included in this group

e.g. $H^{\delta+} - O^{\delta-} - CH$

$CH - O^{\delta-} - H^{\delta+}$

(5) Hydrophobic bonds - the attraction between hydrocarbon or hydrophobic residues caused by the mutual exclusion of water. As many amino acid residues carry a non-polar side chain, the contribution of hydrophobic bonds to the overall protein structure may be of considerable magnitude.

As all of these forces come into play, each amino acid side chain seeks its most thermodynamically stable state. An examination of the side-chains (or R-groups) of the twenty one amino acids most important to protein chemistry (see Appendix 1) reveals that there are many ways the above interactions can come into play in the folding of a single protein chain. Hence it can readily be seen that all amino acids have three potentially active groups of importance to protein chemistry, and all contribute to the ultimate structure of the protein.

Protein structure is usually broken down into four categories.

(a) Primary Structure - the kinds, number and sequence of amino acids in the chain. The principle bond of importance in the peptide bond.

(b) Secondary Structure - these are the areas of more rigidly defined structure within the chain, where the alpha helix, pleated sheet or random coil structures prevail. These more structured areas result from the peptide bond and from hydrogen bonding.

(c) Tertiary Structure - this is the spatial configuration of the entire peptide chain, and all bonds and forces contribute to this aspect of the protein structure.

(d) Quaternary Structure - complex proteins composed of more than one peptide chain have a quaternary structure which is the description of the interaction of the different chains. Here, all bonds previously discussed and other types of covalent bonds and metal interactions can be of importance.

The net result is that a particular protein exists in a specific three-dimensional structure which is characteristic of the protein. There are hydrophobic areas, and areas of hydration, as well as amino acid side chains projecting from the surface, with varying potential chemical reactivity, and the overall protein has a net charge which is counteracted by ions from the suspending solution.

B. Enzymes as Catalysts

It should now be apparent how one molecule can diffuse into the vicinity of an enzyme and be held there because of the attractive forces of the projecting amino acid side chains. Other neighbouring groups may attract another portion of this molecule, causing bond breakage or other changes in the molecule.

This is an enzymatic reaction, for the reactive sites on the enzyme have catalyzed a chemical reaction, converting one molecule into another. Considering the complex nature of the enzyme itself, it is reasonable to expect that many parameters will affect the rate of this catalytic activity. These will be dealt with in the next sub-section (1.1.2.).

Because enzymatic reactions are chemical reactions the basic thermodynamic principles governing all chemical reactions must apply, and it is, therefore, important to realize that, as a catalyst, enzymes do not alter the position of equilibrium, but merely increase the rate of both forward and reverse reactions, by decreasing their activation energies, as shown in Fig. 1.1.1.

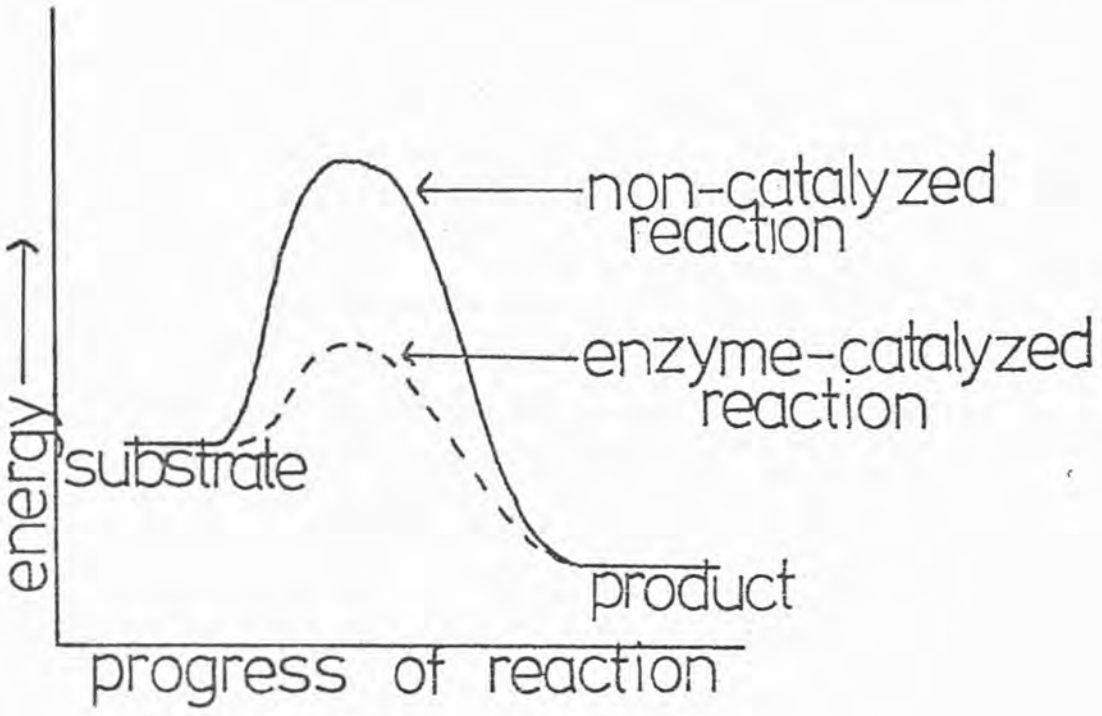


Fig. 1.1.1.

C. Enzymes as specific catalysts

No further explanation is needed to see why an enzyme catalyzes a specific reaction. Considering the nature of the protein, the charges, and the inter-atomic distances in the active site, it is evident that only certain specific compounds will be transformed by any enzyme.

1.1.2. Factors affecting the catalytic activity of enzymes.

The catalytic activity of an enzyme is measured in terms of the reaction catalyzed. The reaction rate is often expressed in International Units (I.U.), one I.U. corresponding to the formation of one μmol of product per minute. In the SI-system, the unit is mol s^{-1}, and this quantity is called a katal (kat).

$$1\ \text{I.U.} = 16.67 \times 10^{-9}\ \text{kat.}$$

In contrast to the determination of the concentration of substrate (where the accuracy of the results of a method can be checked by comparison with a carefully weighed standard), standardization in the determination of the catalytic activities of enzymes can be achieved only on the basis of defined conditions of measurement. The weighing of a crystalline enzyme with a definite catalytic activity would by no means lead to identical accurate results under different conditions of measurement. Some of the factors which affect the catalytic activity of enzymes are listed below:-

(a) Effect of substrate concentration

The basic equations for the reaction of an enzyme and its substrate were developed by Michaelis and Menten (3). In the mechanism for the reaction, a substrate S, combines with the enzyme, E, to form an intermediate complex ES, which subsequently breaks down into products, P, and liberates the enzyme:-

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P$$

It was further assumed that, after a very short time, a steady-state would exist with respect to ES (i.e. the rate of formation of ES would equal the rate of breakdown of ES), and under these conditions $k_1[E][S] = k_2[ES] + k_3[ES]$,

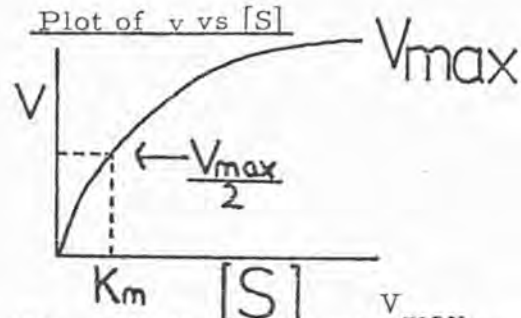
$$\text{or} \qquad \frac{[E][S]}{[ES]} = \frac{k_2 + k_3}{k_1} = K_M \text{ (the Michaelis Constant).}$$

The initial rate of reaction, V, can then be shown to be related to substrate concentrations, $[S]$, by the following equation (Michaelis-Menten equation):-

$$v = \frac{k[E][S]}{K_M + [S]}$$

or

$$v = \frac{V_{max}[S]}{K_M + [S]}$$



It may also be noted that when $[S] = K_M$, V will equal $\frac{V_{max}}{2}$, and hence the value of [S] which is experimentally found to give half the maximum velocity will be equal to the Michaelis constant, K_M. When $[S] \ll K_M$ the rate will be proportional to the substrate concentration, whilst when $[S] \gg K_M$, the rate will be independent of the substrate concentration.

(b) Effect of enzyme concentration

The Michaelis-Menten equation predicts that the initial rate of an enzymatic reaction is proportional to the enzyme concentration, [E]. This is indeed found in the great majority of cases.

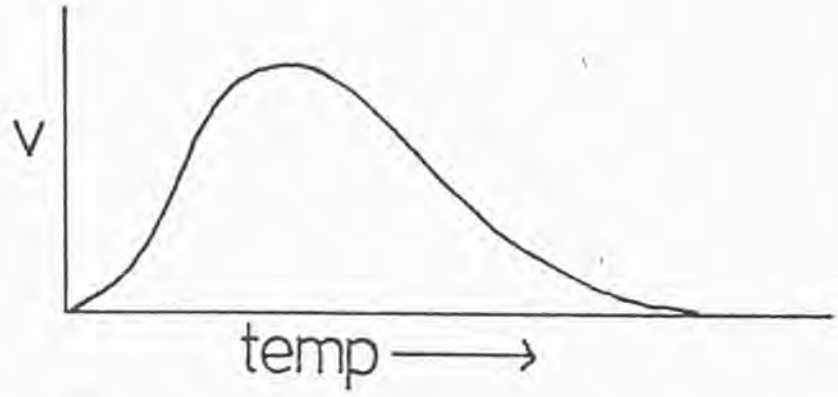
(c) Effect of temperature on enzymatic activity

- (i) on initial activity

  As the temperature is increased, two simultaneous effects occur:

  - The rate of the reaction increases, as is observed in most chemical reactions, and
  - The stability of the protein decreases due to thermal deactivation.

  The net result is a bell-shaped curve, the position of the peak of which is time-dependent.



Therefore the term "temperature optimum" is misleading and inaccurate unless it is interpreted as the optimum temperature for a particular set of reaction parameters.

- (ii) on enzyme stability

As the temperature increases, hydrophobic, ionic and electrostatic bonds are weakened, and increased kinetic energy enhances rotation about bonds, shifting important R-groups from their normal positions, resulting in decreased enzyme activity.

(d) Effect of pH on Enzymatic Activity

The degree of ionization of the surface amino acid residues is a function of the pH of the medium. Again, a bell-shaped curve of rate vs pH is obtained, but it is less influenced by reaction conditions than in the case of temperature. The main factor involved is the state of ionization of the enzyme which may affect the surface charge, the properties of the active site, and the stability of the enzyme. An enzyme may then be said to have an optimum pH. The ionization of the substrate or product may also have to be considered.

(e) Effects of inhibitors on enzymatic activity

These can be of several types:-

Irreversible inhibitors

- The inhibitor combines with the enzyme, and the resultant

complex is stable and enzymatically inactive. A compound which formed a covalent bond with a group in the active site would cause this type of results. In principle, one could "titrate" the active site with such an inhibitor. Since the active concentration is reduced, V_{max} is decreased, but K_M remains unaltered. Reactions of importance are:-

$E + S \rightleftharpoons ES \longrightarrow E + P$ S = Substrate

$E + I \text{---} EI \not\rightarrow$ I = Inhibitor

Reversible inhibitors

- The complex of inhibitor and enzyme is not stable, and may be reversed by substrate. There are various types of reversible inhibition.

Competitive - S and I compete for the same site on the enzyme. The net effect is that if the substrate concentration is very much greater than the inhibitor concentration, the reaction rate will equal V_{max}. However, K_M will increase since substrate concentration must be increased to overcome the effect of the inhibitor. Reactions of importance are:-

$E + S \rightleftharpoons ES \longrightarrow E + P$

$E + I \rightleftharpoons EI$

$EI + S \rightleftharpoons ES + I \longrightarrow E + P$

Non-competitive - Here, the inhibitor does not prevent the formation of ES, but affects the rate at which ES breaks down to form E + P. Reactions of importance are:-

$E + S \rightleftharpoons ES \xrightarrow{k_3} E + P$

$E + I \rightleftharpoons EI$

$EI + S \rightleftharpoons EIS \xrightarrow{k_3'} E + I + P$

$ES + I \rightleftharpoons EIS \xrightarrow{k_3'} E + I + P$ where $k_3 > k_3'$

V_{max} is reduced, but K_M remains unaltered.

Uncompetitive - The inhibitor attaches to ES forming an unreactive ESI. Reactions of importance are:-

$$E + S \rightleftharpoons ES \longrightarrow E + P$$

$$ES + I \longrightarrow ESI \not\longrightarrow$$

V_{max} is decreased, and K_M is increased.

Mixed - Various types of mixed inhibition may occur.

(f) Other factors that can influence enzymatic activity

Many other environmental factors may affect the observed enzymatic activity. These include the ionic strength of the medium (μ), the pressure (especially if one of the reactants is a gas), the buffering species employed, the purity of the substrate and of the enzyme itself, and also the presence or absence of activators. All of these factors must be experimentally determined, or at least arbitrarily chosen and held constant, before any thorough study of an enzyme can be made.

1.1.3. Use of enzymes in analysis

Enzymatic analysis is by no means a new branch of analytical chemistry; Osann detected hydrogen peroxide using peroxidase in 1845 (7). However, only much more recently, when reliable optical measuring instruments have become commercially available, have these methods found wider application. Another step in this development was the measurement of the fluorescence of certain coenzymes, often increasing the sensitivity of the determination by two or three orders of magnitude. However, the use of enzymes in analysis has been somewhat restricted because, being high molecular weight proteins, their stability was limited, necessitating great care in their preparation and

use. On a gram-for-gram basis enzymes are also extremely expensive to prepare in a pure form, and enzymic reactions tend also to be particularly prone to irreproducibility and error due to pH and temperature changes, unless large amounts of these expensive reagents were used to achieve true end-point or equilibrium-type assays, rather than kinetic measurements.

It has only been in relatively recent times, with the advent of techniques such as affinity chromatography, that enzymes have become commercially available in a reasonably pure form, at prices which are not prohibitive.

None-the-less, analytical methods based on the use of enzymes as reagents have been gaining popularity over the past decade, largely due to the fact that the inherent selectivity of enzymes is such that direct determination of a single species in a complex mixture is often possible without prior separation. In principle, enzymatic methods are sufficiently specific so that inherently non-specific detection systems, including such techniques as manometry, pH-stats, conductometers, other electrochemical and potentiometric methods, microcalorimetry and radiochemical techniques have been used, as well as more specific techniques such as polarimetry, spectrophotometry and fluorimetry (8).

The importance of enzymatic analysis in various fields is now manifold, and the following examples are illustrative, rather than comprehensive:-

(a) In food chemistry

Enzymes are being used to an increasing extent for the determination of carbohydrates (mono-, di-, and polysaccharides alone, or in the presence of each other), organic acids such as citrate, isocitrate, malate and D- and L- lactate, and

various alcohols (e.g. ethanol, glycerol, sorbitol) (9).

(b) In pharmacology

Enzymatic methods are being used increasingly in biochemical pharmacology (e.g. analysis of digitoxin in human blood by an enzyme immunoassay method) (9).

(c) In clinical chemistry (9)

The classical metabolites determinable by enzymatic analysis include glucose, triglycerides, cholesterol, uric acid, urea and many others. Here too, the products of thyroid gland function, steroid hormones, insulin, immunoglobulins, viral antigens etc., are detected by means of enzyme immunoassays. (9).

Section 1.2.- Immobilized Enzymes

1.2.1. A brief history, and definition, of immobilized enzymes.

One of the earliest reports of immobilized enzymes was that of Nelson and Griffin in 1916 (10), who reported the adsorption of invertase on charcoal and alumina, and demonstrated that these immobilized enzymes retained their activity. In 1948, Sumner (11), found that urease from jack beans became water-insoluble on standing in 30% alcohol and sodium chloride for 1-2 days at room temperature, and that this water-insoluble urease was still active. Many other reports of water-insoluble enzymes appeared during the next decade, utilizing a variety of supports, and it soon became apparent that it was possible, by choice of a suitable method, to confine many enzymes on, or in, a variety of water-insoluble matrices with retention of a large proportion of their catalytic activity. Such enzymes are referred to by various names, such as "insolubilized enzymes", "fixed enzymes", "matrix-bound enzymes", "solid-supported enzymes" or "gel-entrapped enzymes", according to the nature of binding and

the choice of support.

The term "immobilized enzyme" was recommended at the 1st Enzyme Engineering Conference in 1971, to describe the general case of physical or chemical confinement or localization of enzyme molecules during a continuous catalytic process. The word "continuous" is emphasized in order to limit the definition to a reasonable extent. Although an enzyme contained in a beaker, or vial, could be thought of as an "immobilized enzyme", it is not considered to be that in the context of the definition because the enzyme cannot be employed efficiently in a continuous manner.

1.2.2. Classification of immobilized enzymes.

Immobilized enzymes can be classified according to immobilization procedure, as shown in Fig. 1.2.2.1. Thus, for practical purposes, enzymes can be considered as "native", "soluble but chemically modified" or "insoluble and chemically modified".

Except for the use of an ultrafiltration membrane, immobilized enzymes are water-insoluble, and the enzyme reaction is carried out in a heterogenous medium.

As can readily be seen in Fig. 1.2.2.1., immobilized enzymes can be classified into three broad types, which can often be further sub-divided, according to the actual type of immobilization performed. A very brief and general description of the various types, and sub-types, of immobilized enzymes is given below:-

1. Carrier-bound enzymes

This is the oldest immobilization method for enzymes, and the subject of considerable published material. When enzymes are immobilized in this way, care is required regarding the

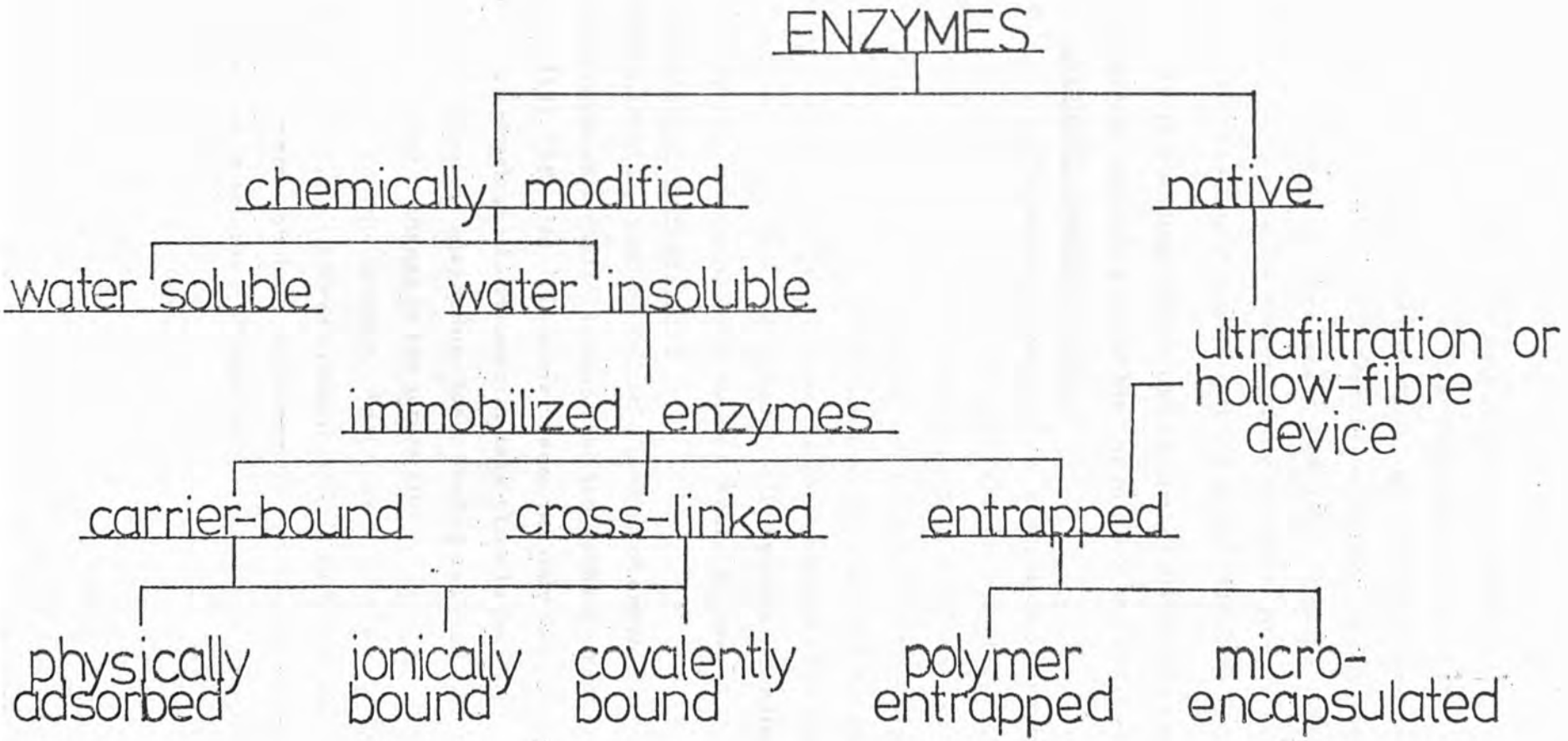

ENZYMES
chemically modified
native
water soluble
water insoluble
ultrafiltration or hollow-fibre device
immobilized enzymes
carrier-bound
cross-linked
entrapped
physically adsorbed
ionically bound
covalently bound
polymer entrapped
micro-encapsulated

Fig. 1.2.2.1.

selection of carriers, as well as binding techniques. Although the selection of the carrier also depends upon the nature of the enzyme itself, pore size, particle size, surface area, and the nature of the surface of the carrier must also be considered. Generally speaking, increases in hydrophilic groups and surface area will increase the amount of the bound enzyme per unit carrier, resulting in higher activity of the immobilized enzyme.

The carrier-binding method can be further divided into three categories, according to the binding mode of the enzyme.

(a) Physically adsorbed enzymes

This method is based on the physical adsorption of enzyme protein on the surface of water-insoluble carriers, e.g. (10), (12), (13). The method causes little or no conformational change of the enzyme protein, or destruction of its active centre. However, this method has the disadvantage that the adsorbed enzyme may leak from the carrier, because the forces binding the protein and carrier are weak, being principally hydrogen-bonding, hydrophobic interaction and van der Waals' forces.

(b) Ionically bound enzymes

This method is based on the ionic binding of enzyme protein to water-insoluble carriers containing ion-exchange residues, e.g. (14), (15), (16). In some cases, not only ionic binding, but also physical adsorption may take place in the binding. As with physical adsorption, ionic binding causes little or no conformational change in the active site of the enzyme, and often yields high enzyme activity. Again, as with physical adsorption, the binding forces between protein and support are less strong than in covalent binding, so leakage of enzyme from the carrier may occur in solutions of high ionic strength, or upon the

variation of pH.

(c) Covalently bound enzymes

This is based on the binding of enzymes to water-insoluble carriers by covalent bonds. Amongst the various carrier-binding methods, most studies have utilized this technique. The functional groups that can take part in the covalent binding of an enzyme to a carrier are:-

(1) α- or ε-amino groups (e.g. terminal amino groups, or lysine residues).

(2) α-, β-, or γ-carboxyl groups (e.g. aspartic acid, glutamic acid, proline or hydroxyproline residues).

(3) sulphydryl groups (e.g. cysteine residues).

(4) hydroxyl groups (e.g. serine, threonine, hydroxyproline residues).

(5) imidazole groups (e.g. histidine residues)

(6) phenolic groups (e.g. tyrosine residues).

The selection of conditions for immobilization by covalent binding is more difficult than in the previous examples. The reaction conditions required are not particularly mild, and, in some cases, covalent binding may alter the conformational structure, and/or the active centre of the enzyme, resulting in a major loss of activity. However, the binding force between the enzyme and carrier is strong, and leakage of the enzyme does not occur even in the presence of substrate or salt solutions of high ionic strength. Amongst the most popular methods are diazo-coupling (to tyrosine residues) e.g. (17-24), coupling to glutaraldehyde-activated supports (via lysine residues and terminal amino groups), e.g. (25-35), and coupling to cyanogen bromide activated celluloses

(also via lysine residues and terminal amino groups), e.g. (36-38), whilst amongst the most popular supports are included controlled porosity glass e.g. (20-21), (23-25), (31), polyacrylamide e.g. (17-18), (29), (34-35) cellulose, and various of its derivatives e.g. (19), (26), (36-38) and nylon, e.g. (32, 33), 39-42). An excellent review of various enzyme immobilization methods, and supports, is given by Mosbach (43).

2. Cross-linked enzymes

This immobilization method is based on the formation of chemical bonds, as in the covalent binding method. However, the immobilization of the enzyme is here performed by the formation of intermolecular cross-linkages between molecules by means of bi- or multifunctional reagents, such as glutaraldehyde e.g.(44-46), or diisocyanates (47). The cross linking agent is chosen to bind functional groups specifically on the protein not involved in the active site, but because the conditions are again relatively severe, the conformation of the active centre of the enzyme may be affected by the reaction leading to significant loss of activity.

3. Entrapped enzymes

(a) Enzymes entrapped within polymer matrices

Enzymes and cells have been entrapped in the interstitial spaces of cross-linked polymers, e.g. (48-50). Entrapment is accomplished by cross-linking after addition of the protein. The entrapped enzyme, once within the cross-linked polymer, cannot escape from the matrix, but, on the other hand, only low molecular weight substrates can reach such an entrapped enzyme.

(b) Microencapsulated enzymes

Proteins and other material can be immobilized within microcapsules prepared from organic polymers e.g. (51, 52). The entrapped macromolecules cannot escape, but low molecular weight materials

can enter and leave the microcapsules by diffusion through the membrane. The membranes are generally prepared by the process of phase separation which is similar to the homogenization of water in oil. One phase is not miscible with the other, but forms a droplet or coaxervate with the other phase when mixed.

(c) Enzymes entrapped by ultrafiltration and hollow-fibre devices

Immobilization of a protein device containing semi-permeable membranes (e.g. standard ultrafiltration equipment) is, in a sense, figurative, since immobilization simply consists of placing the protein in solution on one side of a semi-permeable membrane which is permeable to small molecules only (53). Although the enzyme is still in its native state, such an enzyme would still obey the criteria used to define an immobilized enzyme, as stated earlier.

1.2.3. Properties of Immobilized Enzymes compared to free Enzymes.

The main difference between a free and an immobilized enzyme is that, once immobilized, the enzyme is no longer completely surrounded by an aqueous environment. One can suspend the immobilized enzyme in a solution of substrate, activators or other components at a particular pH and ionic strength, but there is no assurance that the conditions in the medium immediately surrounding the enzyme (its "microenvironment") are the same as those in the external solution. Indeed, they may be quite different.

Any change in a chemical or physical property of an enzyme upon immobilization can be conveniently viewed as being due either to the nature of the water-insoluble support or to some actual alteration of the enzyme itself. It is often extremely difficult,

if not impossible, to ascribe precisely the cause and magnitude of an alteration in a property of an enzyme upon its immobilization.

In the former case the major effects involved are the diffusion of the substrate across a relatively poorly stirred Nernst diffusion layer to the enzyme on the surface of the carrier (external mass transfer effects) or through virtually stagnant conditions inside a pore in the surface of the carrier to an enzyme situated on the inside of the pore (internal mass transfer effects); to electrostatic or other (e.g. hydrophobic) interactions between the carrier and substrate, activators, inhibitors or buffer, and to steric hindrance effects.

All these effects can be summed up in the term "microenvironmental effects", and, in most cases, the changes in the properties observed are apparent changes, which would not be present in the absence of the carrier, preventing the enzyme from diffusing freely into the bulk solution, rather than to any changes in the actual properties of the enzymes.

The latter case, however, involves actual modification of amino acid residues in the active site of the protein, conformational changes of the enzyme protein, and changes in the charge on the enzyme due to its chemical modification. Some of the properties of enzymes often found to be altered upon immobilization are as follows:-

(1) Enzyme activity

The usual way of expressing the activity of an enzyme is in μ-mol of product formed per minute (International Units). Normally this method is also used for expressing the activity of an immobilized

enzyme. Since the activity is dependent on the concentration of the enzyme, anything that affects the effective concentration of the enzyme will affect this parameter. The activity of an immobilized enzyme can vary from nil to apparently higher values (54) than the native, water-soluble enzyme. In general there seems to be no normal or expected range of activities for an immobilized enzyme, the activity obtained being dependent on the particular enzyme, support, method of immobilization and often the individual experimentalist.

(2) Substrate specificity

The relative activities towards two different substrates shown by an enzyme often changes upon immobilization of the enzyme, particularly where one substrate is of a very high molecular weight. This is often due to the slower rate of diffusion of the high molecular weight substrate to the enzyme from the bulk solution, or to greater steric hindrance to the approach of the high molecular weight substrate to the active site of the enzyme (55-56), particularly where the enzyme is immobilized by an "entrapping" method. (48). The opposite effect has also been reported (57) where the high molecular weight substrate was attracted preferentially towards the carrier. In most cases, these effects seem to be apparent, rather than real.

(3) pH-activity

Almost all supports are charged to some degree and in practically all immobilization techniques some charged material is introduced at some time, even if it is only protein. The net charge of a matrix can have a profound influence on the characteristics of an immobilized enzyme due to its effect on the microenvironment of that enzyme.

For example, in the case of an enzyme immobilized to a negatively charged carrier, the hydrogen ion concentration in the domain of this carrier will be greater than that in the bulk solution (the phase measured experimentally with a pH meter). Consequently, this enzyme will experience a hydrogen ion concentration that is higher than that of the bulk solution, particularly in low ionic-strength media, apparently displacing the optimum pH for the enzyme towards bulk solutions of lower hydrogen ion concentration (i.e. more alkaline). The opposite effect (i.e. displacement of pH optimum towards lower pH) is often observed for enzymes immobilized to positively charged supports (58-60).

(4) Temperature-stability

At present, there is considerable confusion in the literature as to whether immobilized enzymes in general have enhanced thermal stabilities. Although there are far more examples of enhanced thermal stability in the literature, this may be due to selective reporting. None-the-less, Melrose (61) has found that of fifty enzymes studied, upon immobilization thirty showed increased temperature stability, eight had less, whilst twelve had a similar stability to that of the free enzyme. It therefore seems likely that the majority of enzymes will exhibit increased temperature stability upon immobilization, provided that a suitable immobilization method is used. This is probably due to a combination of the stabilization of the tertiary structure of the enzyme by binding (covalently-bound or cross-linked enzymes) and to protection from turbulence (all immobilized enzymes).

(5) Michaelis Constant (K_M)

This may increase, remain unaltered, or (occasionally), decrease upon immobilization. As previously mentioned, the

Michaelis constant is equal to the substrate concentration which will give half the maximum velocity. As the immobilized enzyme particles are surrounded by a poorly-stirred Nernst diffusion layer, which, relative to the bulk solution, is depleted in substrate and rich in product, half-saturation of the immobilized enzyme will normally occur at a higher bulk solution substrate concentration than normally required to half-saturate the freely soluble enzyme. This, in turn, will give a higher <u>apparent</u> K_M value of the immobilized enzyme for its substrate. Even so, the <u>apparent</u> K_M for an immobilized enzyme is (in the opinion of the author) a largely meaningless term, unless every condition under which it is measured is rigorously specified. For example, if the thickness of the Nernst diffusion layer was reduced by methods such as reducing the particle size of the support, increasing the stirring rate of the suspended particles, or even solubilizing the water-insoluble derivative, then the <u>apparent</u> K_M value should be considerably reduced and could even approach the <u>true</u> K_M (60, 62-64).

The charge on the support particles may also influence the apparent K_M value (particularly where the substrate is also charged). Opposite charges on the support and substrate will tend to increase the substrate concentration in the microenvironment of the immobilized enzyme, giving a lower apparent K_M value than otherwise expected, whilst the opposite effect would be observed for like charges on the support and substrate (65-66).

Whereas all the previously mentioned factors affected the <u>apparent</u> K_M, due to their influence on the microenvironment of the immobilized enzyme, it is also possible to imagine the chemical modification of the enzyme introducing some conformational

rigidity to the enzyme, and placing a restriction on the ease of achieving the conformational change which may be necessary for catalysis. The active site of the enzyme may also be sterically hindered. These will result in changes in the Michaelis constant which are real, rather than apparent.

(6) Ionic strength

Again, there is no assurance that the ionic strength of the micro-environment is the same as that of the surrounding solution. Goldstein (67) has used this property to reverse charge-charge interactions between charged supports and charged substrates.

(7) Product concentration

Initially this is no problem, but as the reaction proceeds, product must diffuse out of the poorly-stirred Nernst diffusion layer (a relatively slow-moving substrate depleted, product enriched boundary layer in the vicinity of each support particle) into the bulk solution. The product-rich nature of this layer may give rise to greater product inhibition than normally expected, or cause the reverse reaction to occur to such an extent that the net reaction is zero.

In summary it is possible to say that almost every enzyme parameter may be affected by the immobilization process, in a real, or an apparent way.

1.2.4. Choice of support materials

From the preceding discussion it is apparent that choice of support can have a profound effect on the characteristics of the immobilized enzyme bound to, or in the vicinity of, such a support.

Some general requirements of all supports should include

(a) Chemical durability under the operating conditions of the process (e.g. w.r.t. temperature, pH, solvent, ionic strength, etc.).

(b) High available surface area for enzyme attachment (usually means the support will be finely divided and porous).

Additional factors that may, or may not, be important, according to the actual process to be used, include

(c) Mechanical strength and dimensional stability (to avoid compaction and to protect the enzyme structure)

(d) Microbial resistance (to avoid destruction of both enzyme and carrier)

(e) Charge on carrier (for rapid reactions, it is advantageous to use a carrier with charge opposite to that of the substrate)

(f) Regeneration of support (where support material is relatively expensive)

(g) Presence of high concentration of suitable functional groups (when enzyme is to be covalently attached)

(h) Suitable distribution of pore-sizes (particularly when enzyme is to be physically adsorbed, e.g. (68)).

It is evident that there is no universal support of choice, and each support must be chosen to suit the process in which it will be used. Having chosen the support, the method of immobilization will be, to a certain extent, dictated by this choice. A comprehensive review of most supports so far used in the immobilization of enzymes has been given by Mosbach (43).

1.2.5. Advantages and disadvantages of using immobilized enzymes as opposed to free enzymes

Having mentioned some of the characteristics and properties of free and immobilized enzymes, it is necessary to answer the question"Is it worth immobilizing enzymes ?" The answer may be yes, or no, according to the process one has in mind, but some of the advantages and disadvantages of immobilizing enzymes are given below.

Advantages of immobilized enzymes

These include:-

(a) Re-usability of the immobilized enzyme

Unlike most inorganic catalysts enzymes are generally soluble and relatively unstable, and can be used but once in free solution unless elaborate separation techniques are employed. On a milligram basis of pure protein enzymes are one of the most expensive and difficult materials to obtain in reasonable quantity. This re-usability is hence important to the analytical chemist, as it enables him to use relatively large amounts of immobilized enzyme to achieve rapid thermodynamic end-point type assays at low cost. Use of a large excess of immobilised enzyme also gives "pseudo 100%" stability over a long period, often several months or more.

(b) Ease of separation from reactants and products

Industrially, the ease of separation enables greater control over the reaction (e.g. partial hydrolysis of proteins to amino acids), without destroying the enzyme.

(c) Increased storage, temperature and pH stability

This is often achieved by stabilization of the tertiary structure of the enzyme, and by protection from turbulence, as previously

discussed in I.2.3.

Any procedure that can economically extend the life of these expensive biologically active materials needs to be considered.

(d) The product is not contaminated by the enzyme

This is very important in the modification of foodstuffs, and in the pharmaceutical industry. Many of the most suitable enzymes for these transformations are not allowed in foods or injectables, and immobilization of these enzymes removes the need for elaborate separation procedures.

(e) Applicability of immobilized enzymes to continuous flow processes

Continuous flow processes are much more efficient than batch processes, both industrially and analytically.

(f) Increased rates for multi-step reactions

For a multi-step process, a $\longrightarrow$ b $\longrightarrow$ c, immobilized enzymes are often more efficient than soluble enzymes, due to the relatively high concentration of b that accumulates in the microenvironment of the enzyme.

(g) Other analytical advantages

These include more predictable decay rates, elimination of reagent preparation, and minimization of operator error.

(h) In vivo biological models

Most enzymes are not found randomly distributed throughout the body, but bound to certain types of membrane. Immobilization of an enzyme on to a matrix similar to that in which it is naturally found provides the potential to use them as in vitro models of in vivo systems. Indeed, bound enzymes packed consecutively in columns have mimicked seven steps in the glycolytic pathway (61).

Disadvantages of immobilized enzymes

(a) Expense of carrier or reagents for immobilization

These may, or may not,be prohibitive, depending on the actual process.

(b) Low real or apparent activity

This may be due to immobilization procedure (real), or to low mass transfer rates (apparent).

(c) High pressure drops across reactor

As the particle size is decreased to enable more enzyme to be immobilized, so the pressure drop across the column will increase. Again, this may, or may not, be important.

(d) High molecular weight substrates

As mentioned in 1.2.3., immobilized enzymes are often less effective on high molecular weight substrates.

1.2.6. Use of immobilized enzymes in analysis

The advantages of using enzymes, and, more particularly, immobilized enzymes in analysis have been mentioned in 1.1.3., and 1.2.5., respectively, and not surprisingly, reports dealing with the analysis of many important compounds using many different techniques, in conjunction with immobilized enzymes, are common.

Table 1.2.6.1. lists some examples which are meant to be illustrative and not comprehensive, to give some idea of the diversity that immobilized enzymes have found in analysis. Thermal analysis methods are excluded here, but will be dealt with in 1.3.3.

Table 1.2.6.1.

substrate	immobilized enzyme(s)	detection system	references
glucose	glucose oxidase	electrode	(17), (33), (68-74), (93)
glucose	glucose oxidase/peroxidase	spectrophotometer	(33), (41), (75-79)
glucose	glucose oxidase	chemilumiscence detector	(80, 81)
glucose	glucose oxidase	polarimeter	(82)
glucose	glucose oxidase	fluorimeter	(83)
glucose	hexokinase/glucose-6-phosphate dehydrogenase	spectrophotometer	(84)
urea	urease	electrode	(71), (85-94)
urea	urease	spectrophotometer	(41), (75, 76), (79), (95)
urea	urease/α-ketoglutarate	fluorimeter	(96)
uric acid	urate oxidase	electrode	(34)
uric acid	urate oxidase/peroxidase	spectrophotometer	(41), (79)
acetylthiocholine	acetyl cholinesterase	electrode	(97)
acetylthiocholine	acetyl cholinesterase	spectrophotometer	(98)
L-amino acids	L-amino acid oxidase/peroxidase	electrode	(99)
L-amino acids	L-amino acid oxidase	electrode	(18)
D-amino acids	D-amino acid oxidase	electrode	(100)
tyrosine	tyrosine decarboxylase	electrode	(92)
penicillin	penicillinase	electrode	(71), (101-104)
ethanol	alcohol oxidase	electrode	(105, 106)
lactic acid	lactate dehydrogenase	electrode	(70)
lactic acid	lactate dehydrogenase	fluorimeter	(107)
nitrate	nitrate reductase	spectrophotometer	(108)
creatine	creatine phosphokinase/pyruvate kinase/ lactate dehydrogenase	fluorimeter	(109)
cholesterol	cholesterol oxidase/peroxidase	fluorimeter	(110)
cholesterol esters	cholesterol esterase/cholesterol oxidase/ peroxidase	spectrophotometer	(111, 112)
D-arabinose	D-arabinase dehydrogenase	spectrophotometer	(113)

(continued ...)

Table 1.2.6.1. (continued)

substrate	immobilized enzyme(s)	detection system	references
galactose	galactose oxidase	electrode	(114)
hypoxanthine	xanthine oxidase	polarographic	(115)
indole-3-acetate	indole-3-acetic acid oxidase/peroxidase	fluorimeter	(116)
amylose	β-fructofuranosidase	fluorimeter	(117)

Section 1.3. Thermal Analysis

1.3.1. General Thermometric Methods

Although many papers appear in the literature on thermal analysis, most of them can be placed into one of the three broad categories shown in Table 1.3.1.1.

Table 1.3.1.1.

Designation	Property measured	Apparatus
Thermogravimetric analysis (T.G.A.)	change in weight	thermobalance
Differential thermal analysis (D.T.A.) (or differential scanning calorimetry)	heat evolved or absorbed	DTA apparatus or differential scanning calorimeter
Thermometric titrimetry (T.T.)	change in temperature	Isoperibol titration calorimeter
Direct injection enthalpimetry (D.I.E.)	change in temperature	isoperibol solution calorimeter

---

Thermogravimetric analysis (T.G.A.)

This is a technique whereby the weight of a sample can be followed over a period of time while its temperature is being changed (118) (119). The balance is calibrated, preferably each time it is used, by placing a known weight on the pan to give a reference mark. It must be remembered, however, that the thermogravimetric method, as ordinarily carried out, is dynamic, the system is never at equilibrium, and hence the temperatures of distinctive features on the curves are somewhat different as observed on different instruments, or on the same instrument at different rates of temperature scanning, or with

different size samples etc. Obviously, the thermobalance used to measure the weight of the sample must be very sensitive, and stringent precautions against convection effects interfering with the balance must be taken.

Most balances intended for precise work have provision for automatic recording of weight either against time or, directly against temperature.

Differential thermal analysis

This is a technique by which phase transitions or chemical reactions can be followed by observation of the heat absorbed or liberated. It is especially suited to studies of structural changes within a solid at elevated or low temperatures, where few other methods are available. Much of the quantitive work is now carried out on an instrument manufactured by Perkin-Elmer, called a Differential Scanning Calorimeter (D.S.C.), although Technical Equipment Corporation also manufacture a Deltatherm Dynamic Adiabatic Calorimeter (D.A.C.), and Du Pont, a Differential Thermal Analyser (D.T.A.).

Whereas the two latter designs employ essentially adiabatic calorimeters, the Perkin-Elmer D.S.C. is a calorimeter of the isothermal type. Two sample holders, (containing a sample, and also an identical, empty, reference), are provided with their own reactive heaters. When the differential thermocouple surrounding both starts to register a voltage, an automatic control loop sends just enough power into which ever of the two samples is cooler to counteract the trend, and keep the two temperatures within a very small fraction of a degree of equality. A second

electronic loop forces the temperature of the cells to increase linearly with time. The recorder traces out the electrical power which has to be delivered to one or other cell to maintain isothermal conditions. The resultant peak area is then an exact measure of the energy supplied to (or absorbed by), the unknown sample.

Whereas the previous methods described have been applied mainly to the analysis of solid materials, and to the measurement of properties of the sample over a wide temperature range, two distinct analytical methods based upon the measurement of small temperature changes engendered by the enthalpy change accompanying a reaction in the solution phase have been developed. The earlier, and more widely used of these techniques, is often termed "thermometric titrimetry" (120), whilst a more recent technique has been named direct injection enthalpimetry (D.I.E.) (121). A comprehensive and rigorous review of these techniques has been given by Carr, (122).

Thermometric titrimetry (T.T.)

This method utilizes variations of temperature to indicate titration end-points, in precisely the same fashion as variations of conductance or of limiting current are used to locate end-points in conductometric and amperometric titrations, and has been used successfully to determine species such as acids (123), bases (124), redox species (125), precipitants (126), and complex-forming species (127). In addition, the method has been applied to a number of interesting organic analytical problems, including diazotization of anilides (128), hypochlorite oxidation of sulpha-drugs (129), and organo-aluminium reactions (130).

One of the major features of thermometric methodologies is the range of problems to which they are applicable, due to the

generality of heat changes in chemical reactions.

The two major requirements which must be met in order for T. T. to be successfully applied are

(a) A reasonably large reaction enthalpy (say, at least 20 kJ mol^{-1})

(b) The reaction must be relatively fast.

The essential equipment for T. T. is relatively simple in comparison to the sophisticated apparatus encountered in electrochemical and spectrochemical analysis, and can be divided into four categories

(1) Temperature measuring devices (e.g. thermistors)

(2) Associated circuitry (e.g. Wheatstone bridges and recorders)

(3) Calorimetric vessels and thermostats

(4) Ancillary equipment for calibration and automatic determination of end points.

Direct injection enthalpimetry

This utilizes a measurement of a net temperature change, or some related variable, as a direct measure of the concentration of the sample species. As previously stated, the technique is based on the measurement of heat evolved, rather than on location of an end point, when a sample A is completely consumed by an excess of reagent (R).

e.g. $aA + rR \rightleftharpoons pP$

A reasonably large excess of R is often employed to drive the equilibrium to completion, and to help ensure a relatively short half life for the process.

In general, although thermometric methods are not without serious limitations, they possess a variety of advantages over the more familiar electro-analytical and spectrochemical methods.

For example:-

(a) The nature of the solvent is virtually irrelevant

(b) The method can, in principle, be applied to reactions of any type

(c) Data interpretation is generally simple

(d) The apparatus is often simple, and easily constructed.

On the other hand, some of the major limitations placed on thermometric measurements by the chemical reaction are

(a) The reaction enthalpy and solubility of the unknown must be such as to produce an easily measurable temperature change

(b) The added reagent (titrant), must be about 100 times as soluble as the unknown, so that mismatch in temperatures of the sample and titrant will cause minimal errors, and also minimize the changes in heat capacity of the system

(c) The reaction must proceed to completion in a time period which is short compared to the rate of heat loss from the calorimetric vessel and the rate of addition of the titrant

(d) If the process is carried out by titration, the equilibrium constant must be such that >99.9% completion is achieved with a modest (25-50%) excess of titrant.

1.3.2. Thermal analysis using free enzymes.

The calorimetric technique was one of the earliest methods to be applied to the quantitative study of chemical reactions (131). Its potential for monitoring biochemical and biological processes was recognized very early, but a wider use of calorimetry for these purposes was not possible until the development of the micro-calorimetric technique (132, 133), and of small semi-conductor devices called thermistors (134), allowing very sensitive heat measurements to be carried out on small sample volumes.

Table 1.3.2.1.

substrate determined	environment	enzyme(s) used	reference
glucose	in serum, plasma and whole blood, with prior deproteinization + solution	hexokinase/glucose-6-phosphate dehydrogenase	(135-142)
glucose	solution	glucose oxidase	(143)
urea	urine + solution	urease	(144-146)
uric acid	solution	urate oxidase	(147)
hydrogen peroxide	solution	peroxidase	(144)
penicillin G, ampicillin, phenoxymethyl penicillin	solution	penicillinase	(148)
pyruvate	solution	lactate dehydrogenase	(149)

Calorimetry is, by principle, a non-specific technique, which makes it suitable as a general monitor for complex processes, such a cell metabolism. On the other hand, in combination with the highly specific reactions of enzymes, calorimetry offers a very general principle of detection, totally independent of the optical properties of the system investigated, and with the additional advantage of eliminating the need for auxiliary enzymes and their co-factors.

The use of the direct-injection enthalpy technique previously described is suited ideally to the determination of a substrate by an enzyme, and has been utilized for a variety of purposes by many authors, as can be seen from Table 1.3.2.1.

In addition to the determination of substrates, studies on enzyme activities have been carried out on glucose oxidase (143), cholinesterase (143), alkaline phosphatase (143), lactate dehydrogenase (143) and urease (146).

The area of instrument design may be divided into two broad classes, based upon the fundamental principle of heat measurement employed in the instrument.

(a) Isoperibol calorimeters e.g. (135-137), (145), (147-149). If one attempts to determine the amount of heat liberated in a reaction by causing the heat to raise the temperature of the system, minimizing heat loss to the surroundings by enclosure in a constant-temperature environment and this temperature rise is measured it is termed isoperibol calorimetry. For this type of determination, a thermistor is generally used as the detector. Although thermistors can take several forms, the most frequently used consist of a small piece of semi-conducting material, the resistance of which R_{T_1} at a temperature T_1 (K)

can be related to the resistance(R_{T_2}) at any other temperature T_2(K) by the equation:-

$$R_{T_1} = R_{T_2} \exp\left(\frac{B}{T_1} - \frac{B}{T_2}\right)$$ where B is a constant for a given thermistor

Consequently, although the variation of resistance with temperature over a large temperature range is exponential, over a very small range, it can be regarded as approximately linear.

Therefore, if a thermistor is equilibrated in a solution at a constant temperature before the reaction, its resistance at this temperature can be balanced against a Wheatstone bridge circuit so that no current flows through the circuit. After the reaction, any small increase in temperature of the solution will be reflected by a corresponding lowering of the resistance of the thermistor, which will cause a disbalance potential to be registered in the Wheatstone bridge circuit. This can be amplifed and fed to a chart recorder.

The calorimeter must, for obvious reasons be well insulated from its surroundings, often by a vacuum container plus other types of insulation. The solution added to the vessel as reagent, generally enzyme, is fairly concentrated, to minimize the effect of any temperature mismatch of the solutions, and to minimize the heat capacity of the system. With care, the system is quite sensitive, although claims by McGlothlin and Jordan to be able to detect a temperature rise of 0.00001^{o}C (10^{-5} ^{o}C)(136) are, in the opinion of the author, ambitious. Most calorimeters appear to be able to measure to 0.001 (10^{-3o}C) with reasonable accuracy. An in-depth treatment of this type of calorimeter has been given by Carr (150).

(b) Heat flow calorimeters e.g. (138-143), (146)

The alternative approach to heat measurements makes use of the fact that isoperibol measurements oppose the natural tendency of heat to flow from an area of higher temperature to one of lower temperature. Thus, if one is able to place a heat-sensing device in the path of the heat flow, measurements are possible. For enzyme catalyzed reactions it has been found that the heat flow system works well, due in part to the fact that the detector does not liberate heat into the system. Thorough theoretical treatments of the use of heat-flow calorimeters in biochemical and biological systems have been given by Prosen (142), Wadso (151), Beezer and Tyrrell (152), and Beezer (153).

1.3.3. Thermal analysis using immobilized enzymes

Some of the advantages of using immobilized enzymes, as opposed to soluble enzymes, have been given in 1.2.5. In the case of thermal analysis, the re-usability, the increased stability, the applicability to continuous flow processes, the elimination of reagent preparation and minimization of operator error when using immobilized enzymes are particularly important. (See 1.2.5. for a fuller discussion of these, and other points).

The enthalpy change associated with the reaction of an immobilized enzyme can be measured by two different principles (see 1.3.2.), based on the flow technique (i.e. the measurement of a temperature rise with a thermistor as sensor in an essentially adiabatic reaction cell containing the immobilized enzyme, or the measurement of a heat flux from the cell, to an infinite heat sink, across a series of thermocouples).

Whereas the heat-flux technique appears to be slightly more sensitive,

it requires a longer time per analysis (due mainly to the relatively high time-constant of the calorimeter, compared to that of thermistor) and is also very much more expensive to build or purchase than the relatively inexpensive thermistor/adiabatic flow calorimeter assembly. Consequently many papers (see Table 1.3.3.1) have appeared in the literature utilizing the combination of immobilized enzymes and an adiabatic flow cell with a thermistor as a temperature sensor. By contrast, the combination of immobilized enzymes in conjunction with a heat-leak type calorimeter in thermal analysis has only been reported in the literature on three occasions (154-156).

Most of the papers appearing in the literature on the combination of immobilized enzymes and thermal analysis, up to mid 1980, are listed in Table 1.3.3.1, and can be divided broadly into the following three categories:-

(a) packed bed reactor/heat leak type microcalorimeter
(b) packed bed reactor/adiabatic calorimeter/thermistor
(c) enzyme bound thermistor.

Additionally, a new method, thermometric enzyme linked immunosorbent assay (TELISA), has been applied to the assay of endogenous and exogenous compounds in biological fluids, based on the previously established enzyme linked immunosorbent assay technique (ELISA), but in this case utilizing the enzymic heat formation, which is measured using a packed-bed/adiabatic microcalorimeter/thermistor (or "enzyme thermistor") detection system (180).

A theoretical treatment of some analytical aspects of immobilized enzyme columns, used in conjunction with thermistors, has been

Table 1.3.3.1.

substrate	immobilized enzymes(s)	category type (see above)	references
glucose	glucose oxidase/ catalase	packed bed/ heat-leak microcalorimeter	(154) (156)
glucose	glucose oxidase/ catalase	packed bed/ adiabatic microcalorimeter/ thermistor	(156-161)
glucose	glucose oxidase/ catalase	enzyme-bound thermistor	(162, 163)
glucose	hexokinase	packed bed/ adiabatic microcalorimeter/ thermistor	(164)
glucose	hexokinase	enzyme-bound thermistor	(163)
glucose	saccharomyces cerevisiae (whole cells)	packed bed/ adiabatic microcalorimeter/ thermistor	(49)
urea	urease	packed bed/ heat-leak microcalorimeter	(156)
urea	urease	packed bed/ adiabatic microcalorimeter/ thermistor	(157), (166 -169)
urea	urease	enzyme-bound thermistor	(162)
N-benzoyl L-arginine ethyl ester (BAEE)	trysin	packed bed/ heat-leak microcalorimeter	(155)
B.A.E.E.	trysin	packed bed/ adiabatic microcalorimeter/ thermistor	(157), (170, 171)
penicillin	pencillinase	packed bed/ adiabatic microcalorimeter/ thermistor	(157)
lactose	β -galactosidase/ glucose oxidase/ catalase	packed bed/ adiabatic microcalorimeter/ thermistor	(158) (161)
hydrogen peroxide	catalase	batch microcalorimeter containing immobilized enzyme	(172)
hydrogen peroxide	catalase	vessel-less microcalorimeter	(173)
hydrogen peroxide	catalase	enzyme-bound thermistor	(162)
A. T. P.	apyrase	packed bed/ adiabatic microcalorimeter/ thermistor	(170, 171)
uric acid	urate oxidase	packed bed/ adiabatic microcalorimeter/ thermistor	(158)

(continued...)

Table 1.3.3.1. (continued)

substrate	immobilized enzyme(s)	category type (see above)	reference
cholesterol esters	cholesterol esterase/ cholesterol oxidase/ catalase	packed bed/ adiabatic microcalorimeter/ thermistor	(158)
cholesterol	cholesterol oxidase/ catalase	packed bed/ abiabatic microcalorimeter/ thermistor	(158)
sucrose	invertase/ glucose oxidase/ catalase	packed bed/ abiabatic microcalorimeter/ thermistor	(174)
ascorbic acid	ascorbic acid oxidase	packed bed/ adiabatic microcalorimeter/ thermistor	(174)
oxalic acid	oxalic acid decarbonylase	packed bed/ adiabatic microcalorimeter/ thermistor	(175)
creatinine	creatinine iminohydrolase	packed bed/ adiabatic microcalorimeter/ thermistor	(176)
cellobiose	β-glucosidase/ glucose oxidase/ catalase	packed bed/ adiabatic microcalorimeter/ thermistor	(177)
cephalosporins	cephalosporinase	packed bed/ adiabatic microcalorimeter/ thermistor	(178)
phenol	tyrosinase	packed bed/ adiabatic microcalorimeter/ thermistor	(179)
cyanide	injectase	packed bed/ adiabatic microcalorimeter/ thermistor	(179)
cyanide	rhodanase	packed bed/ adiabatic microcalorimeter/ thermistor	(179)

given by Schifreen et al (169), and a review of some of the work mentioned in Table 1.3.3.1. has been provided by Danielsson(177).

Section 1.4. The scope of the present work

The majority of the work presented in this thesis is concerned with the analytical applications of immobilized enzymes using thermal detection systems, although Chapter 4 describes work carried out using electrodes and spectrophotometers as detectors.

The ultimate aims were to devise analytical techniques for certain species, based on the well-recognized advantages of immobilized enzymes (Chapter 1, Section 2.5), in combination with a detection system based on a universal property of all chemical reactions (i.e. an enthalpy change), and to understand the relative significance of, and where possible to optimize factors such as particle size of support, flow rate, sample dispersion and choice of buffer etc. In principle, this combination should be applicable to any enzyme-catalysed reaction, and this has generally been shown to be so (Chapter 3). Where a reaction was believed to be quantitative, it was possible to measure the overall enthalpy of reaction, the results showing good agreement with those obtained using soluble enzyme methods. Hence,it was possible to study the effect of buffer on overall reaction enthalpy, and also, for a series of semi-synthetic penicillins, to correlate qualitatively their enthalpy of hydrolysis with the structure of the side-chain.

Chapter 3 also compares the capabilities of the analytical techniques developed, using the commercial heat leak type LKB 10700-1 microcalorimeter (flow-through mode) and the far less expensive "home-made" semi-adiabatic flow micro-calorimeter. The relative success of this technique for determination of many enzyme substrates (Chapter 3) prompted

a study of enzyme inhibitors (Chapter 5), and enzyme activators (Chapter 6), and attempts are described to develop analytical techniques for such species.

Occasionally it was realized that this combination (immobilized enzymes and thermal detectors) could find applications outside of analysis, and the possibility is advanced that such techniques could be used as an initial screen of pharmacological activity for new drugs in the cholinergic nervous system (Chapter 5, Section 2), or in the study of enzyme mechanisms (Chapter 6, Section 1.1).

CHAPTER 2

Section 2.1.

Preparation of immobilized enzymes covalently bound to finely divided supports.

2.1.1. Preparation of aminoethyl cellulose (A.E.C.) - enzyme derivatives.

All aminoethyl cellulose - enzyme derivatives were prepared by essentially the same technique. All chemicals were obtained from Sigma Chemical Company, Poole, U.K. Aminoethyl cellulose (A.E.C., 0.5 g) was washed with buffer, (0.1M N-ethyl morpholine HCl (N.e.m. HCl) at pH 8.5 (2 x 20 cm^3) and then suspended in a further 20 cm^3 of the same buffer. To this was added 25% aqueous glutaraldehyde (1.0 cm^3), and the mixture stirred for 5 min, during which time the white colour of the A.E.C. turned yellow. The actual reaction is complicated, and still not completely understood (181); however, at least some Schiff base is believed to be formed:-

$$\text{Cellulose derivative}\text{—}NH_2 + OHC(CH_2)_3CHO \longrightarrow \text{Cellulose derivative}\text{—}N{=}CH(CH_2)_3\text{-}CHO$$

The resultant suspension was filtered off and washed thoroughly with N.e.m. buffer (at least 3 x 20 cm^3), to remove all traces of unreacted glutaraldehyde which could subsequently cause cross-linking of the enzyme and so lower the resultant activity. The aldehyde derivative was re-suspended in N.e.m. buffer (10 cm^3), the enzyme added in appropriate quantity (see individual enzymes, Chapter 3), and the mixture stirred in iced water for 2 h :-

$$[\text{-}N{=}CH(CH_2)_3CHO + NH_2\text{—enzyme}\rightarrow]\text{-}N{=}CH(CH_2)_3\text{-}CH{=}N\text{-enzyme}$$

Any unreacted aldehyde groups were blocked either by suspension in 0.1M trishydroxymethyl aminomethane (THAM) or ethanolamine buffers (pH 8.0) at 4°C overnight. Alternatively, it was found equally acceptable to block the aldehyde groups by reduction with a little $NaBH_4$ (in phosphate buffer, pH 6.5, at 0°C), until the colour of the A.E.C. - enzyme derivative had faded to pale yellow or white. This latter technique served also to reduce the Schiff base to an amine probably imparting increased resistance to hydrolysis of the derivative. The resultant immobilized enzyme was stored in buffer, at it pH optimum.

2.1.1. Preparation of controlled porosity glass (C.P.G.)- enzyme derivatives.

Controlled porosity glass (C.P.G.), 200-400 mesh, (96% SiO_2, 3% B_2O_3, 1% Na_2O), with mean pore diameters 1944Å (± 4.9%) and 85Å (± 19%), and surface areas 13.3 $m^2 g^{-1}$ and 216.9 $m^2 g^{-1}$ respectively, were purchased from B.D.H. (U.K.).
3-Aminopropyltriethoxysilane was purchased from Aldrich Chemical Co. Inc., Milwaukee, U.S.A.
All other chemicals were obtained from Sigma (U.K.).

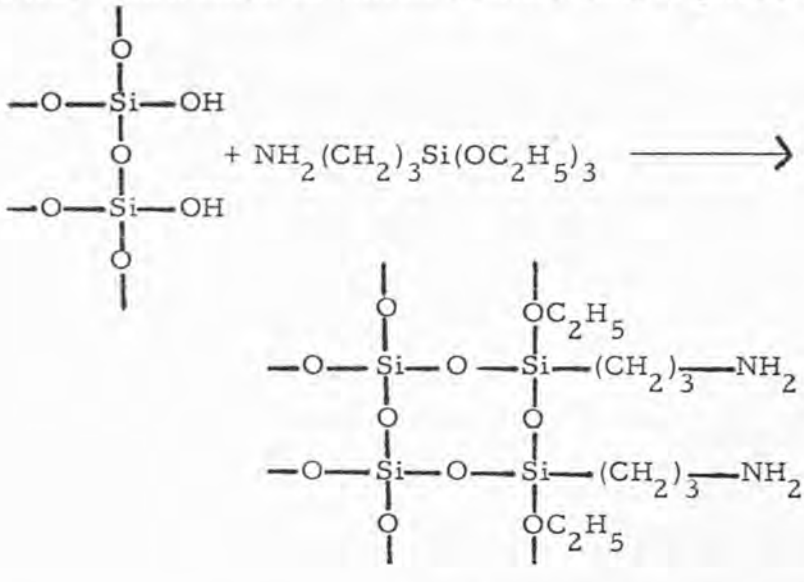
The enzyme could be easily coupled to the controlled porosity glass, via a four-stage synthesis:-

(a) Preparation of the carrier

C.P.G. (1 g), was refluxed in approximately 18% HNO_3 (conc., 70% HNO_3, diluted 1:3 with distilled water), at 80-90°C for 2 h, and then rinsed to neutrality with distilled water, the overall effect being to clean and hydrate the surface of the silica.

(b) Preparation of the alkylamine derivative

To the C.P.G., treated as in (a), was added 50 cm^3 of a 1% v/v solution of 3-aminopropyltriethoxy silane in dry acetone. This was allowed to evaporate to dryness (with occasional stirring), at room temperature, and then heated at 115°C in an oven overnight, to yield an alkylamine derivative of silica, which could be stored as such until required for use:-



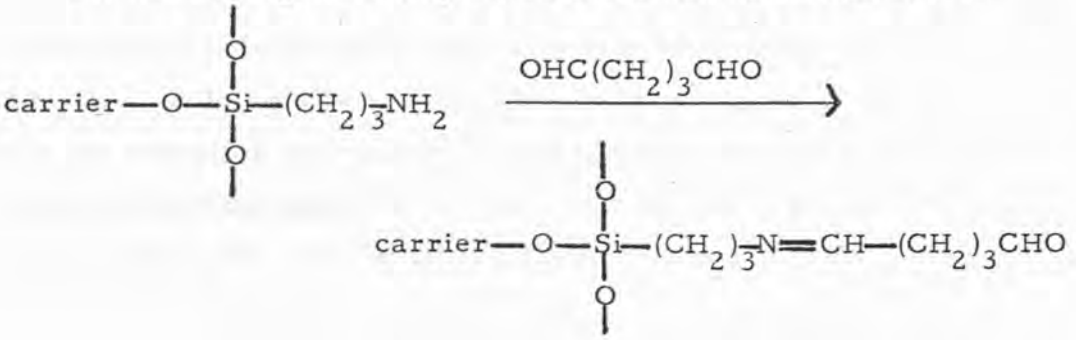
alkyl amine glass

(c) Preparation of the aldehyde derivative

This reaction was essentially the same as in the prepartion of the aldehyde derivative of aminoethyl cellulose (see 2.1.1.), except that, due to the solubility of silica in alkalis , neutral conditions, and a longer coupling time, were employed.

To the alkylamine glass as prepared in (b) was added 25 cm^3 of 2.5% aqueous glutaraldehyde, in 0.1M phosphate buffer, at pH 7.0. The entire reaction mixture was placed in a

vacuum desiccator and partially evacuated, to remove any gas entrapped in the pores. Coupling was allowed to proceed for 1 h. The product (orange for 1944 Å pore size, red for 85 Å pore size), was exhaustively washed with buffer to remove all traces of glutaradehyde, as described in Section 2.1.1.



(d) Coupling of the enzyme to the active (aldehyde) site

To the glutaraldehyde activated carrier, as prepared in (c), was added the appropriate enzyme, and just enough 0.1M phosphate buffer (pH 7.0), to cover the surface of the support and enzyme. The mixture was allowed to stand in a refrigerator at 4°C for 12-24 h, thoroughly rinsed with buffer to remove any uncoupled enzyme, and treated with THAM, ethanolamine or sodium borohydride to block unreacted aldehyde groups, as described in Section 2.1.1.

The actual choice of blocking agent did not appear critical, and all three appear to perform satisfactorily. The glass-immobilized enzyme was stored in 0.1M phosphate buffer. containing 10mM EDTA, at pH 7.0, until required for use.

Section 2.

Preparation of immobilized enzymes covalently bound to, or entrapped within, the vicinity of other solid supports.

2.2.1. Preparation of enzymes covalently bound to the inside of nylon tubing.

The method used was very similar to that described by Morris et al (32). All chemicals were obtained from Sigma (U.K.), with the exception of 1-chloro-2:3 epoxypropane (Fisons), boron trifluoride diethyl etherate, N-ethyl morpholine and hexamethylene diamine (B.D.H.) and the nylon tubing (Portex Ltd., Hythe, Kent).

Stage 1. Preparation of triethyoxonium tetrafluoroborate

The procedure used followed closely the method of Meerwein (182). 1-chloro-2:3 epoxypropane (6.25 g) was added dropwise to a refluxing solution of boron trifluoride diethyletherate (12.5 g) in 125 cm^3 of dry ether), and vigorously stirred with a magnetic stirrer. When all the former reagent had been added the mixture was refluxed for a further hour, and then allowed to cool to room temperature with continuous stirring. The precipitate of triethyloxonium tetrafluoroborate was washed thoroughly with dry ether (2 x 150 cm^3), the excess of ether being decanted off, and the compound dissolved in dichloromethane (50 cm^3) to give an approximate 20% (w/v) solution. The triethyloxonium tetrafluoroborate was always used with 24 h of its preparation:-

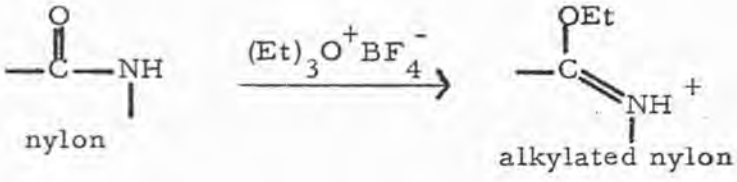
$$4(C_2H_5)_2OBF_3 + 2(C_2H_5)_2O + 3ClCH_2\underset{\diagdown O \diagup}{CH - CH_2} \longrightarrow$$

$$3\,C_2H_5O^+\,BF_4^- \;+ B\,(O\underset{\displaystyle CH_2Cl}{CH}CH_2OC_2H_5)_3$$

triethyl oxonium
tetrafluoroborate

Stage 2. Activation of the nylon tubing

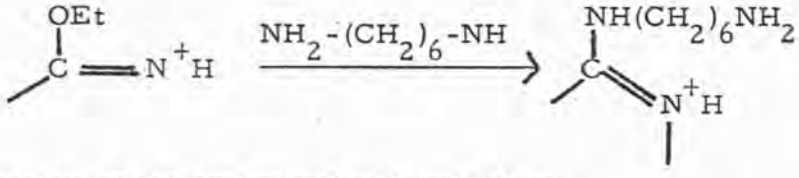
Nylon tubing (1mm internal diameter) was activated by drawing the approx. 20% solution of triethyloxonium tetrafluoroborate in dichloromethane into the tube by means of a syringe, with appropriate attachments to accommodate the nylon tubing, sealing the ends of the tube, and incubating for 60 min at room temperature:-



Stage 3. Conversion to amine-substituted nylon

Alkylation in Stage 2 was terminated by flushing the tube through with dichloromethane for 1-2 min.

The tube was then perfused with 0.5M hexamethylene diamine, pH 9.5, until at least 10 cm^3 had been drawn through the tube over a period of about 2 min. Again the ends of the tube were sealed, and incubated for 30 min. Finally, the tube was perfused with distilled water from a persitaltic pump, for 3 h (at 120 $cm^3 h^{-1}$), yielding amine-substituted nylon.



Stage 4. Activation of the amine substituted nylon

5% glutaraldehyde (v/v) in 0.1M boric acid at pH 8.5 (NaOH), was pumped through the tube for 15 min (2 cm^3 min^{-1}), washed for 15 min with 0.1M N-ethylmorpholine HCl (pH 8.5) (2 $cm^3 min^{-1}$), filled with the appropriate enzyme solution in

the N. e. m. HCl buffer, and incubated for 2 h at 4°C after sealing the ends of the tube.

The tube was washed free of any uncoupled enzyme by perfusion with 0. 1M THAM (pH 8. 0), containing 0. 25 M NaCl for 3 h (which also served to block any unreacted aldehyde groups). When not in use, tubes were stored containing buffer at the pH optimum of the immobilized enzyme, [0. 1M THAM + 10mM EDTA (pH 7) or 0. 1M phosphate + 10mM EDTA (pH 7)]

Section 2.2.2.

Preparation of enzymes entrapped near the surface of a glass (pH) electrode - an "enzyme electrode".

All organic chemicals used were purchased from Sigma (U.K.). Two techniques were used: a gelatin-entrapping method, based on that described by Durand et al (97), and a cross-linking method, based on that described by Tran-Minh et al (93).

(a) Gelatin-entrapping method

Gelatin (600 mg) (Sigma 225 bloom) was solubilized in distilled water (10 cm^3), at 60°C for 30 min, and then allowed to cool to 25°C.

To a 0.5 cm^3 aliquot of this gelatin solution at 25°C, a known mass of enzyme was added (see Chapter 4, Section 1.1. for each particular enzyme), and stirred until dissolved. The resulting solution was painted on to the sensitive part of the electrode, with a small paint brush, and allowed to dry at room temperature for 4 h, followed by immersion in 2.5% glutaraldehyde solution (in 0.1M phosphate buffer, pH 7.0) for 15 min.

The electrode was rinsed in the same buffer, and introduced into a 10 mg cm^{-3} glycine solution (in 0.1M phosphate + 10 mM EDTA, at pH 7.0) and left overnight. When not in use the enzyme electrodes were stored in this buffer.

(b) Cross-linking method

The appropriate enzymes were mixed with a 3-4 fold (w/v) excess of an inert protein, human serum albumin (HSA), and dissolved in such a volume of 0.1M phosphate buffer as to give an approximately 3-5% (w/v) of protein in solution.

To this solution was added 0.1 cm^3 of 25% glutaraldehyde solution, and the mixture stirred rapidly. The electrode was repeatedly "painted" with the mixture, using a small paint brush, until it suddenly thickened and solidified (approximately 30 sec. to 1 min.) The electrode coating was allowed to dry for 15 min, then dipped into a 10 mg cm^{-3} glycine solution (in 0.1M phosphate + 10 mM EDTA buffer, pH 7.0), where it was allowed to remain until required, and in which the electrode was stored when not in use.

CHAPTER 3

Determination of enzyme substrates using the combination of immobilized enzymes with thermal sensing devices.

Section 3.1 Heat-leak flow microcalorimeter

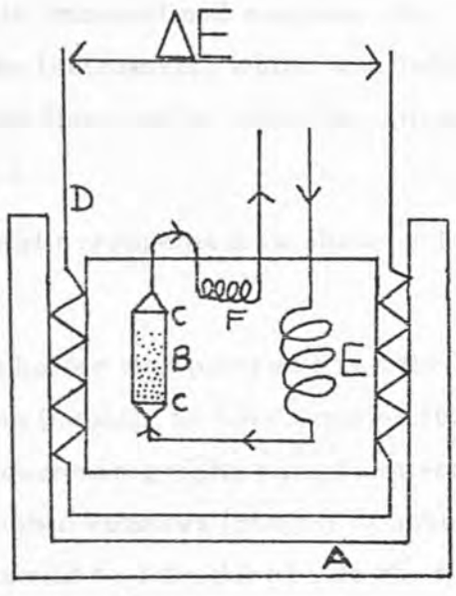
3.1.1. The LKB 10700-1 microcalorimeter

For many years the use of calorimetry in analysis has been commonplace (see Chapter 1, Section 3.1). It is, however, only recently that the availability of sensitive commercial microcalorimeters has permitted exploration of biochemical and microbiological applications of microcalorimetry in analysis. The flow microcalorimeter that has been most widely used in these applications is the LKB 10700-1 microcalorimeter. This calorimeter, of the heat-leak type, contains two operational calorimetric cells, a batch cell and a flow-through cell. In the course of this study, only the latter cell was used, and hence only this will be described. In the flow-through cell, the main features of which are shown in Fig. 3.1.1.1 , the species of interest is pumped through a small microcolumn situated within the cell (in this study, containing the immobilized enzyme), and the instrument measures the rate of heat flow from the microcolumn, (caused by the reaction process), via the thermopiles, to an infinite heat sink generating a voltage, ΔE, over the thermopiles which is directly proportional to the heat flow. This voltage is amplified by a Keithly 150B Null-voltmeter, to a level suitable for display on a 100 mV chart recorder.

The temperature in the region of the cells was maintained constant within $\pm$ 0.001 ^{o}C by an LKB air thermostat, in which

the cells were housed, (the reaction cells and thermostat being collectively referred to as the LKB assembly). Under these conditions, the sensitivies of heat measurements were $< 1 \mu W$ (continuous heat effect), or $>$ 1mJ (heat pulse).

A=infinate heat sink
B=microcolumn(cont. immobilized enzyme)
C= teflon filter papers



D= thermopiles
E= thermal equilibration coil
F= secondary heat exchanger

Fig. 3.1.1.1.

The inner dimension of the microcolumn were approximately 6mm x 17mm, corresponding to a total volume of approximately 0.5 cm^3. Immobilized enzyme could be easily confined within this microcolumn by means of teflon filter papers situated over the inlet and outlet. A short heat exchanger following the microcolumn ensured complete heat transfer from the liquid. The flow was generated by a peristaltic pump, with flow rate of between 0.072 and 0.43 $cm^3 min^{-1}$ depending on the system being studied.

After filling with immobilized enzyme, the microcolumn was inserted into the instrument, which was left to equilibrate with a constant buffer flow until a stable baseline was recorded (at least 2-3 h.).

The experimental arrangement is shown schematically in Fig. 3.1.1.2.

As can be seen buffer was pumped from the reservoir by a peristaltic pump (usually an LKB, model 10200), through an "Altex" liquid chromatography sample injection valve, where highly reproducible volumes (500 µl) of substrate solution (in the buffer) could be introduced into the flowing buffer stream, and then to the LKB thermostat where buffer and sample were thermally equilibrated before reaching the microcolumn containing the immobilized enzyme. Here the substrate was rapidly converted to product and the resultant enthalpy change registered as a "heat pulse" on the chart recorder.

Alternatively, the substrate solution could be continuously pumped into the microcalorimeter by including the substrate

FIG. 3.1.2.

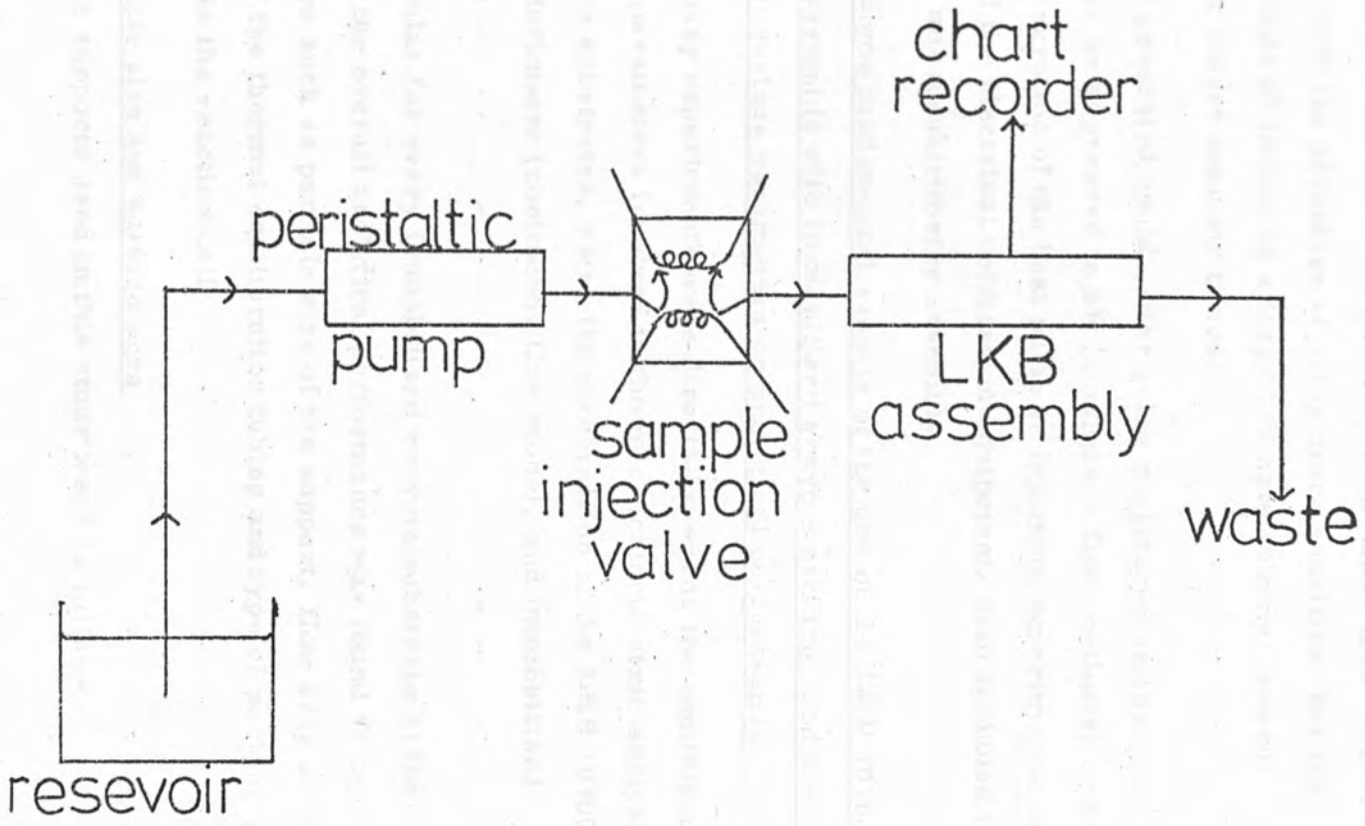


chart
recorder
peristaltic
pump
sample
injection
valve
LKB
assembly
waste
resevoir

solution in the reservoir, until a quasi steady-state was registered on the chart recorder. In general, continuous flow methods were found to have the advantage of being more sensitive, but the disadvantage of requiring a larger sample volume, and of requiring longer analysis times.

The heat generated could, after being registered on the chart recorder, be expressed in μW (continuous flow methods) or mJ (after integration of the heat pulse in injection experiments) by means of the electrical calibration equipment, also included in the LKB microcalorimeter assembly.

3.1.2. Some fundamental aspects of the use of the LKB 10700-1 assembly with immobilized enzyme packing, and the effect of various parameters on analytical performance.

Preliminary experiments were directed towards the optimization of many parameters involved in the microcalorimetric analysis of enzyme substrates, using the combination of the LKB 10700-1 microcalorimeter (continuous flow mode), and immobilized enzymes.

In particular for every immobilized enzyme/substrate system studied, the overall analytical performance was found to depend on factors such as particle size of the support, flow rate used, length of the thermal equilibration tubing and type of packing of support in the reaction cell.

(a) Particle size and surface area

The three supports used in this study were as follows:-

	"mesh" size	surface area	conc. of functional groups
aminoethyl cellulose	medium ($\gg$ 75 μm dia.)	unknown ($\ll 13.3 m^2 g^{-1}$	300 μ-equivs per gram (given by Sigma)
controlled porosity glass	200-400 (37-75 μm dia.)	$13.3 m^2 g^{-1}$	approx. 50μ equivs.* per gram
controlled porosity glass	200-400 (37-75 μm dia.)	$216.9 m^2 g^{-1}$	approx. 300-500 * μ equivs per gram

*these surface concentrations of functional groups were estimated by the method given in appendix 2.

The effect of flow rate on the peak area obtained on passage of a 0.5 cm^3 pulse of 0.5×10^{-3} M glucose over the enzymes glucose oxidase and catalase co-immobilized on each of the three supports listed above is given in Fig. 3.1.2.1.

The glass supported enzymes in both cases showed peak height (and hence sensitivity), to increase with increasing flow rate, whilst peak areas remained essentially independent of the flow rate. This would be the result expected if all, or even a constant fraction of, the substrate was converted to product. However, with aminoethyl cellulose supported enzymes, the reverse was found (i.e. peak height appeared to be largely independent of flow rate, whilst peak area increased with increasing flow rate.

The independence of peak height on flow rate could be explained by postulating that insufficient enzyme was present to convert all the substrate to product (i.e. that the reaction was zero order with respect to substrate), as could the dependence of peak

Fig. 3.1.2.1. Effect of flow rate on peak height and peak area for the passage of a 0.5 cm^3 pulse of 5×10^{-4} M glucose over the enzymes glucose oxidase and catalase immobilized to the following supports:-

support	flow rate ($cm^3 min^{-1}$)	peak ht (units)	peak area (units)	duration of peak/min.
aminoethyl cellulose	0.15	37.5	2775	24
	0.15	37.5	2775	24
	0.11	41	3731	26
	0.08	43	4859	32
	0.04	40	6480	48
controlled porosity glass ($13.3 m^2 g^{-1}$)	0.15	140.5	6190	18
	0.15	140.5	6210	18
	0.11	104	6290	21
	0.08	76	6250	25
	0.04	39	6320	31
controlled porosity glass ($216.9 m^2 g^{-1}$)	0.15	143	6280	18
	0.11	107	6320	20
	0.08	78	6320	25
	0.04	40	6370	30

area on flow rate (i.e. on the residence time of the substrate in the reaction cell). However, increasing the substrate concentration whilst keeping the flow rate constant would not, if this were so, be expected to affect either the peak height or the peak area. That this was not so was easily shown, indeed, doubling the concentration of substrate to 1×10^{-3}M resulted in almost a doubling of both peak height and peak area. Furthermore the binding capacity of aminoethyl cellulose was greater than that of the 13.3 $m^2 g^{-1}$ surface area controlled porosity glass, where the effect was not observed (see Fig. 3.1.2.1.). The probable explanation is that an immobilized enzyme reaction, unlike the case where both species are in solution, can be envisaged of occurring in three stages:-

$$S + E \xrightarrow{k_1} ES \xrightarrow{k_2} EP \xrightarrow{k_3} E + P$$

k_1: diffusion of substrate to enzyme

k_2: conversion to product by enzyme

k_3: diffusion of product away from enzyme

S = substrate

E = immobilized enzyme

P = product

It appear likely that for the relatively large particles of amino-ethyl cellulose, the mass transfer rate of substrate to immobilized enzyme (k_1) is the rate limiting step. This would plausibly explain all the observed facts (i.e. the independence of peak height on flow rate, and the dependence of peak area on both flow rate and substrate concentration). As a consequence it was realised that the particle size of the support was often more important than the concentration of functional groups on its surface. Many finely divided supports, some with mean particle diameters even less than 37Å, are in use in various forms of chromatography, and hence are easily

obtainable. However, the combination of the ease of activation of controlled porosity glass, with relatively inexpensive and easy to handle reagents, and its non-biogradability (in contrast to most organic polymers), appeared to make this the support of choice, provided that the enzyme immobilized was compatible with pH values of less than 10.

(b) Effect of sample dispersion in the thermal equilibration tubing and flow rate.

It is important to consider the properties of two idealized limiting reactors, namely, the perfect plug flow reactor and the ideal, continuously-stirred reactor. A perfect plug reactor is one which is operated such that there is no axial mixing of the reactant (in this case, substrate), plug with the fluid in front or behind. The sample flows through the column or packed bed with an infinitely sharp front and tail. It is easy to show that the extent of conversion in such a reactor is identical with that which occurs in a pure batch reactor in a period of time equal to the residence time in the flow reactor. Since there is no mixing of fluid, there is no dilution to consider, in marked contrast to the other extreme limit of behaviour, the perfectly well-mixed reactor. In this type of reactor it is assumed that any sample or fluid entering the reactor is instantaneously mixed with all other fluid in the reactor. Physically, this is equivalent to an exponential dilution.

These two limiting models are valuable in the following regards. First, with any real reactor, no heat flux greater than that which would occur in a plug flow reactor is possible, nor can the duration of the heat pulse (on which the overall analysis time may depend) be reduced to less than that obtained in a perfect

plug flow reactor. Further, no matter how poorly designed the actual reactor, the response time cannot be longer than that of a well mixed reactor. Clearly any real reactor lies between the above limits, and thus maximum sensitivity occurs with the perfect plug flow reactor, whilst maximum analysis time will occur with a perfectly well-mixed reactor. Additionally, the shortest analysis time should also occur with a perfect plug flow reactor, although additional factors can also act to broaden the peak, which may be more significant than the actual heat pulse itself, such as sluggish heat transfer to the detector, slow response of the detector or slow electronic response of the measuring and recording system.

In connexion with this, it becomes obvious that the longer a sample pulse of substrate remains in the thermal equilibration tubing between the sample injection valve, and the microcalorimeter itself, the more axial dispersion would occur by diffusion mechanisms, and hence the less like a perfect plug the sample would appear as it entered the reaction cell itself.

To minimize this sample dispersion, (i.e. residence time in the thermal equilibration tubing), it was found advantageous to use as short a piece of thermal equilibration tubing as possible, and as high a flow rate as was consistent with reasonable baseline noise (see also next Section).

The net effect of reducing the thermal equilibration tubing, and increasing the flow rate, is shown in Table 3.1.2.2.

TABLE 3.1.2.2.

Influence of length of thermal equilibration tubing, and flow rate, on the sensitivity (α peak height), and analysis time, for a 0.5 cm^3 pulse of 5×10^{-4} glucose using glass-immobilized glucose oxidase and catalase.

flow rate ($cm^3 min^{-1}$)	length of equilibration tubing (M)	peak height	analysis time(min)
0.15	3	120.5	21
0.15	1	140.5	18
0.32	1	165.0	15

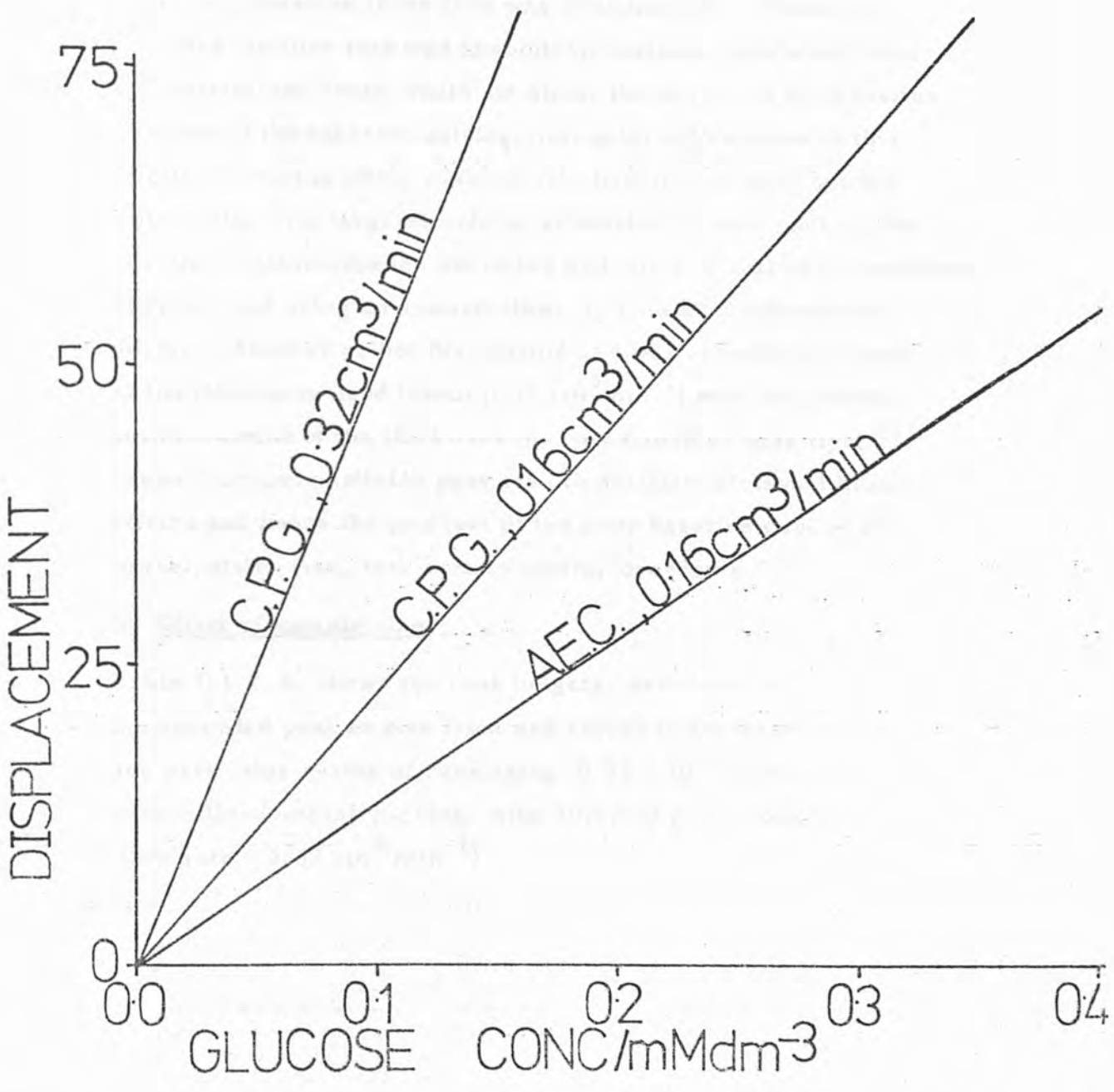
In each of the above experiments, the peak area, measured by a planimeter, remained approximately constant ($\pm$ 5%). Although it may be thought that it would be advantageous to increase the flow rate, and decrease the length of equilibration tubing still further, this was not always the case. Increasing the flow rate produced a noisy baseline (see next Section), whilst decreasing the equilibration tube-length further often gave rise to considerable baseline drift and other noise, due to incomplete temperature equilibration of buffer and sample. Hence the actual optimum flow rate and length of equilibration tubing had to be judged in terms of a compromise between, on the one hand, slightly greater sensitivity and slightly shorter analysis time, and on the other, quality of baseline.

It did not in any case appear possible to reduce analysis times significantly below 15 min, due to the high time-constant of the microcalorimeter itself, and therefore most analyses were carried out using a flow rate of 0.32 $cm^3 min^{-1}$, with a one metre length of equilibration tubing.

In continuous injection analysis, it was obviously even more advantageous to use as high flow rates as possible, as the steady-state displacement was directly proportional to the flow rate, because more moles of substrate per sec. reached the reaction cell. The length of equilibration tubing used did not, of course, affect the sensitivity of the continuous-injection type of analysis.

Fig. 3.1.2.3. illustrates the effect of flow rate and support, on the steady-state displacement when a continuous pulse of glucose was passed over the immobilized enzyme system glucose oxidase/ catalase.

FIG. 3.1.2.3. Plot of steady-state displacement vs glucose conc using different supports and flow rates



(c) Type of packing of immobilized enzyme in reaction cell.

Although various typesof perstaltic pump were used to deliver buffer and sample to the microcalorimeter, everyone was found to cause a slight pulsing in the flow stream that could not be completely removed by the use of pulse-suppressors. As the solutions were forced through the tightly packed bed of immobilized enzyme in the reaction cell of the microcalorimeter, a frictional heating effect was produced, shown by a fall in the heat flux generated if the flow was switched off. However, provided the flow rate was absolutely constant, this would also be constant and hence would not affect the quality of the baseline. Because of the inherent pulsing, irregular differences in this frictional heating effect resulted in a baseline of poor quality. This could, to a large extent, be alleviated by only packing the reaction column between one-third and one-half full of immobilized enzyme, and using an upward-flow, as if in a "fluidized-bed" design. Whether or not the enzyme bed was actually fluidized at the flow rates used (about 0.32 $cm^3 min^{-1}$) was not proven, but the motion of the fluid over the (presumably) less tightly packed support particles gave rise to smaller frictional heating effects and hence the problem of the poor baseline quality due to the pulsing was, to a certain extent, overcome.

(d) Effect of sample size

Table 3.1.2.4. shows the peak heights, and times taken, for the recorded peak to rise from and return to the baseline, in one particular series of runs using 0.95×10^{-3} urea, and immobilized urease packing, with different pulse volumes. (flow rate = 0.32 $cm^3 min^{-1}$).

TABLE 3.1.2.4.

vol. of pulse/μ l	peak height(units)	analysis time/min
100	6.0	11
200	14.0	13
500	31.0	15
flat-topped peak (3500)	108.0	25

Based on the above table, it appeared that the maximum sensitivity consistent with reasonable analysis time was to use a 500 μ l pulse. Under the conditions of the above runs, it can be seen that sample volume, and peak height are approximately directly proportional in the range 100 μ l $\rightarrow$ 500 μ l.

Consequently, all pulse experiments were conducted using a 500 μ l pulse of sample. Of course, where maximum sensitivity was required, this could be achieved (at the expense of analysis time) by using a "continuous pulse" as described earlier.

(e) Correlation between peak height and peak area

Under both steady-state and pulse experiments, the heat flux generated is simply equal to the number of moles of substrate converted to product per unit time multiplied by the enthalpy change per mole of reaction.

In the steady-state (continuous injection) mode, total enthalpy change is proportional to the displacement of the baseline.

In the case of the pulse experiments, however, the total enthalpy change is proportional to the area of the peak, necessitating in the absence of an integrator on the chart recorder, the use of a

a planimeter. However, provided that flow rate and sample volume were maintained constant, it was empirically observed that the maximum peak height appeared to be directly proportional to the concentration of substrate.

In order to test this hypothesis, the correlation coefficient (r^2) between maximum peak height and peak area (as measured with a planimeter) was calculated for 13 different peaks obtained during the hydrolysis 500 μ l of penicillin G by glass-immobilized penicillinase, (in the concentration range 0.000125 $\longrightarrow$ 0.006M) with a constant flow rate of 0.32 $cm^3 min^{-1}$, using an HP65 programmable calculator, and found to be 0.984. Hence it was decided to take the peak height as being proportional to peak area. The actual data used are given in Table 3.1.2.5.

A further study to confirm the hypothesis, used the mean peak area, and mean peak heights, of three peaks obtained by the hydrolysis of 0.5 cm^3 of urea by glass immobilized.

In this study a correlation coefficient (r^2) of 0.999 was obtained, using the results listed in Table 3.1.2.6.

TABLE 3.1.2.5.

Correlation between peak height and peak area for the hydrolysis of 0.5 cm^3 of penicillin G by glass-immobilized pencillinase (flow rate = 0.32 cm^3 min^{-1})

conc. of penicillin G. (M)	peak area (planimeter units)	peak ht. (mm)
0.000125	1.0 (± 0.1)	10.5 (± 0.25)
0.000125	1.1 (± 0.1)	10.5 (± 0.25)
0.000250	2.0 (± 0.1)	20.0 (± 0.25)
0.000250	2.0 (± 0.1)	19.0 (± 0.25)
0.000250	2.2 (± 0.1)	20.0 (± 0.25)
0.000500	3.9 (± 0.1)	39.0 (± 0.75)
0.001000	7.8 (± 0.1)	78.0 (± 0.75)
0.001000	7.2 (± 0.1)	75.0 (± 0.75)
0.002000	14.0 (± 0.2)	140 (± 2.5)
0.002000	14. 0 (± 0.2)	145 (± 2.5)
0.004000	26.0 (± 0.2)	280 (± 2.5)
0.004000	28.0 (± 0.2)	280 (± 2.5)
0.006000	42.0 (± 0.3)	415 (± 5.0)

$r^2 = 0.984$

TABLE 3.1.2.6.

Correlation between mean peak height and mean peak area for the hydrolysis of 3 identical 0.5 cm^3 samples of urea, at 6 different concentrations, by glass-immobilized urease.

conc. of urea (M)	mean peak area	mean peak ht.
15.6×10^{-3}	25.0	58.0
7.8×10^{-3}	13.0	29.0
3.9×10^{-3}	6.0	14.5
1.95×10^{-3}	3.0	7.5
0.98×10^{-3}	1.6	3.8
0.49×10^{-3}	0.8	1.8

$r^2 = 0.999$

Section 3.2. Determination of enzyme substrates using the LKB 10700-1 microcalorimeter.

Previous work on the application of immobilized enzymes in thermal analysis has already been mentioned in Chapter 1. Section 3.3. However, most workers appear to have used devices similar to that of Mosbach and co-workers' "enzyme thermistor" (170), where a thermistor is used as the heat-sensing device.

The purpose of this work was to utilize the more elaborate LKB 10700-1 assembly to compare the analytical performance of the two methods.

3.2.1. Determination of penicillin G, and comparison of the enthalpy of hydrolysis of several semi-synthetic penicillins, using the combination of immobilized pencillinase and the LKB 10700-1 microcalorimeter.

The most commonly quoted methods of penicillin are those based on the procedure described by Alicino (183), in which unconsumed iodine is back titrated after incubation with the hydrolized penicillin. Hydrolysis can be done either with alkali, or the enzyme pencillinase. This technique, although selective for the intact penicillin molecule, has limitations in that the stoichiometry may vary with experimental conditions (184), including time, pH and iodine reagent concentration (185). Furthermore, the presence of unsaturated side chains in the penicillin molecule interfers with iodine uptake, which makes careful blank titrations necessary. Enzymatic analysis provides a more selective assay. Completely enzymatic procedures, in which a physical property related to the progress of the pencillinase-catalyzed hydrolysis of penicillin

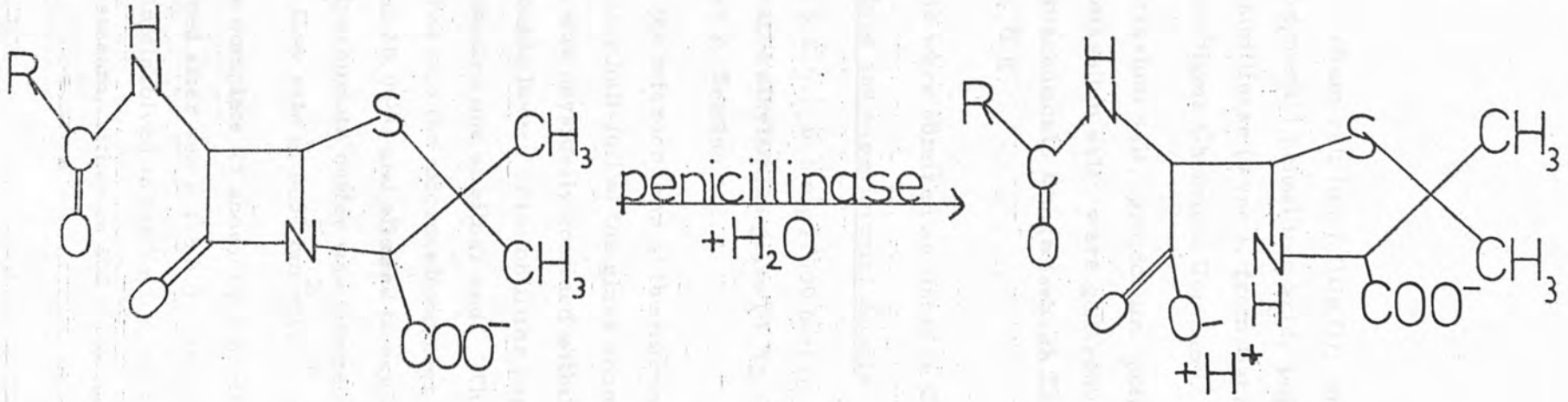
is monitored, have been reported, such as calorimetric methods (186) and potentiometric methods (71, 101, 104).

The calorimetric/enthalpimetric approach to pharmaceutical analysis has the distinct advantage that a high level of non-reacting excipients can be present without significantly affecting the precision or accuracy of the results (187), and the combination of immobilized enzyme hydrolysis with enthalpimetric instrumentation would seem to provide the basis for a realistic alternative method of assay.

Mosbach and co-workers (157) have reported such an analysis technique using their "enzyme thermistor" device, and Grime and Tan (148) have utilized thermal analysis techniques with soluble enzymes. However, despite the obvious potential little other work appears to have been reported in the literature on similar techniques utilizing the enzyme penicillinase, in combination with thermal sensors.

The term penicillinase denotes an enzyme which catalytically hydrolyses the amide bond in the β-lactam ring of 6-amino-penicillanic acid and its N-acyl derivatives. Such derivatives are commonly referred to as penicillins, and the overall enthalpy change occurring as a result of this hydrolysis, and subsequent protonation of the buffer, was used as the basis for the analytical technique.

FIG. 3.12.1. ENZYMATIC HYDROLYSIS OF PENICILLINS



R. penicillin

R. penicilloic acid

Reagents

benzyl penicillin, sodium salt (penicillin G); ampicillin trihydrate; phenoxymethyl pencillinic acid, potassium salt (penicillin V); penicillinase (Type I, from Bacillus cereus) were obtained from Sigma Chemical Company, UK.

phenethicillin, potassium salt; propicillin, potassium salt; and azidocillin, potassium salt; were generously donated by Beecham Pharmaceuticals Ltd, (Research Division), Worthing, Sussex, U K.

All other materials were obtained as listed in Chapter 2.

Immobilization procedure and experimental details

Penicillinase (E.C. 3.5.2.6.), 0.5 mg (1000 units), was coupled to glutaraldehyde activated alkylamine glass (0.5g, 216.9 $m^2 g^{-1}$) as described in Chapter 2, Section 1.2.

After immobilization, the microcolumn of the microcalorimeter was packed approximately half-full of the glass bound enzyme (see 3.1.2.(c)), which was physically confined within the microcolumn by two double layers of teflon filter papers, (obtained from LKB) situated one at either end. The microcolumn was then inserted into the microcalorimeter which was maintained at a nominal 25.00°C and allowed to equilibrate overnight, whilst the appropriate buffer was pumped through the microcolumn, at a flow rate of 0.32 $cm^2 min^{-1}$.

When equilibration was complete as shown by a horizontal baseline (usually obtained after some 4-5 h.), the substrate, sodium benzyl penicillin, dissolved in the buffer, was introduced into the flowing buffer stream, either in 500 μ l pulses by means

of the Altex Injection valve, or as a pulse large enough (experimentally about 7 cm^3) to give a flat-topped peak (or "steady-state" displacement).

On applying the former technique, a calorimetric response of the type depicted in Fig. 3.2.1.2. was obtained, the peak area or, as shown in Sec. 3.1.2. (e), the peak height, being directly proportional to the concentration of benzyl penicillin in the pulse. The sampling frequency was about 4 per hour. On applying the alternative technique of substrate administration, a flat-topped peak (or "steady-state" displacement) was obtained, as depicted in Fig. 3.2.1.3., where the steady-state displacement of the baseline was directly proportional to the concentration of benzyl penicillin.

Tables and plots of peak height vs. concentration of benzyl penicillin, and of steady-state displacement vs. concentration of benzyl penicillin, using 0.1M THAM + 0.01 M EDTA (adjusted to pH 7.2 with conc. HCl) as buffer, are given in Tables 3.2.1.4, 3.2.1.5 and Figures 3.2.1.6 and 3.2.1.7., as are the correlation coefficients (r^2).

(THAM = Tris(Hydroxymethyl)Amino Methane)
Provided neither buffer nor flow rate was altered, the reproducibility of results on a day-to-day basis was $\pm$ 2%, or better, with no significant trend to give results consistently higher, or lower, than would have been expected from the original calibration plot, provided the solutions of benzyl penicillin were always freshly made-up, to avoid any non-enzymatic hydrolysis.

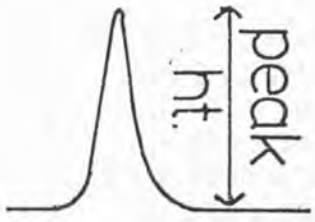


Fig. 3.2.1.2.

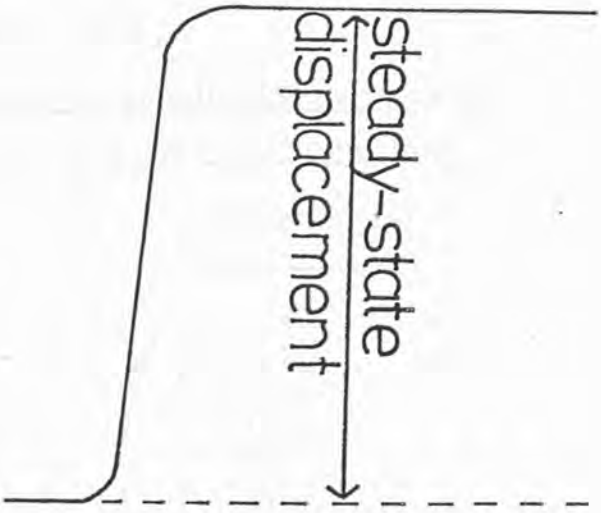


Fig. 3.2.1.3.

The activity of the immobilized enzyme, as shown by the peak height obtained by the hydrolysis of 500 μ l of a 10^{-3}M benzyl penicillin solution, remained constant over the period of study (two weeks). However, this was almost undoubtedly due to the fact that an excess of enzyme over that required to hydrolyze the 10^{-3}M pulse had been immobilized rather than to quantitative stability of the enzyme. Nonetheless, this "apparent" 100% stability was analytically extremely useful, in that rapid, quantitative end-points (hence, maximum sensitivity), were easily achieved, and that sources of error often apparent in kinetic measurements (e.g. slight variations in temperature, pH and the presence of slight amounts of inhibitor or activators) were eliminated.

As can be seen from Tables 3.2.1.4 and 3.2.1.5, the analytically useful ranges appear to be from approximately 6×10^{-5} to 3×10^{-2}M(pulse) and from 2×10^{-5} to 6×10^{-3}M (continuous flow).

TABLE 3.2.1.4.

Table of peak ht vs conc. of benzyl penicillin (500 μ l pulse) in 0.1M THAM buffer, pH = 7.2 (flow rate = 0.32 $cm^3 min^{-1}$

conc. of benzyl penicillin (mol dm^{-3})	peak ht (units)
0.0625×10^{-3}	5.4
0.0625×10^{-3}	5.1
0.125×10^{-3}	10.5
0.125×10^{-3}	10.5
0.0250×10^{-3}	20
0.250×10^{-3}	19
0.250×10^{-3}	20
0.500×10^{-3}	37.5
0.500×10^{-3}	39
1.000×10^{-3}	78
1.000×10^{-3}	75
2.000×10^{-3}	140
2.000×10^{-3}	145
4.000×10^{-3}	280
4.000×10^{-3}	280
6.000×10^{-3}	415
33.333×10^{-3}	2310
33.333×10^{-3}	2310
66.667×10^{-3}	4550
66.667×10^{-3}	4550
100.000×10^{-3}	6500
100.000×10^{-3}	6500
133.333×10^{-3}	8400
133.333×10^{-3}	8400

in concentration range $0 \longrightarrow 33 \times 10^{-3}$M .

$r^2 = 0.998$

0.0625 mM $\lesssim$ R $\lesssim$ 33mM where R = analytically useful range

TABLE 3.2.1.5.

Table of steady-state displacement vs. conc. of benzyl penicillin (continuous pulse) in 0.1M THAM pH = 7.2 (flow rate = 0.32 cm^3 min^{-1})

conc. of benzyl penicillin (mol dm^{-3})	displacement (units)
0.020×10^{-3}	6.2
0.020×10^{-3}	6.5
0.050×10^{-3}	16.0
0.050×10^{-3}	16.5
0.100×10^{-3}	27.0
0.100×10^{-3}	32.5
0.125×10^{-3}	33.0
0.200×10^{-3}	39.0
0.200×10^{-3}	66.0
0.500×10^{-3}	162
0.500×10^{-3}	165
1.000×10^{-3}	325
1.000×10^{-3}	325
6.667×10^{-3}	2145
6.667×10^{-3}	2145
42.000×10^{-3}	13050

for concentration range $0 \longrightarrow 6.7 \times 10^{-3}$M

$$r^2 = 0.999$$

$$0.02\text{mM} \leqslant R \leqslant 6.7\text{mM}$$

where R = analytically useful range

FIG. 3.12.6. Plot of peak ht. vs. penicillin conc (0·5 cm^3 pulse), as in table 3.12.4.

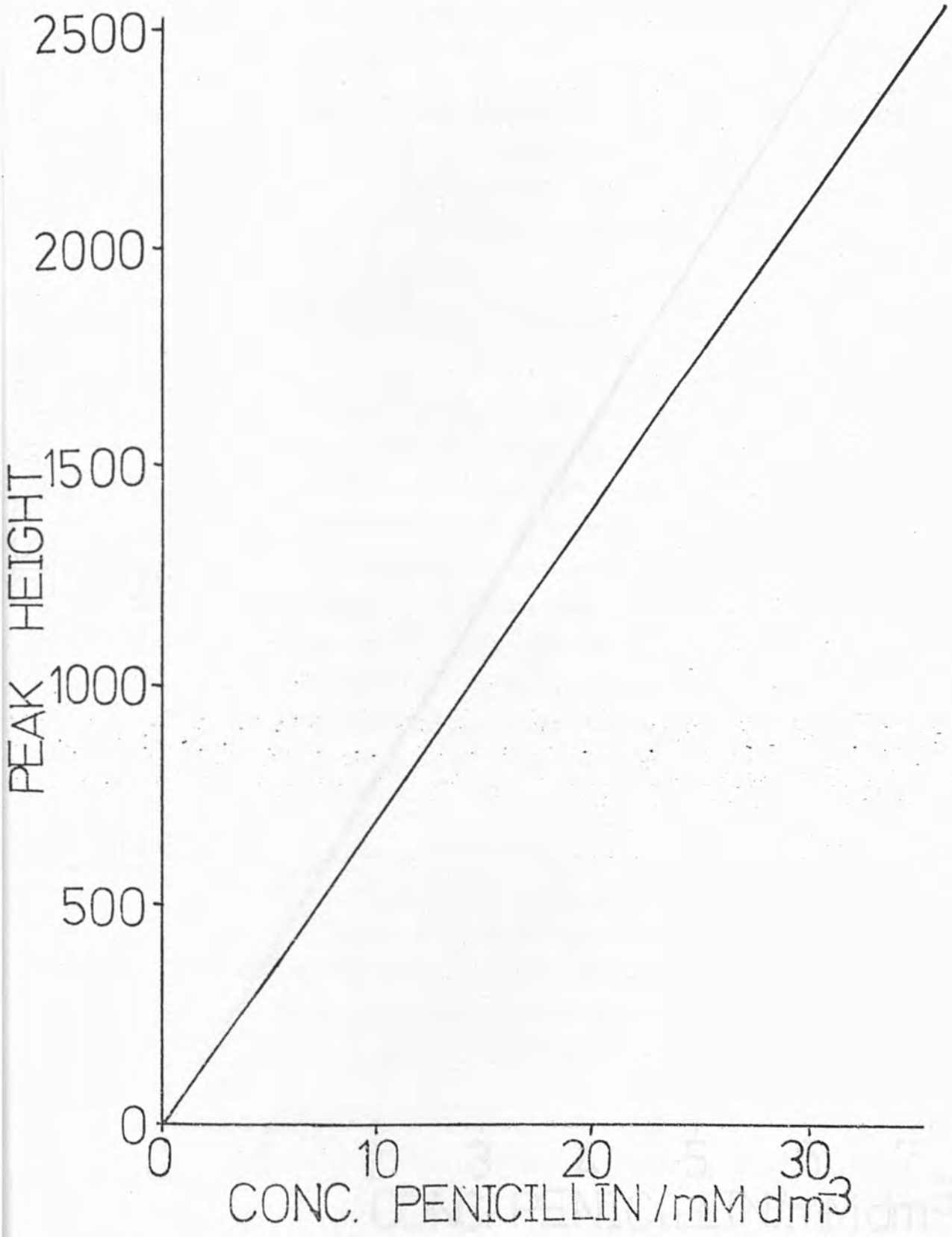
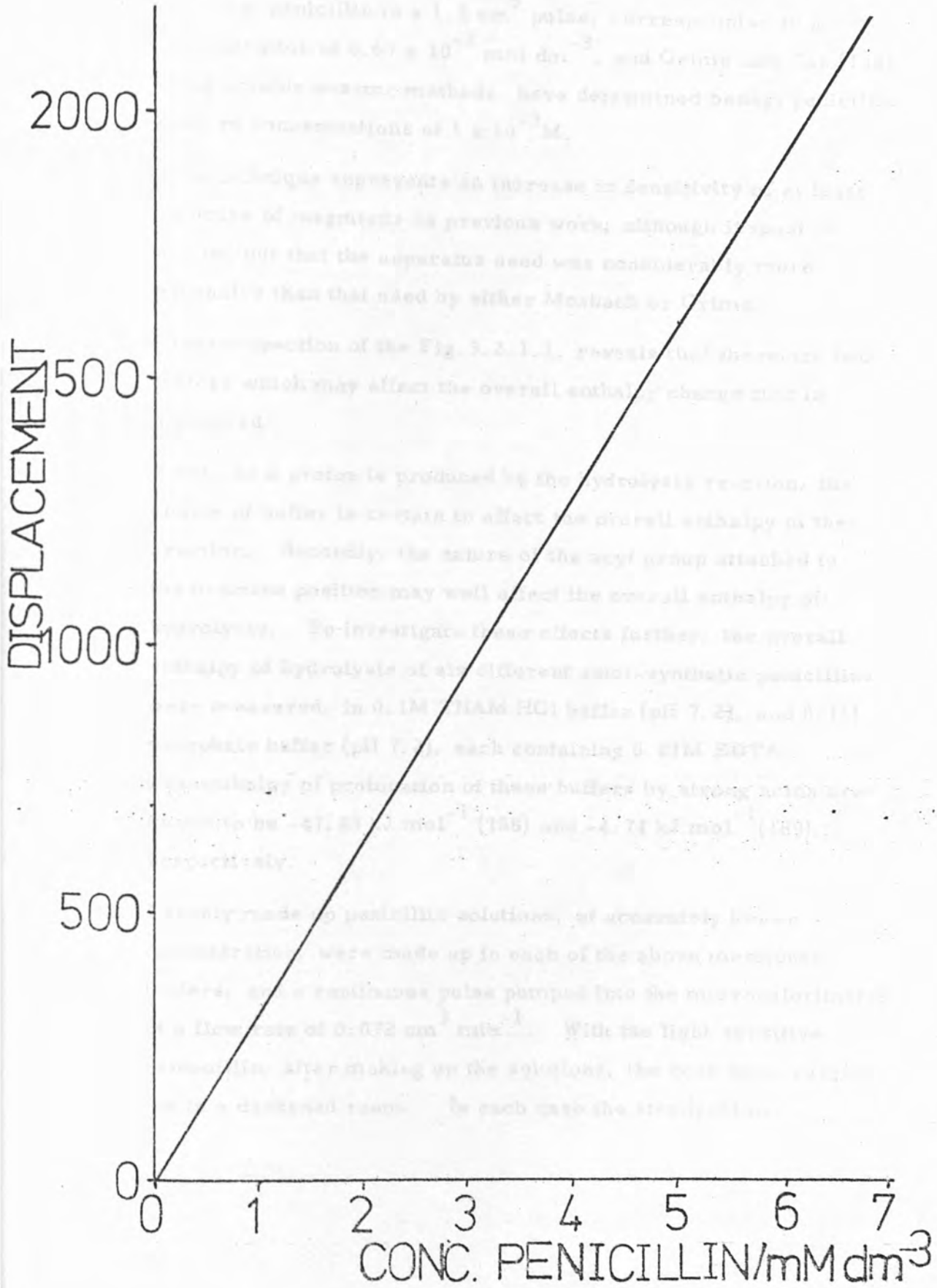


FIG.3.12.7 Plot of displacement vs. penicillin conc.,(as in table 3.12.5.)



Mosbach and co-workers (157) have detected down to 10^{-6} mol of benzyl penicillin in a 1.5 cm^3 pulse, corresponding to a concentration of 0.67×10^{-3} mol dm^{-3}, and Grime and Tan (148), using soluble enzyme methods, have determined benzyl penicillin down to concentrations of 1×10^{-3}M.

This technique represents an increase in sensitivity of at least an order of magnitude on previous work, although it must be pointed out that the apparatus used was considerably more expensive than that used by either Mosbach or Grime.

Close inspection of the Fig. 3.2.1.1. reveals that there are two factors which may affect the overall enthalpy change that is measured.

First, as a proton is produced by the hydrolysis reaction, the choice of buffer is certain to affect the overall enthalpy of the reaction, Secondly, the nature of the acyl group attached to the 6-amino position may well affect the overall enthalpy of hydrolysis. To investigate these effects further, the overall enthalpy of hydrolysis of six different semi-synthetic penicillins were measured, in 0.1M THAM HCl buffer (pH 7.2), and 0.1M phosphate buffer (pH 7.2), each containing 0.01M EDTA. The enthalpy of protonation of these buffers by strong acids are known to be -47.48 kJ mol^{-1} (188) and -4.74 kJ mol^{-1} (189), respectively.

Freshly made up penicillin solutions, of accurately known concentration, were made up in each of the above mentioned buffers, and a continuous pulse pumped into the microcalorimeter at a flow rate of 0.072 cm^3 min^{-1}. With the light sensitive azidocillin, after making up the solutions, the runs were carried out in a darkened room. In each case the steady-state

displacements obtained when accurately known electric currents (I) were passed through a 49.988 Ω resistor situated in the microcalorimeter itself, under identical conditions (e.g. flow rate), were also measured, and hence a plot of power (I^2 x 49.988) against steady-state displacement was drawn (Fig. 3.2.1.10) from which the overall enthalpy of reaction for each semi-synthetic penicillin in each buffer could easily be calculated (Table 3.2.1.9).

The results are summarized below:-

TABLE 3.2.1.9.

(Flow rate = 0.072 $cm^3 min^{-1}$ (= 1.2 x 10^{-6} $dm^3 sec^{-1}$)

Compound	"R" group	0.1M THAM buffer (pH 7.2)				0.1M phosphate (pH 7.2)			
		5 x 10^{-3}M disp (μV)	ΔH HYD /kJ mol^{-1}	5 x 10^{-3}M disp (μV)	ΔH HYD /kJ mol^{-1}	5 x 10^{-3}M disp (μV)	ΔH HYD /kH mol^{-1}	5 x 10^{-3}M disp (μV)	ΔH HYD /kJ mol^{-1}
benzyl penicillin	$C_6H_5CH_2$—	34.0	113.3	102	113.3	23.25	77.5	71.0	78.9
		34.5	115.0	102	113.3	22.50	75.0	70.5	78.3
phenoxymethyl penicillinic acid	$C_6H_5OCH_2$—	36.5	121.7	110	122.2	25.5	85.0	77.5	86.1
		37.0	123.3	108.5	120.6	26.0	86.7	77.5	86.1
ampicillin	$C_6H_5CH(NH_2)$—	37.5	125.0	112.5	125.0	27.0	90.9	79.5	88 3
		37.5	125.0	114.0	126.7	26.75	89.2	79.0	87.8
phenthicillin	$C_6H_5OCH(CH_3)$—	35.0	116.7	105.0	116.7	25.0	83.3	72.5	80.6
		35.5	118.3	105.0	116.7	24.5	81.2	73.5	81.2
propicillin	$C_6H_5OCH(C_2H_5)$—	34.5	115.0	103.5	115.0	23.75	79.2	72.0	80.0
		34.5	115.0	105.0	116.7	24.25	80.8	72.0	80.0
azidocillin	$C_6H_5CH(N_3)$—	36.5	122.5	109.5	121.7	25.5	85.0	76.5	85.0
		36.5	121.7	111.0	123.3	26.5	88.3	76.5	85.0

The enthalpy change per mol of reaction was calculated using the slope of the plot in Fig. 3.2.1.10 (μ V ≡ 19.995 μ W), the flow rate in litres per second (1.2 x 10^{-9}) and the concentration, using the expression

$$\Delta H\ (kJ\ mol^{-1}) = \frac{\text{power generated } (\mu W)}{\text{conc.}\ (\text{mol } dm^{-3}) \times \text{flow rate } (dm^3 sec^{-1}) \times 10^6} = \frac{\text{displacement} \times 19.995 \times 10^{-6}}{\text{conc.}\ (\text{mol.}\ dm^{-3}) \times \text{flow rate } (dm^3 sec^{-1}) \times 10^6}$$

TABLE 3.2.1.9 (cont'd)

Mean values for enthalpies of hydrolysis of semi-synthetic penicillin in O.1M THAM HCl (pH 7.2) and O.1M phosphate (pH 7.2)

Compound	"R" group	THAM mean ΔH_{HYD}/k J mol^{-1}	Phosphate mean ΔH_{HYD}/kJ mol^{-1}
benzyl penicillin	$C_6H_5CH_2$—	113.7	77.4
phenoxymethyl penicillin acid	$C_6H_5OCH_2$—	122.0	86.0
ampicillin	$C_6H_5\underset{\displaystyle NH_2}{\underset{|}{C}}H$—	125.4	88.8
penethicillin	$C_6H_5O\underset{\displaystyle CH_3}{\underset{|}{C}}H$—	117.1	81.6
propicillin	$C_6H_5O\underset{\displaystyle C_2H_5}{\underset{|}{C}}H$—	115.4	80.0
azidocillin	$C_6H_5\underset{\displaystyle N_3}{\underset{|}{C}}H$—	122 3	85.8

From these results and experience in the uncertainties involved in other similar "steady-state" determinations, the uncertainty is assessed as approximately ± 2.0 kJ/mol for each mean value.

Grime and Tan (148) have measured the enthalpy of hydrolysis of three of these sem-synthetic penicillin in THAM buffer, pH 7.5 using soluble enzyme techniques, and a comparison is shown in Fig. 3.2.1.11.

FIG. 3.2.1.10. Plot of steady-state displacement vs power generated in heater during electrical calibrations

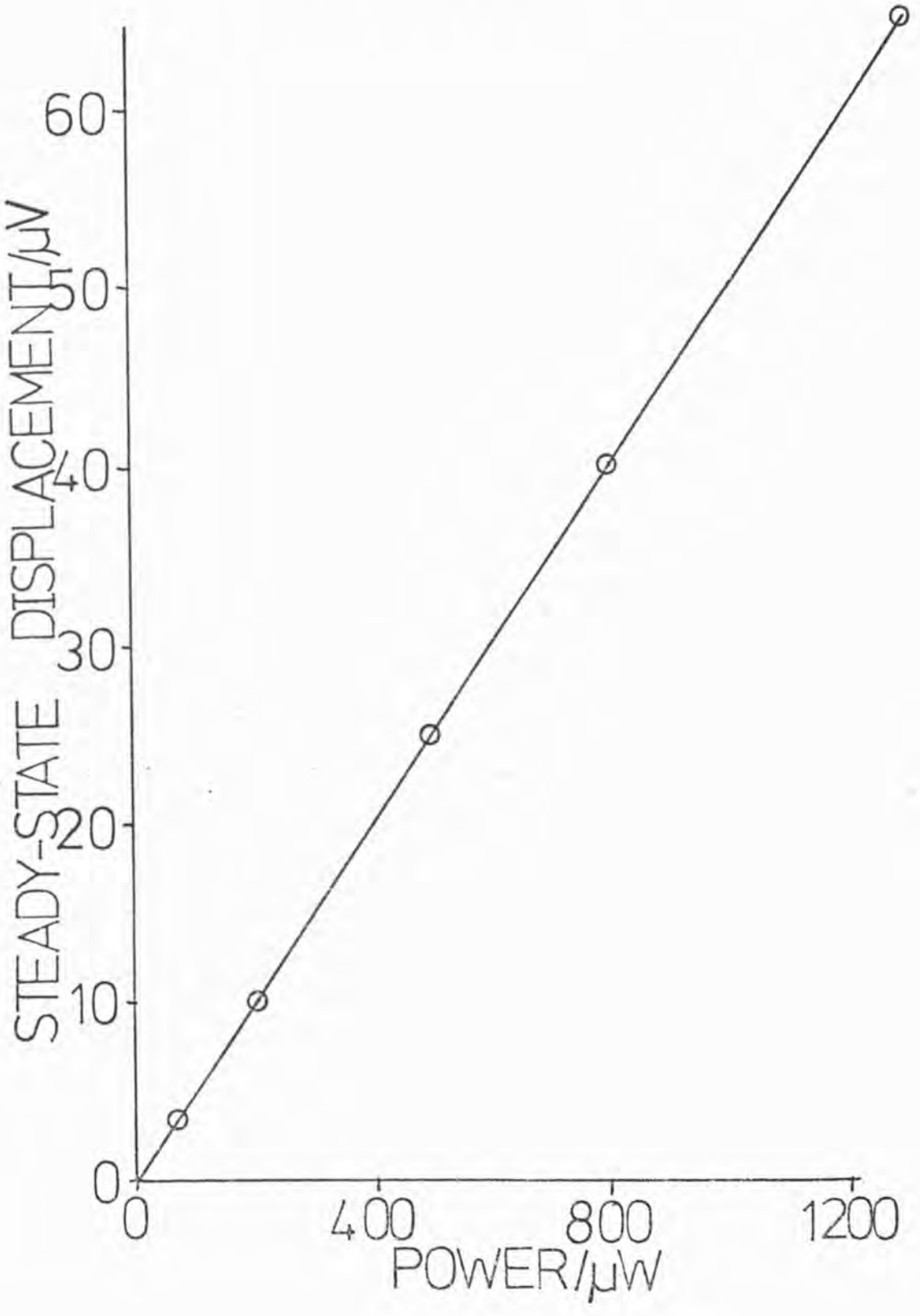


Fig. 3.2.1.11

Comparison of the values obtained for the enthalpy of hydrolysis of certain semi-synthetic penicillins in THAM buffer.

Compound	ΔH_{HYD}/kJ mol^{-1} This study	Grime and Tan(148)
benzyl penicillin	113.7 ± 2.0	114.7 ± 0.6
phenoxymethyl penicillin acid	122.0 ± 2.0	120.5 ± 1.3
ampicillin	125.4 ± 2.0	125.8 ± 1.2

The excellent agreement of these results lead to confidence in the accuracy of the other results, which do not yet appear in the literature.

A brief inspection of the structure of the R group in each penicillin and its enthalpy of hydrolysis indicates that substitution of hydrogen by carbon on the side chain carbon α- to acyl group decreases the enthalpy of hydrolysis, the decrease being greater along the series $H < CH_3 < C_2H_5$

On the other hand, substitution of a group capable of donating electrons by a resonance mechanism increases the enthalpy of hydrolysis. ($NH_2 > N_3 > H$).

That the enthalpy of hydrolysis of each of the penicillins was invariably greater in THAM buffer than in phosphate buffer, at the same pH, was undoubtedly due to the greater enthalpy of protonation of THAM (a nitrogen-base buffer) than phosphate (an oxygen-base buffer), by the protons liberated as a result of the hydrolysis.

Although the difference in enthalpy of hydrolysis in each buffer (approximately 36 kJ mol^{-1}) is less than the difference in protonation enthalpies of the two buffers by strong acids (42.5 kJ mol^{-1}) this is hardly surprising in view of the fact that protonation enthalpies decrease with decreasing acid strength, and penicillin acids would not be expected to ionize as readily as hydrochloric acid or other "strong" acids.

In conclusion, it would appear that the enthalpimetric technique described represents a realistic alternative method of penicillin assay for any penicillase sensitive penicillin. Indeed, the penicillin studied most (benzyl penicillin), would appear to give the least sensitivity. Certainly, this is for determination of enthalpies of hydrolysis of penicillins, the method of choice as Mosbach's enzyme thermistor device (157) cannot be accurately electrically calibrated, whilst Grime and Tan's soluble enzyme method, essentially based on the direct injection enthalpimetry (DIE) technique (see Chapter 1. Section 3.1.) is much slower, and consumes more enzyme than does this technique.

3.2.2. Determination of urea, and the measurement of the enthalpy of hydrolysis of urea in THAM and phosphate buffers at pH 7.2, using glass-immobilized urease and the LKB 10700-1 assembly.

Determination of urea is the most widely used index to protein metabolism and renal function. Methods for its determination have most often been based on spectrophotometric or fluorometric measurements after directly coupling urea to diacetyl monoxime type compounds (190-192) or on the urease-catalyzed hydrolysis of urea to ammonia and carbon dioxide (96, 193). Improved sensitivity and specificity can be obtained with enzymatic spectrophotometric (194) or fluorometric (195) procedures using coupled reactions catalyzed by urease and glutamate dehydrogenase. More recently, various procedures have been presented, taking advantage of the larger number of samples that can be analyzed by a given amount of enzyme using enzyme electrodes (71, 85-94).

The lack of interference by other species in heat measurements, coupled with the very high specificity of the enzyme urease, together with the potential advantages of immobilization, suggest that the use of thermal analysis in conjunction with immobilized urease, could prove a useful technique for urea analysis in complex fluids such as serum or urine.

This has already been recognized by Mosbach and co-workers (157,168) and by Bowers and co-workers, (166, 167, 169) who have used "enzyme thermistor" devices to determine urea in various types of samples. Schmidt et al (156) have used a heat-leak microcalorimeter similar to that used in this work for the determination of urea. Urease catalyzes the hydrolysis of urea to ammonia and carbon dioxide:-

$$NH_2.CO.NH_2 \xrightarrow[H_2O]{\text{urease}} 2NH_3 + CO_2$$

Reagents

Urease (Type VII, from Jack Beans) was obtained from Sigma Chemical Co., U.K.

Urea (99 + % pure) was obtained from B.D.H. Poole, U.K.

All other reagents were obtained as listed in Chapter 2.

Immobilization procedure and experimental details

Urease (3.6 mg, 250 units) was coupled to glutaraldehyde-activated alkylamine glass (0.5 g, 216.9 $m^2 g^{-1}$ surface area) as described in Chapter 2, Section 1.2.

The rest of the immobilization procedure and experimental details were identical to those described in 3.2.1. (analysis of penicillin G).

Tables, plots of peak height vs. concentration of urea, and of steady-state displacement vs. conc. of urea, using 0.1M phosphate buffer + 0.01M EDTA as buffer (at pH 7.2), are shown in Tables 3.2.2.1 and 3.2.2.2., and figures 3.2.2.3 and 3.2.2.4.

Correlation coefficients (r^2) between conc. of urea and peak height are also given in the tables.

TABLE 3.2.2.1.

Table of peak height vs. conc. of urea (500 µl pulse) in 0.1M phosphate buffer (pH = 7.2) (flow rate = 0.32 $cm^3 min^{-1}$)

conc. of urea (mol dm^{-3})	peak height(units)
0.122×10^{-3}	4.5
0.244×10^{-3}	9.0
0.488×10^{-3}	16.5
0.488×10^{-3}	15.0
0.975×10^{-3}	33.0
0.975×10^{-3}	33.0
1.950×10^{-3}	63.0
1.950×10^{-3}	67.5
3.900×10^{-3}	130.5
3.900×10^{-3}	130.5
7.800×10^{-3}	256.5
7.800×10^{-3}	261.0
15.60×10^{-3}	510.0
15.60×10^{-3}	517.5
31.20×10^{-3}	1026.0
31.20×10^{-3}	1035.0

$r^2 = 0.999$

$0.12 \text{ mM} \leqslant R \leqslant 31 \text{ mM}$

where R = analytically useful range

TABLE 3.2.2.2.

Table of steady-state displacement vs. conc. of urea (continuous pulse) in 0.1M phosphate buffer (pH = 7.2) (flow rate = 0.32 cm^3 min^{-1})

conc. of urea (mol dm^{-3})	peak height (units)
0.0195×10^{-3}	3.5
0.0195×10^{-3}	3.0
0.039×10^{-3}	5.5
0.039×10^{-3}	5.0
0.078×10^{-3}	10.0
0.078×10^{-3}	9.0
0.156×10^{-3}	16.0
0.156×10^{-3}	17.0
0.312×10^{-3}	36.5
0.312×10^{-3}	36.0
0.625×10^{-3}	71.5
0.625×10^{-3}	71.0
1.250×10^{-3}	140.5
1.250×10^{-3}	141.0
2.500×10^{-3}	274.0
2.500×10^{-3}	277.0
5.000×10^{-3}	525.0

$r^2 (0.0195 \times 10^{-3} \longrightarrow 2.5 \times 10^{-3}\ M) = 0.999$

$0.02mM \leqslant R \leqslant 2.5mM$

FIG. 3.2.2.3. Plot of peak ht. vs. urea conc (0.5 cm^3 pulse), as in table 3.2.2.1.

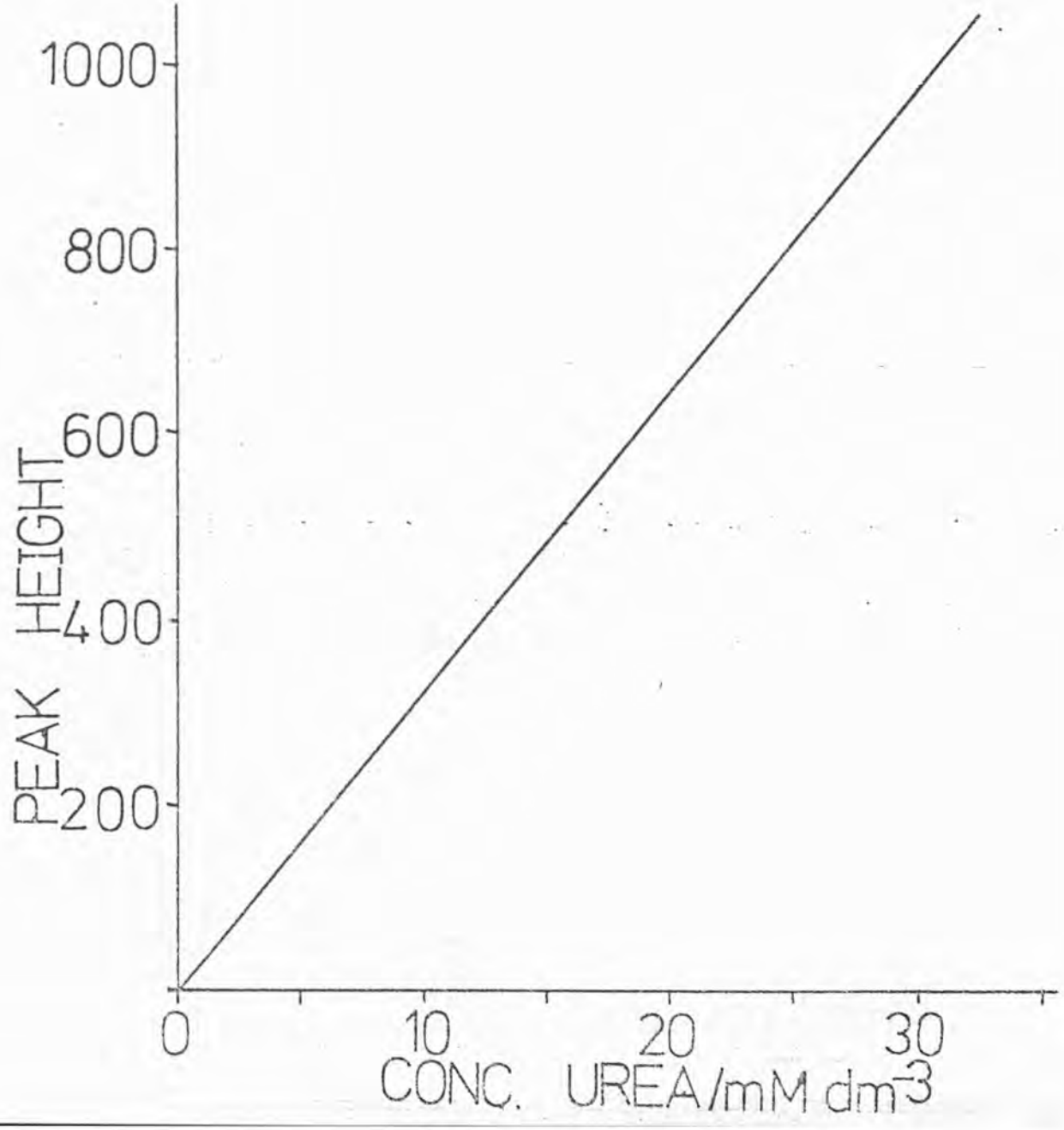
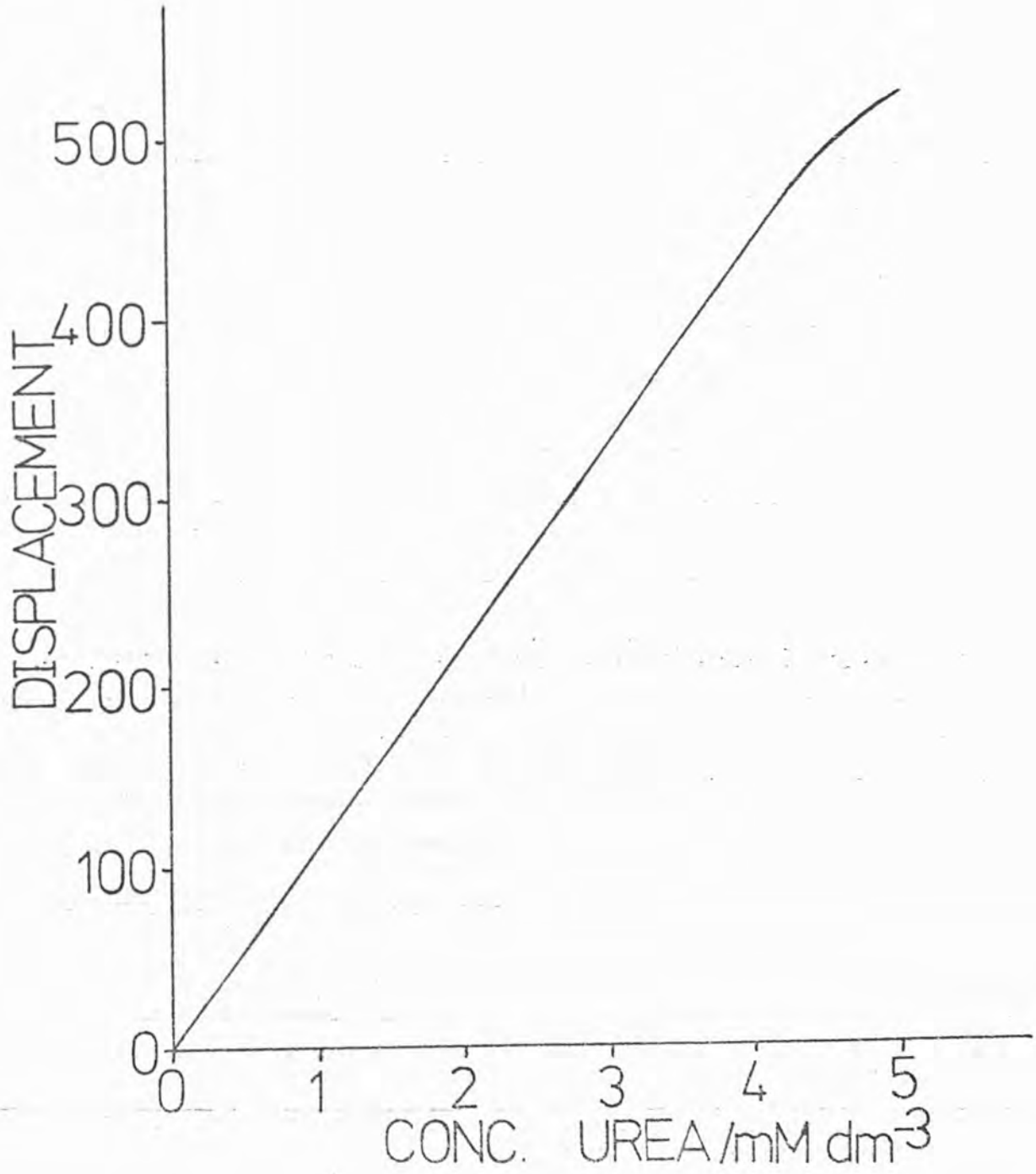


FIG. 3.2.2.4. Plot of steady-state displacement vs. conc. of urea (as in table 3.2.2.2.)



As can be seen from Tables 3.2.2.1 and 3.2.2.2, the analytically useful range for 500 µ l urea samples using this method is from approximately 120 µ M up to at least 31.2 mM (depending on the activity of the immobilized urease), the lower limit corresponding to the determination of 60×10^{-9} moles of urea (about 3.6 µ g).

Using a continuous pulse of urea, concentrations lower than 20×10^{-6}M can be determined. However, it is felt that only samples with a concentration of at least 40×10^{-6}M could be determined with reasonable reliability ($\pm$ 4%).

For concentrations of 0.5×10^{-3}M or over (500 µ l pulse), or 0.2×10^{-3} (continuous pulse) the day-to-day reproducibility of identical samples appears to be $\pm$ 2%. As with glass-immobilized penicillinase, the stability of the immobilized enzyme, as measured by the peak height of a 500 µ l pulse 1×10^{-3}M urea, appeared quantitative throughout the two week duration of this study.

The sensitivity obtained using this method appears to be at least twice that obtained by Schmidt et al (156) using a very similar piece of apparatus, probably due to the fact that phosphate buffer was used throughout all determinations whereas Schmidt and co-workers used THAM buffers. Further discussion is given on this fact later in the Section.

Bowers and co-workers (166,167) and Mosbach and co-workers (160), have determined urea in concentrations as low as 1.0×10^{-3} and 0.02×10^{-3}M respectively, using "enzyme-thermistor"-type devices.

The techniques utilized in this study give sensitivity over an order of magnitude higher than obtained by Bowers and Carr et al, and at least as good as obtained by Mosbach et al.

Discussion

The classical products of hydrolysis of urea by urease are ammonia, and carbon dioxide, in accordance with the following equation:-

$$NH_2.CO.NH_2 \xrightarrow[H_2O]{\text{urease}} 2NH_3 + CO_2 \qquad (1)$$

This can then be followed by two reversible, pH-dependent reactions:-

$$2\ H^+\ \text{buffer} + 2NH_3 \rightleftharpoons 2\ \text{buffer} + 2NH_4^+ \qquad (2)$$

and $$\text{buffer} + CO_2 + H_2O \rightleftharpoons HCO_3^- + H^+\ \text{buffer} \qquad (3)$$

As the measured overall enthalpy of reaction will include a term from each of the three reactions, and as the position of equilibrium, and hence the enthalpy change, of reactions (2) and (3) are pH-dependent a variation in the enthalpy of hydrolysis of urea with pH and with nature of the buffer is expected. This has indeed been observed by Jespersen (196), who found that the overall enthalpy of hydrolysis of urea by soluble urease was greater in 0.05M phosphate buffer at pH 7.5 (61.30 kJ mol^{-1}) than at pH 6.7 (48.24 kJ mol^{-1}), and that the overall enthalpy of hydrolysis of urea in 0.05M phosphate buffer (pH 7.5), was also greater than that obtained in 0.05M THAM buffer (18.70 kJ mol^{-1}), also at pH 7.5. However, Jespersen also points out (196) that the reason for the unexpectedly large difference in enthalpy change between the two buffers may be due to the formation of ammonium carbamate as the major hydrolysis product, in the case of THAM buffer.

Schmidt et al, however, (156), using apparatus similar to that used in this study, have determined the enthalpy of hydrolysis of urea in THAM buffer (pH 7.0), as being 7.11 kJ mol^{-1}, less than half the value reported by Jespersen. In this study the enthalpies of hydrolysis of urea by glass-immobilized urease have been determined in 0.1M phosphate buffer (pH 7.2), and 0.1M THAM (pH 7.2), and are compared with reported values.

Urea solutions were made up by dissolving an accurately weighed sample of urea in an accurately measured volume of either 0.1M phosphate buffer, or 0.1M THAM buffer, both at pH 7.2, and continuously pumping a pulse of the solution into the LKB assembly at a flow rate of 0.072 cm^3 min^{-1}. The steady-state displacements obtained were calibrated electrically as shown in Fig. 3.2.1.10, and the overall molar enthalpy of reaction calculated as described in Chapter 3, Section 2.1. The results are summarized in Table 3.2.2.5.

TABLE 3.2.2.5.

Determination of overall molar enthalpy of hydrolysis of urea in 0.1M phosphate buffer (pH 7.2) and in 0.1M THAM buffer (pH 7.2)
Flow rate = 0.072 $cm^3 min^{-1}$

urea conc.	0.1M phosphate buffer, pH 7.2		0.1M THAM pH 7.2	
mol dm^{-3}	displacement μV	ΔH_{HYD} (kJ mol^{-1})	displacement μV	ΔH_{HYD} (kJ mol^{-1})
20×10^{-3}	67	55.8	-	-
	68	56.7	-	-
10×10^{-3}	34	56.7	12.0	20.0
	35	58.3	12.5	20.8
5×10^{3}	17	56.7	6.0	20.0
	17.5	58.3	5.5.	18.3
	mean	57.1(± 1.0)	mean	19.8(±0.4)

The uncertainties of mean value are assessed at about ± 2%.

The value for the overall enthalpy of hydrolysis of urea in phosphate buffer (pH 7.2) appears, allowing for the pH effect, to be in good agreement with that determined by Jespersen (196), as shown in Table 3.2.2.6.

TABLE 3.2.2.6

Comparison of the overall molar enthalpy of hydrolysis of urea in phosphate buffer (pH 7.2 - this study), with that in phosphate buffer at pH 7.5 and 6.7 (Jespersen's study (116).

	pH 7.5 (Jespersen)	pH 7.2 (this study)	pH 6.7 (Jespersen)
overall molar enthalpy of hydrolysis of urea/kJ mol^{-1} in phosphate buffer	61.30± 0.79	57.1± 1.0	48.24± 0.88

A comparison of the overall enthalpy of hydrolysis of urea in THAM buffer as measured in this study (pH 7.2), Jespersen's study (pH 7.5) and Schmidt et al, (pH7.0), is given in Table 3.2.2.7.

TABLE 3.2.2.7

Comparison of the overall molar enthalpy of hydrolysis of urea in THAM buffer in this study with that obtained by Jespersen (196) and Schmidt et al (156).

	pH 7.2 (this study)	pH 7.5 (Jespersen)	pH 7.0 (Schmidt et al)
overall molar enthalpy of hydrolysis of urea/kJ mol^{-1} in THAM buffer	19.8(± 0.4)	18.70(±0.63)	7.11(± 0.84)

Comparison of values for the overall enthalpy of hydrolysis of urea in THAM buffer obtained in the three studies show Schmidt's to be lower than both this study and Jespersen's. This may be due to the incomplete reaction, due to the choice of support material. It has been emphasized in Chapter 3, Section 1.2, how the choice of support material for the immobilized enzyme is critical, due to the small amount which can be accommodated into the LKB microcolumn. Schmidt appears to have used Enzite^{R}, obtained from Miles-Seravac, and mentions no attempts in his paper either to optimize the support or to show that 100% reaction had occurred.

Although the sensitivity of this technique to urea determinations appears to be only about half of that obtained in the analysis of penicillin (see 3.2.1), due to the lower overall enthalpy of hydrolysis, the technique would still appear suitable for urea determinations in complex or coloured biological fluids, e.g. urine. Although no attempts have been made in this study to use biological fluids such as serum or urine, owing principally to the difficulty in obtaining accurate standards from elsewhere, Borresbaeck and Börjeson (197) have described the use of an "enzyme-thermistor" device, containing glass-immobilized urease in the assay of serum samples.

3.2.3. Determination of hydrogen peroxide, using glass-immobilized catalase in conjunction with the LKB 10700-1 microcalorimeter assembly.

Little work appears to have been reported where an immobilized enzyme(e.g. either catalase or peroxidase), has been used to determine hydrogen peroxide using a continuous-flow microcalorimeter.

Forrester et al (172), have described a batch microcalorimeter which utilizes immobilised catalase whilst Pennington (173) has designed a "vessel-less" microcalorimeter, where catalase was bound directly to the surface of the detector (a Peltier cooler), where the sample was introduced directly on to the enzyme through a microsyringe. It was hence decided to investigate the feasibility of hydrogen peroxide determinations, using the techniques described in the previous sections, with immobilized catalase.

Catalase, a highly active enzyme occurring widely in nature, catalyzes the disproportionation of hydrogen peroxide into water and oxygen:-

$$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2$$

Reagents

Catalase (from Bovine Liver) was obtained from Sigma Chemical Company, U.K.

Hydrogen peroxide was obtained from B.D.H., Poole, U.K. as an approximately 30% w/v solution in water.

All other reagents were obtained as listed in Chapter 2, or from B.D.H. as laboratory reagent grades.

Standardization of hydrogen peroxide

1.0 cm^3 of the 30% solution was diluted to 100 cm^3 in a graduated flask with distilled water. To a 10.00 cm^3 aliquot of this solution was added potassium iodide (0.5 g) and 1M sulphuric acid (50 cm^3) After standing for 15 min the liberated iodine was titrated with 0.10044M standardized sodium thiosulphate (using starch as indicator), from a 10.00 cm^3 burette.

TABLE 3.2.3.I

Titration of liberated iodine from 10.00 cm^3 H_2O_2 + excess KI + H_2SO_4 with 0.10044M sodium thiosulphate

vol. of H_2O_2 soln. cm^3	original burette reading/cm^3		final reading cm^3	total vol. of 0.10044M $Na_2S_2O_3$ required for equivalence
10.00	10.00	-	0.00	16.63
	7.56	-	0.93	
10.00	10.00	-	0.00	16.61
	8.41	-	1.80	
			mean =	16.62

The reaction can be written:-

$$H_2O_2 + 2H^+ + 2I^- \longrightarrow I_2 + 2H_2O$$

$$I_2 + 2Na_2S_2O_3 \longrightarrow 2NaI + Na_2S_4O_6$$

therefore $H_2O_2 = 2Na_2S_2O_3$

therefore $[H_2O_2] = \frac{1}{2} \times 0.10044 \times \frac{16.62}{1000} \times \frac{1000}{10} = 0.0835$ mol. dm^{-3}

This solution was diluted with buffer (o.1M phosphate, pH 7.0), by a factor of 10, and then by a further factor of 10, to obtain a solution of concentration 835×10^{-6} mol dm^{-3}. Solutions of other concentrations were obtained by appropriate dilution of this starting solution. When not in use, the standardized hydrogen peroxide solutions were stored in the dark at 4°C. All runs were carried out within 12 h of making up the standardized hydrogen peroxide solution.

Immobilization procedure

Catalase (E.C.1.11.1.6), 10 mg (18500 units) was coupled to glutaraldehyde activated alkylamine glass (0.5g, 13.3 $m^2 g^{-1}$ surface area), as described in Chapter 2, Section 1.2. The rest of the immobilization procedure and experimental details, were identified to those described in 3.2.1. (analysis of penicillin G), except that the buffer was 0.1M phosphate + 0.01M EDTA, at pH 7.0, flow rate = 0.32 $cm^3 min^{-1}$.

Tables and plots of peak height (500 μ l pulse injections) and steady-state displacement (continuous injection) vs. conc. of hydrogen peroxide are shown as Tables 3.2.3.2, 3.2.3.3, and Figs 3.2.3.4 and 3.2.3.5. The correlation coefficients (r^2) are also included.

TABLE 3.2.3.3.

Table of peak height (units), vs. conc. of hydrogen peroxide (500 μ l pulse).

conc. H_2O_2/mol dm^{-3}	peak height (units)
0.104×10^{-3}	1.75
0.104×10^{-3}	1.75
0.208×10^{-3}	4.0
0.208×10^{-3}	4.0
0.208×10^{-3}	4.5
0.416×10^{-3}	9.0
0.416×10^{-3}	9.5
0.416×10^{-3}	9.5
0.835×10^{-3}	19.5
0.835×10^{-3}	20.0
0.835×10^{-3}	21.0

$r^2 = 0.997$

TABLE 3.2.3.4.

Table of steady-state displacement(units) vs. conc. of hydrogen peroxide (continuous injection)

conc. hydrogen peroxide ($mol\ dm^{-3}$)	peak height (units)
0.026×10^{-3}	2.0
0.052×10^{-3}	4.5
0.104×10^{-3}	10.0
0.208×10^{-3}	21.0
0.416×10^{-3}	45.0
0.835×10^{-3}	94.0

$r^2 = 0.999$

Despite the apparently satisfactory correlation coefficients for both sets of results, inspection of the plots revealed a distinct curvature, particularly marked in the case of the continuous injection series of runs.

This curvature is almost certainly due to the non-enzymatic decomposition of hydrogen peroxide by trace of transition metal ion contaminants (especially Co^{2+}) before the samples reach the enzyme. Despite thorough washing of all glassware used, and the inclusion of EDTA in the buffer, this effect could not be eliminated, only minimized, and was further shown by the appearance of small bubbles of, presumably, oxygen at the interface of solution and glassware a short time after the solution had been placed in the vessel.

As a result, it was felt that any attempt to measure quantitatively the enthalpy of disproportionation of hydrogen peroxide into water and oxygen would be unprofitable, due to the uncertainties involved in concentration of substrate actually reaching the immobilized

FIG. 3.2.3.4. Plot of peak ht. vs. conc. H_2O_2 (05cm^3 pulse), as in table 3.2.3.2.

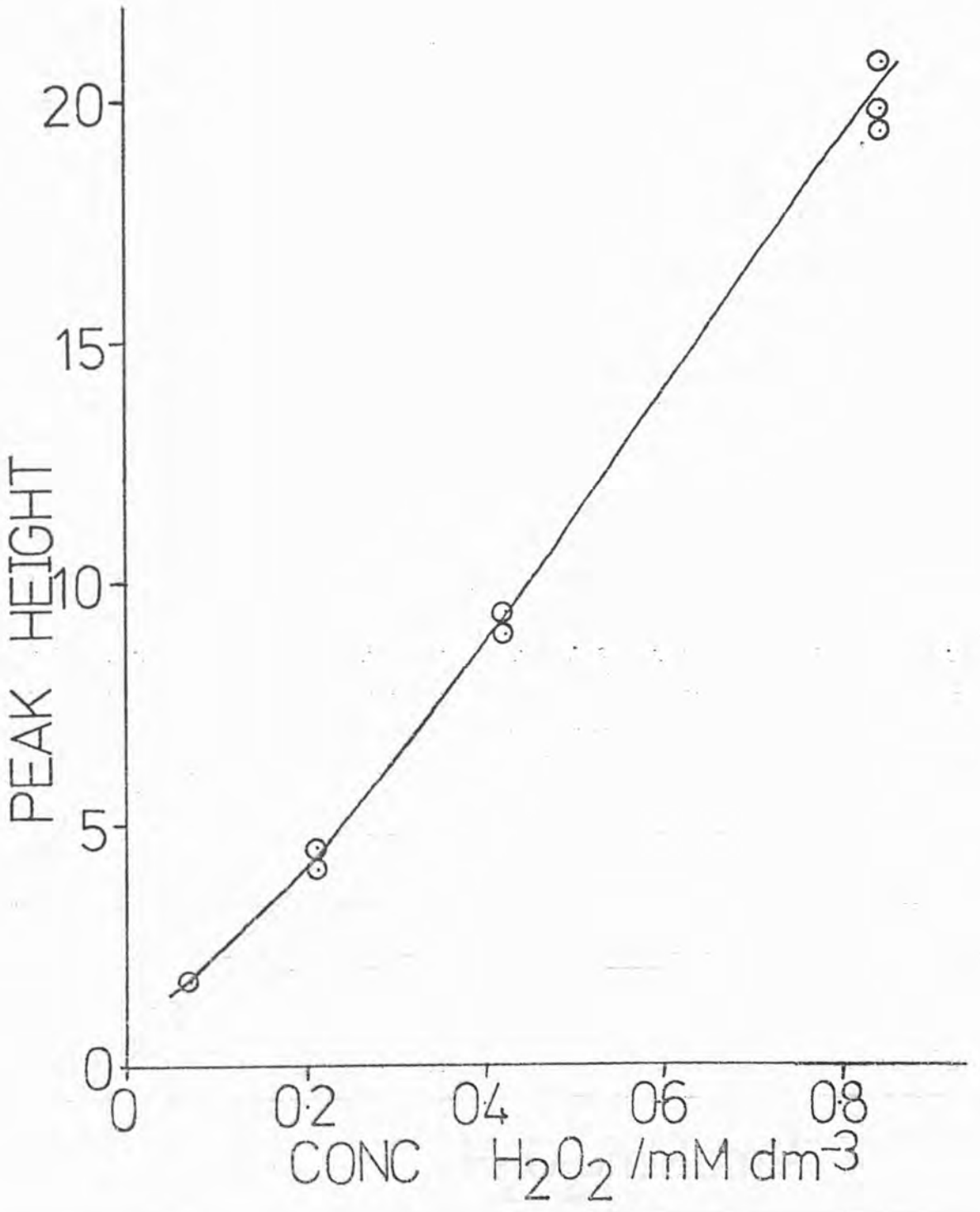
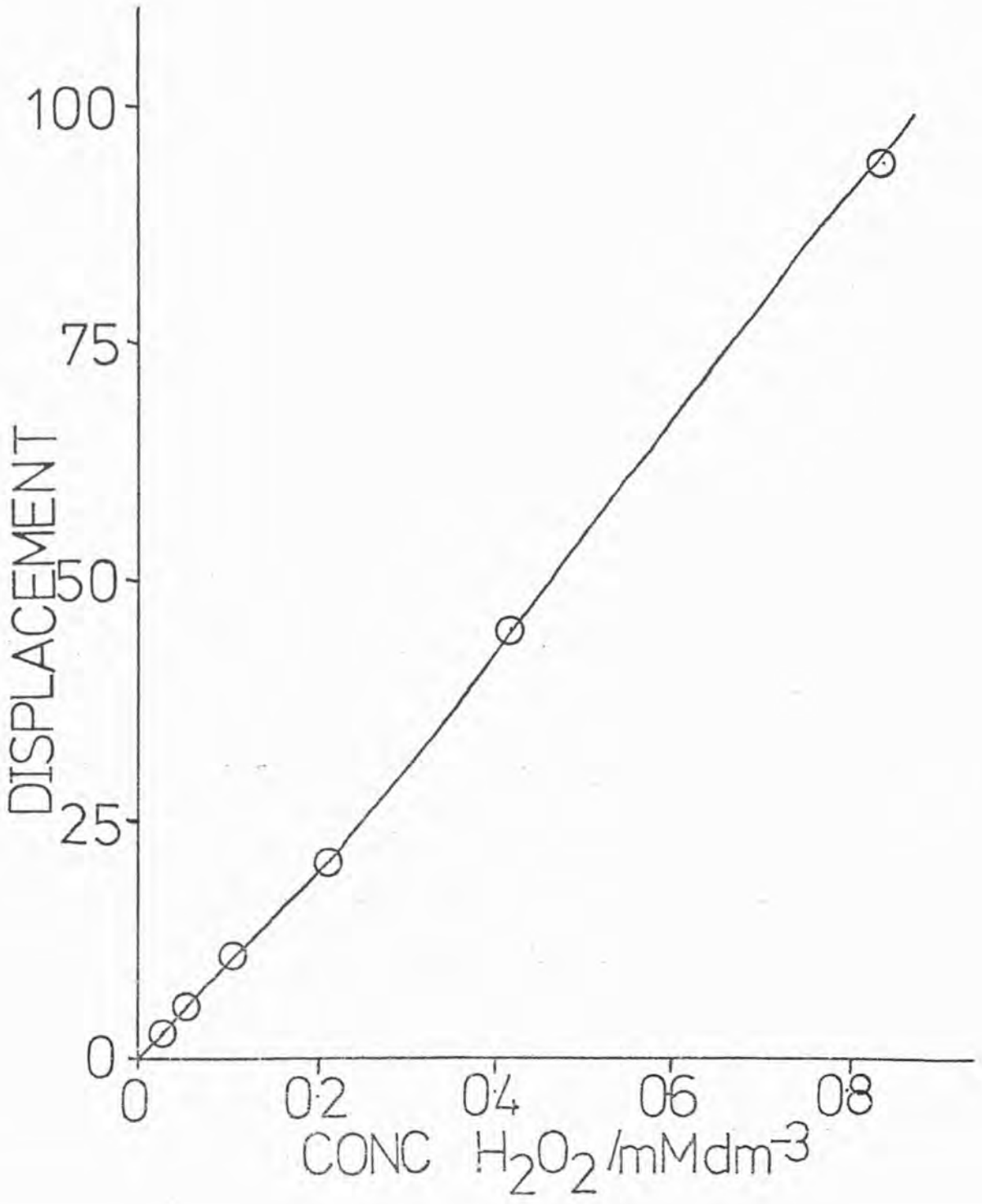


FIG. 3.2.3.5. Plot of steady-state displacement vs. conc. H_2O_2, as in table 3.2.3.3.



catalase in the microcolumn.

3.2.4. Determination of glucose using glass-immobilized glucose oxidase and catalase in conjunction with the LKB 10700-1 microcalorimeter

The determination of glucose is one of the most routinely performed of clinical analyses, and of great importance in the food industry (9).

Various methods for the determination of glucose in biological materials have been comprehensively reviewed (198, 199). With few exceptions they involve the measurement of absorbance or fluorescence by products of either a direct glucose reaction, or a coupled secondary reaction. Such measurements tend to be affected by matrix materials (e.g. proteins), or reagents with interfering spectral characteristics. Electrochemical methods include determination (by rate potentiometry or constant-current coulometry) of the hydrogen peroxide produced during aerobic oxidation of glucose in the presence of glucose oxidase. These procedures are likewise prone to protein interferences caused by blocking of electrode surfaces. Because of these difficulties, protein is generally removed from serum samples before the determinative step; however, this entails propagation of errors inherent in any multi-step procedure.

The non-interference of inert substances in microcalorimetry, together with the high specificity towards glucose of the enzyme glucose oxidase, and the potential advantages of immobilization, would appear to make the thermal techniques which have already been described in this thesis extremely suitable for glucose determination. Mosbach and co-workers have already reported the use of their "enzyme thermistor" techniques (157-160), and

heat-leak type microcalorimeters, such as that used in this study have been utilized by Johansson (154), and Schmidt et al (156). However, particularly in the case of Johansson, it will be shown later how his conditions were far from being optimized, mainly due to the choice of support material.

The co-immobilization of glucose oxidase (specific for β-D-glucose), and catalase (to decompose the inhibitory hydrogen peroxide so formed, and to give a larger overall enthalpy change), can be regarded as giving the following reaction scheme, as shown in Fig. 3.2.4.1 :-

$$\beta\text{-D-glucose} \xrightarrow[O_2]{\text{glucose oxidase}} \text{D-glucono-}\delta\text{-lactone} + H_2O_2 \quad (1)$$

$$\text{D-glucono-}\delta\text{-lactone} \underset{-H_2O}{\overset{+H_2O}{\rightleftharpoons}} \text{D-gluconic acid} \quad (2)$$

$$H_2O_2 \xrightarrow{\text{catalase}} H_2O + \tfrac{1}{2}O_2 \quad (3)$$

Fig. 3.2.4.1.

Reagents

Glucose oxidase (Type VII from Aspergillus niger), and catalase (from Bovine liver) were obtained from Sigma Chemical Co. U.K.

Anhydrous D-glucose (puriss) was obtained from Koch-light Laboratories, Colnbrook, Bucks, U.K.

All other reagents were obtained as listed in Chapter 2.

Immobilization procedure

Glucose oxidase (E C. 1.1.3.4.), 20 mg (4200 units), and catalase (E.C.1.11.1.6.), 1 mg (1850 units), were coupled to gluteraldehyde-activated alkylamine glass (0.5 g, 216.9 $m^2 g^{-1}$ surface area), as described in Chapter 2, Section 1.2.

All other experimental details were identical to those described in

Chapter 3 , Section 2.1. (analysis of penicillin G).
Tables and plots of peak height (500 μ l pulses), and of steady-state displacement (continuous pulse) vs. conc. extraction of glucose are shown in Tables 3.2.4.2 and 3.2.4.3., and Figs. 3.2.4.4 and 3.2.4.5., for O.1M phosphate buffer, containing 0.01M EDTA at pH 5.8.
Correlation coefficients (r^2) were calculated.

The shape of the plots in Fig. 3.2.4.4. and 3.2.4.5. shows that at low concentrations of glucose, the plot is linear, but that this linearity is lost rather abruptly at higher concentrations, (approx. 1.25×10^{-3} for 500 μ l pulses of glucose, and at approx. 0.5×10^{-3} for continuous injections).

This could have been due either to a limiting activity of glucose oxidase immobilized in the microcolumn, or to depletion of oxygen (a co-substrate of glucose oxidase - see Fig. 3.2.4.1.) in the buffer solution. The former seems unlikely since (a) this effect had never previously been encountered at such low concentrations, and (b) had never been so abrupt. Hence it appears likely that the plateau was in fact due to depletion of dissolved oxygen in this buffer. This was substantiated by the fact that the linear range of the plot could be approximately doubled by saturating the buffer solution with oxygen just prior to it being introduced into the LKB assembly.

As before, the day-to-day reproducibility of identical samples was about $\pm$ 2%, but solutions of glucose were found to deteriorate if left for more than a few days, presumably due to the action of bacteria. To avoid erroneous results due to bacterial decomposition, glucose solutions were eitherfreshly prepared (i. e. within 24 h of use), or made up in a saturated

TABLE 3.2.4.2.

Table of peak height (500 μ l pulse) vs. concentration of glucose in phosphate buffer, pH 5.8, flow rate = 0.32 $cm^3 min^{-1}$

conc. of glucose/mol dm^{-3}	peak height (units)	
0.039×10^{-3}	4.0	
0.039×10^{-3}	4.0	
0.078×10^{-3}	8.5	
0.078×10^{-3}	8.0	
0.156×10^{-3}	18.5	
0.156×10^{-3}	18.5	
0.156×10^{-3}	18.0	
0.312×10^{-3}	37.5	
0.312×10^{-3}	38.0	
0.625×10^{-3}	74.0	
0.625×10^{-3}	72.5	
1.250×10^{-3}	152.0	
1.250×10^{-3}	150.5	
2.500×10^{-3}	169.5	284.0 *
2.500×10^{-3}	169.5	285.5 *
5.000×10^{-3}	176.5	332.0 *
5.000×10^{-3}	177.0	330.5*

$r^2(0.039 \times 10^{-3} \longrightarrow 1.250 \times 10^{-3}M) = 0.999$

* in buffer saturated with O_2

$0.04mM \leq R \leq 1.25mM$ in

$0.04mM \leq R \leq 2.0mM$ (in O_2 saturated solution)

where R = analytically useful range

TABLE 3.2.4.3.

Table of steady-state displacement vs. conc. of glucose (continuous pulse) in phosphate buffer, pH 5.8, flow rate $0.32\ cm^3\ min^{-1}$

conc. of glucose/mol dm^{-3}	displacement (units)	
0.009×10^{-3}	4.5	
0.018×10^{-3}	9.5	
0.018×10^{-3}	9.5	
0.039×10^{-3}	18.0	
0.039×10^{-3}	18.5	
0.078×10^{-3}	36.5	
0.078×10^{-3}	36.5	
0.156×10^{-3}	72.0	
0.156×10^{-3}	72.0	
0.312×10^{-3}	147.0	
0.312×10^{-3}	145.5	
0.625×10^{-3}	250.0	293.5*
0.625×10^{-3}	251.5	293.5*
1.250×10^{-3}	298.0	504.5*
1.250×10^{-3}	296.5	503.0*

$r^2(0.009 \times 10^{-3} \longrightarrow 0.312 \times 10^{-3}M) = 0.999$

* in buffer saturated with O_2

$0.009\ mM \leqslant R \leqslant 0.31mM$

$0.009\ mM \leqslant R \leqslant 0.75\ mM$ (in O_2 saturated solution)

FIG. 3.2.4. Plot of peak ht. vs. conc. of glucose (0.5 cm^3 pulse), as in table 3.2.2.

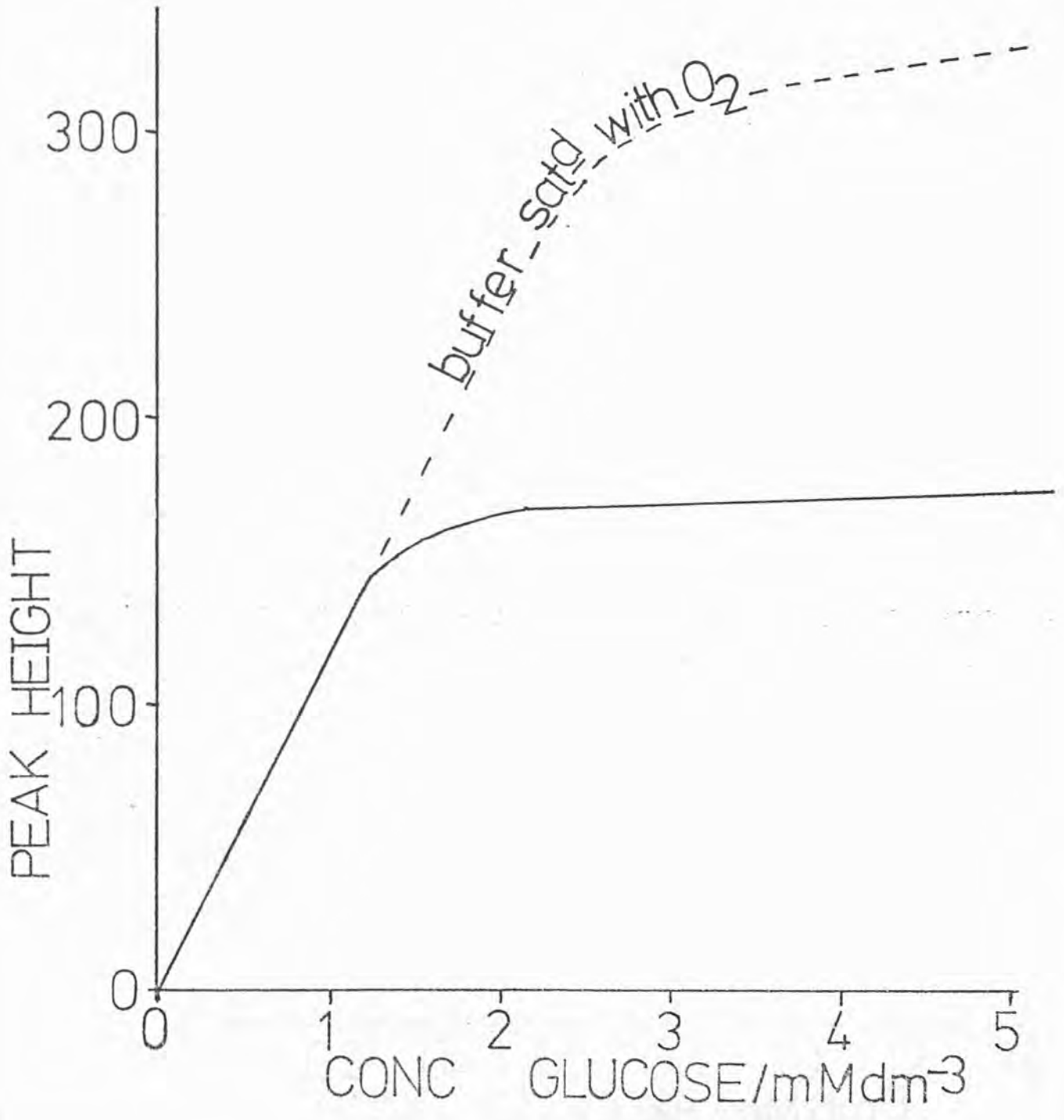
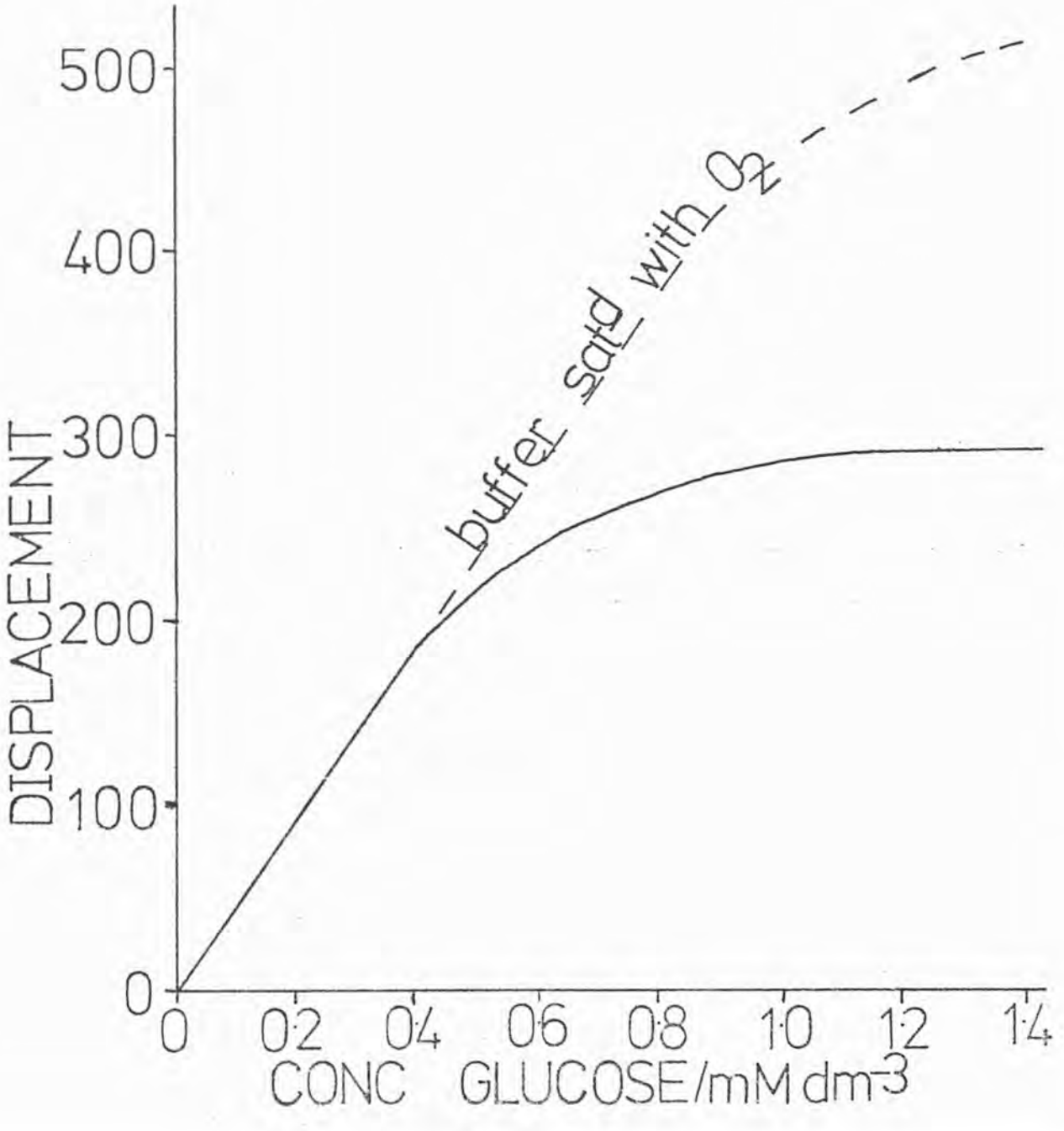


FIG. 3.2.4.5. Plot of steady-state displacement vs conc of glucose, as in table 3.2.4.3.

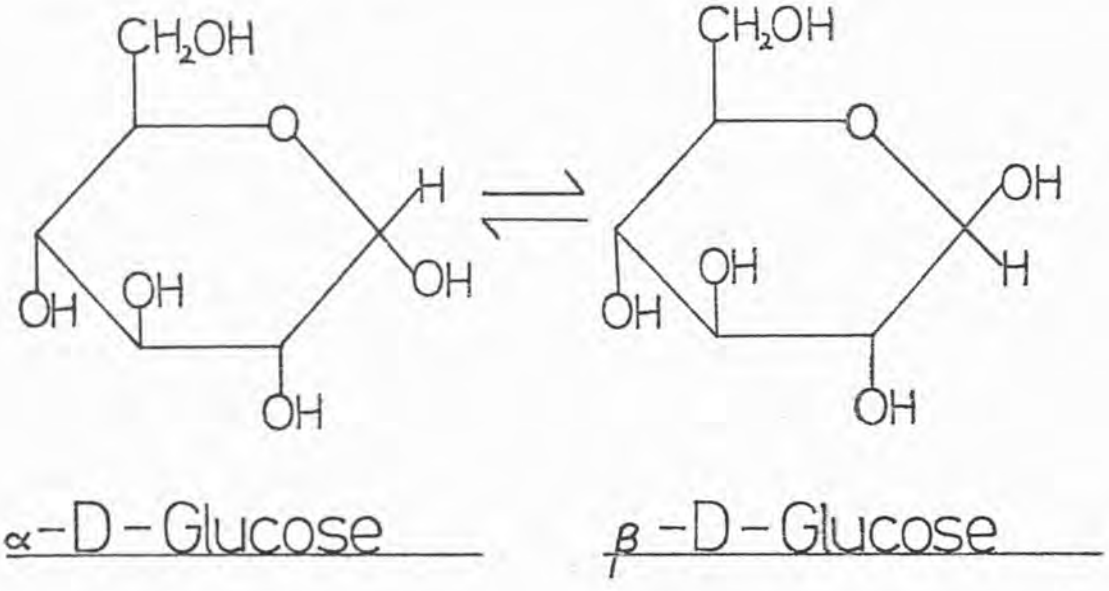


solution of benzoic acid, and adjusted to the desired concentration by dilution with the appropriate volume of buffer beforehand.

The immobilized enzyme in the microcolumn, as in previous studies, showed apparent quantitative stability for a period of at least two weeks, as shown by the peak height of a 500 μ l pulse of 0.5×10^{-3}M glucose.

Effect of mutarotation of D-glucose

D-glucose, when in the form of a 6-membered ring (pyranose form), can exist in two anomeric forms, the α-form, and β-form, as shown in Fig. 3.2.4.6.



α-D-Glucose β-D-Glucose

Fig. 3.2.4.6.

In aqueous solution, the ratio of the α- to β- forms is approximately 36:64 (200), although in the crystalline state this equilibrium no longer holds, the α-form predominating. As the enzyme glucose oxidase is specific for β-D-glucose many workers, (e.g. Mosbach et al (160), have left standard glucose solutions to stand for 24 h to ensure complete equilibrium in the mutarotation.

In the 0.1M phosphate buffer used in this study, no difference was found in the peak heights or steady-state displacements obtained from freshly made-up glucose solutions, and glucose solutions left to stand for 24 h.

Indeed, a freshly prepared 0.625×10^{-3}M solution of (initially) pure α-D-glucose, was observed to give over 95% of the steady-state displacement by the time it reached the immobilized glucose oxidase/catalase in the microcalorimeter (40 min after dissolving the α-D-glucose) given by a solution of D-glucose of idential concentration that had been allowed to stand for 24 h prior to being introduced into the microcalorimeter showing that, under the conditions of the experiments, mutarotation was fairly rapid.

Some workers (e.g. Schmidt et al (156)), have quoted a measured overall enthalpy change for the reaction scheme, as illustrated in Fig. 3.2.4.1.

However, in the opinion of the author, such figures are meaningless unless it can be established that either no mutarotation at all is occurring during the residence time of the glucose solution in the immobilized enzyme microcolumn, in which case the reaction would only be 64% complete, but could easily be corrected for 100% reaction, or that mutarotational equilibrium was established so

quickly that, as β-D-glucose was removed from the equilibrium, the α-form converted so rapidly to the β-form, that it too could be oxidized by the enzyme before it was swept out of the micro-column. In such a case the reaction would be quantitative.

The result obtained with the pure α-D-glucose in this study suggests that the actual rate of mutarotation may be somewhere intermediate between these two extremes, and that in the relatively short time for which the glucose is in contact with the immobilized glucose oxidase (approx. 2-5 min, depending on the flow rate), between 64% and 100% of the stoichiometric reaction is occurring.

Provided this (unknown) extent of reaction remained constant, the analytical value of this technique would not be affected; however, any quoted overall enthalpy changes for the reaction would, obviously, be unreliable.

The major drawback to techniques such as this in analysis would appear to be the limited linear range, due to the dual substrate nature of the enzyme glucose oxidase requiring dissolved oxygen from the buffer as co-substrate. There would appear to be little that can be done to circumvent this difficulty, as even saturation of the buffer solution with oxygen produces only a limited effect. However, the overall enthalpy change appears large enough to enable very accurate measurements to be made in the region 0.1×10^{-3}M to 0.5×10^{-3}M, so dilution of a sample to within this concentration range may be one answer. The weakness in this technique is that, although uncertainties in this low concentration region may be small, when scaled to the original concentration, absolute uncertainties become much greater, although the percentage uncertainty remains unaltered.

Other enzymes that could be used in glucose determinations (e.g. hexokinase, E.C.2.7.1.1.), although having the advantage of greater linear ranges, are inherently much less specific, making their use in complex media subject to more uncertainty. There is the further disadvantage of requiring expensive cofactors (e.g. $NADP^+$), making the cost per analysis much greater. It is felt by the author that, except at low concentrations of glucose ($\leqslant 1 \times 10^{-3}$M), the technique described may not be as generally applicable to glucose determinations as to determinations of species, such as penicillins or urea. The sensitivity of the techniques appears similar to that obtained by Mosbach et al (158, 160), slightly better than that obtained by Schmidt et al (156), and considerably better than that of Johansson (154), who used an identical microcalorimeter.

The lower sensitivity obtained by Johansson under seemingly identical conditions can probably be again ascribed to a poor choice of support material (in this case, polyacrylamide gel), probably giving incomplete conversion of all the β-D-glucose present because of low mass transfer rates of glucose to the enzyme bound in relatively large-diameter, gel-like polyacrylamide.

3.2.5. Determination of sucrose and lactose using the LKB 10700-1 microcalorimeter in conjunction with the appropriate immobilized enzymes.

Little work appears in the literature on the use of the combination of immobilized enzymes and microcalorimetry in the determination of the disaccharides, lactose and sucrose. Mosbach and co-workers have briefly described an "enzyme-thermistor" unit capable of lactose analysis (158) and continuous monitoring of lactose concentration (161). A paper on sucrose analysis (174) was presented on the 12th FEBS Meeting (Dresden) in 1978 by some members of this group, but this appears to be the sum total of published work on these topics up to June 1980, despite the commercial availability of enzymes acting specifically on sucrose and lactose.

Consequently, it was attempted to use the techniques previously described in this chapter to determine these disaccharides specifically, both alone and in the presence of each other, and glucose. Preliminary work showed that the hydrolysis of the disaccharide with the appropriate enzyme will, per se, only produce an extremely small heat change, so it was necessary to use secondary enzymes to increase the overall enthalpy change. The reaction schemes used for lactose and sucrose are shown in Figs. 3.2.5.1 and 3.2.5.2., respectively:-

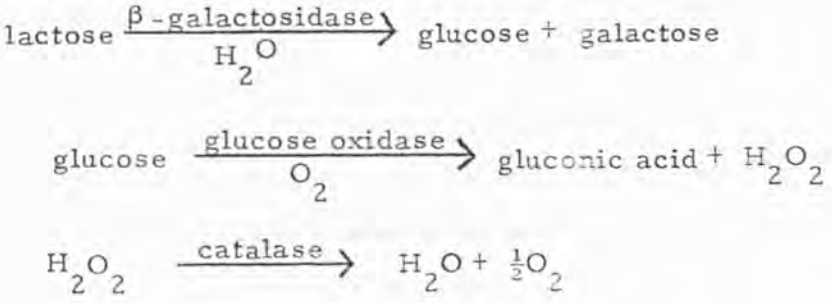


Fig. 3.2.5.1.

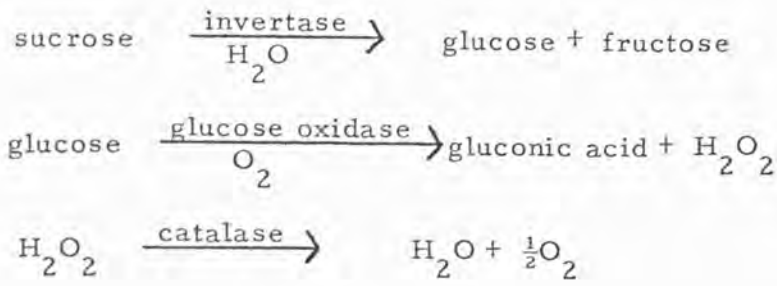


Fig. 3.2.5.2.

Reagents

Glucose oxidase (Type VII, from Aspergillus niger), Catalase (from Bovine liver), β-galactosidase (grade VIII, from E.Coli) and invertase (grade VII, from Bakers Yeast), were all purchased from Sigma Chemical Co.,U.K.
Lactose (Analar grade) and Sucrose (Analar grade) were obtained from B.D.H., Poole, U.K.
Glucose (puriss) was obtained from Koch-light Laboratories, Colnbrook, Bucks, U.K
All other reagents were obtained as listed in Chapter 2.

Immobilization procedure

β-glactosidase (E.C.3.2.1.23.), (1.5 mg, 1000 units) or invertase (E.C.3.2.1.26), (10 mg, 4000 units) were coupled to glutaraldehyde-activated alkylamine glass (0.5 g, 216.9 $m^2 g^{-1}$), along with glucose oxidase (E.C.1.1.3.4.), (20 mg, 4200 units) and catalase (E.C.1.11.1.6), (1 mg, 1850 units). All other experimental details were identical to those described in Chapter 3, Section 2.1, (analysis of penicillin G), except that the buffer solutions used were,for β-galactosidase/glucose oxidase/catalase 0.1M phosphate + 0.001M $MgCl_2$ + 0.001M dithiothreitol, at pH 6.5,wheras for invertase/glucose oxidase/

catalase, the buffer solution was composed of 0.1M phosphate + 0.01M EDTA, at pH 5.5.

Tables and plots of peak height (500 μ l pulse samples), and of steady-state displacement (continuous pulse) vs. conc. of lactose and sucrose, are shown in Tables 3.2.5.3. and 3.2.5.4., and Figs. 3.2.5.5. and 3.2.5.6., using the appropriate enzyme system and buffer in each case.

Correlation coefficients (r^2) were also reported.

The plots obtained for both sucrose and lactose are, not surprisingly, almost identical to those obtained in the previous section for glucose, as the reactions which produce virtually all the enthalpy change are identical in all three cases. Indeed, when identical concentrations of sucrose and glucose in the same buffer (0.1M phosphate + 0.01M EDTA, pH 5.8) were passed through the microcolumn containing glass immobilized invertase, glucose oxidase and catalase, identical displacements, within experimental uncertainty limits, were obtained, adding further evidence to the view previously expressed that hydrolysis of the disaccharide, per se, does not produce a large enthalpy change. Provided substrate concentrations remained within the linear region of the plots in Fig. 3.2.5.5. and 3.2.5.6. reproducibility and precision appeared normal ($\pm$ 2%), and quantitive stability of both sets of immobilized enzymes was observed for the period of study (approximately 1 week in each). Sensitivity appeared to be about an order of magnitude greater than that obtained by Mosbach and co-workers, probably because they failed to immobilize sufficient units of enzyme to bring about maximum conversion as suggested by the fact that their plots were linear up to about 3.5×10^{-3}M.

TABLE 3.2.5.3.

Table of peak height (500 μ l pulse) vs. conc. of sucrose or lactose, using the appropriate glass-immobilized enzymes, flow rate = 0.32 cm^3 min^{-1}

substrate conc./mol dm^{-3}	peak height of sucrose buffer = 0.1M phosphate + 0.01M EDTA, pH 5.5	peak height of lactose buffer = 0.1M phosphate + 0.001M $MgCl_2$ + 0.001M dithiothreitol pH = 6.5
0.050×10^{-3}	5.5	5.5
0.050×10^{-3}	6.0	5.5
0.100×10^{-3}	12.0	12.0
0.100×10^{-3}	13.0	12.0
0.200×10^{-3}	25.0	23.0
0.200×10^{-3}	24.5	24.0
0.400×10^{-3}	51.0	47.0
0.400×10^{-3}	49.5	49.0
0.800×10^{-3}	98.0	96.0
0.800×10^{-3}	99.5	97.5
1.600×10^{-3}	162.5	159.0
1.600×10^{-3}	165.0	157.5
3.200×10^{-3}	175.0	170.0
3.200×10^{-3}	177.5	170.0
6.400×10^{-3}	181.0	176.0
6.400×10^{-3}	182.5	177.5

r^2(0.050×10^{-3}M $\rightarrow$ 0.800×10^{-3}M = 0.999

0.05mM $\leqslant$ R $\leqslant$ 1.25mM

where R = analytically useful range

r^2(0.050×10^{-3}M $\rightarrow$ 0.800×10^{-3} M)= 0.999

0.05mM $\leqslant$ R $\leqslant$ 1.25mM

TABLE 3.2.5.4.

Table of steady-state displacement (continuous pulse) vs. conc. of sucrose or lactose, using the appropriate glass-immobilized enzymes, flow rate = 0.32 cm^3 min^{-1}

substrate conc./mol dm^{-3}	sucrose displacement buffer = 0.1M phosphate + 0.01M EDTA pH = 5.5	lactose displacement buffer = 0.1M phosphate + 0.001M dithiothreitol. pH 6.5
0.010×10^{-3}	4.5	4.0
0.020×10^{-3}	8.5	8.5
0.020×10^{-3}	9.5	8.0
0.040×10^{-3}	17.5	17.0
0.040×10^{-3}	18.0	16.5
0.080×10^{-3}	37.5	35.0
0.080×10^{-3}	36.5	35.0
0.160×10^{-3}	74.5	70.5
0.160×10^{-3}	72.5	71.5
0.320×10^{-3}	142.5	139.5
0.320×10^{-3}	144.0	141.0
0.640×10^{-3}	248.5	243.0
0.640×10^{-3}	251.5	244.5
1.280×10^{-3}	286.0	273.0
	$r^2(0.01 \times 10^{3}M \rightarrow 0.32 \times 10^{-3}M) = 0.999$	$r^2(0.01 \times 10^{-3}M \rightarrow 0.32 \times 10^{-3}M) = 0.999$
	$0.01mM \leqslant R \leqslant 0.4mM$	$0.01mM \leqslant R \leqslant 0.4mM$

where R = analytically useful range

FIG 3.255. Plot of peak ht. vs. conc. of sucrose (0.5 cm^3 pulse), as in table 3.253.

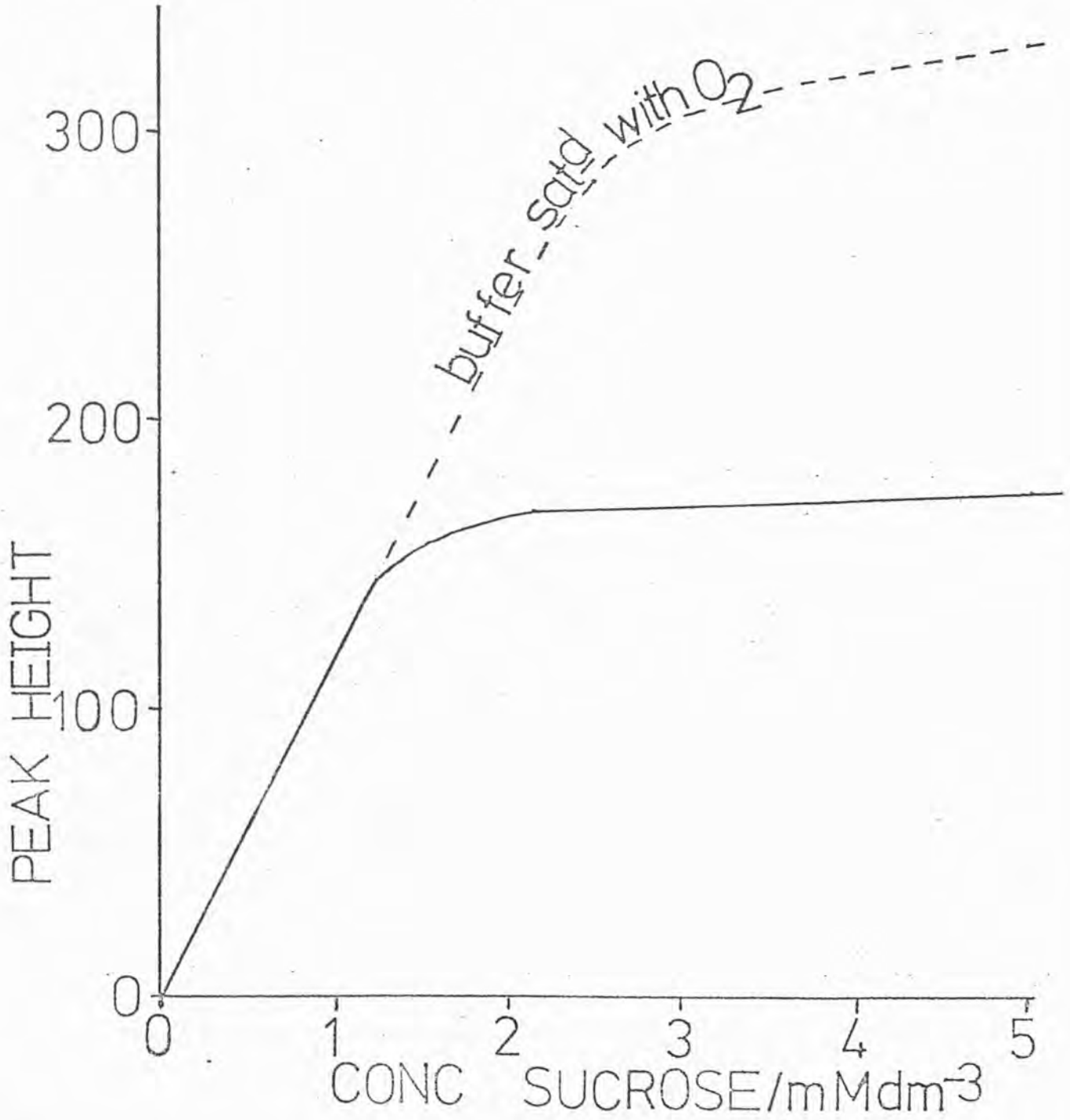
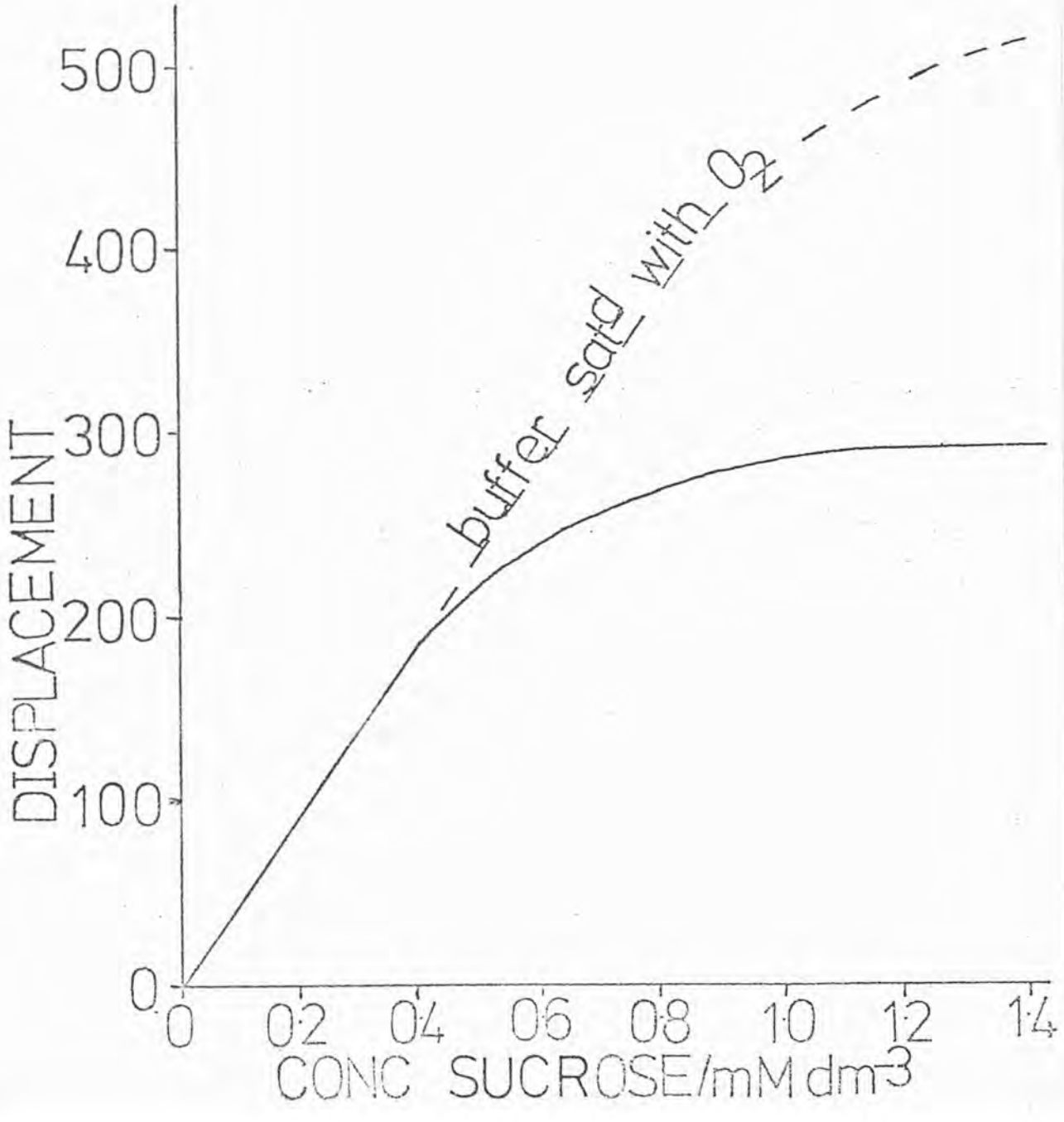


FIG. 3.2.5.6. Plot of steady-state displacement vs conc of sucrose, as in table 3.2.5.4.



The advantages and disadvantages inherent in sucrose and lactose determinations by such a technique are very similar to those of glucose as discussed in the previous section.

3.2.6. Determination of uric acid using glass-immobilized uricase and catalase in conjunction with the LKB 10700-1 microcalorimeter.

Determination of serum uric acid, a major end product of purine metabolism in man, is most helpful in the diagnosis of gout. Elevated levels of uric acid are found in patients with familial idiopathic hyperuricemia and in decreased renal function. Spectrometry of uric acid, based on its oxidation to allantoin by the enzyme uricase, requires a high-quality ultra violet spectrophotometer, and may still be subject to spectral interference. Coupling the reaction to a second enzyme and an oxidation-reduction step, to produce a readily detectable absorbance change in the visible part of the spectrum is prone to chemical interferences (203). By the use of the combination of immobilized uricase and catalase, in combination with microcalorimetry, it was hoped to avoid these problems.

Mosbach and co-workers (158), have very briefly reported a preliminary study of uric acid determinations in the range 1×10^{-3}M to 4×10^{-3}M, using their "enzyme thermistor" apparatus, but apart from this brief mention, no other reports appear in the literature on the determination of uric acid using the combination of immobilized enzymes and thermal methods.

The co-immobilization of uricase and catalase can be regarded as giving the reaction scheme shown in Fig. 3.2.6.1.

uric acid + O_2 + H_2O $\xrightarrow{\text{uricase}}$ allantoin + H_2O_2 + CO_2

H_2O_2 $\xrightarrow{\text{catalase}}$ H_2O + $\frac{1}{2}O_2$

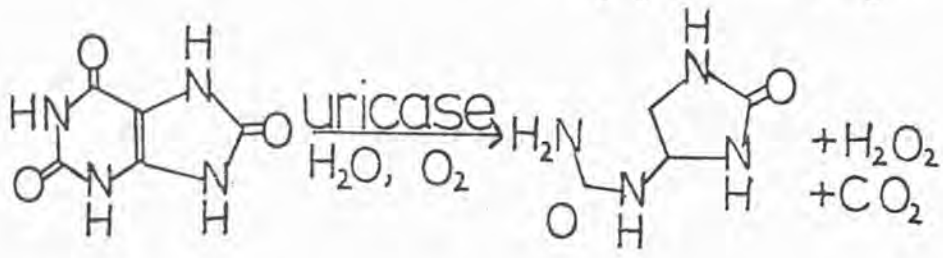


Fig. 3.2.6.1.

Reagents

Catalase (E.C.1.11.1.6, from bovine liver), and uric acid (99% pure) were obtained from Sigma Chemical Co., U.K. Uricase (E.C. 1.7.3.3., from hog liver) was obtained from Boehringer Mannheim Ltd, Lewes, Sussex, U.K.

All other reagents were obtained as listed in Chapter 2.

Immobilization procedure

Uricase (2 mg, 18 units) and catalase(1 mg, 1850 units) were coupled to glutaraldehyde-activated alkylamine glass (0.1 g, 216.9 $m^2 g^{-1}$ surface area), as described in Chapter 2, Section 1.2. All other experimental details were identical to those described in Chapter 3, Section 2.1. (analysis of penicillin G), except that, due to the low activity of the immobilized uricase obtained, a lower flow rate was used to prolong the contact time between substrate (uric acid) and immobilized enzyme, and so give greater conversions to product.

Tables and plots of peak height (500 μ l pulses) and steady-state displacements (continuous pulse) vs. conc. of uric acid are shown in Tables 3.2.6.2. and 3.2.6.3., and Figs. 3.2.6.4. and 3.2.6.5., using a buffer of 0.1M THAM + 0.01M EDTA, at pH 8.0. Correlation coefficients (r^2) were calculated in the usual way.

The shape of the plots in Figs. 3.2.6.4. and 3.2.6.5. show a much more gentle curve, extending over a much greater concentration range, than those obtained for glucose, using a similar dual-substrate natured enzyme (Figs. 3.2.4.4. and 3.2.4.5.). The interpretation placed upon this is that is was not possible to immobilize enough enzyme on to the column to obtain 100% conversion of substrate to product, and that this effect was more pronounced at higher concentrations. The relative expense of uricase (approx. £6 per mg), renders immobilization of a greater activity of enzyme less attractive. Nonetheless, the sensitivity obtained was well over an order of magnitude better than that obtained by Mosbach and co-workers (158).

The within-day reproducibility of identical samples was very good ($\pm$ 2%), but, presumably because no excess of enzyme was employed, as in the other cases mentioned, the apparent stability of the immobilized enzyme was much poorer, with an estimated half-life of about a week, as shown by the peak-height obtained by passage of a 500 μ l pulse of 0.4×10^{-3} uric acid through the column.

TABLE 3.2.6.2.

Table of peak height (500 μ l pulse) vs. conc. of uric acid in THAM buffer, pH 8.0. flow rate = 0.072 $cm^3 min^{-1}$

Conc. of uric acid/mol dm^{-3}	peak height (units)
0.050×10^{-3}	4.0
0.050×10^{-3}	4.5
0.100×10^{-3}	8.0
0.100×10^{-3}	7.5
0.200×10^{-3}	14.5
0.200×10^{-3}	14.0
0.400×10^{-3}	26.5
0.400×10^{-3}	25.5
0.800×10^{-3}	48.5
0.800×10^{-3}	47.0
1.600×10^{-3}	70.5
1.600×10^{-3}	72.0

$r^2 = 0.972$

TABLE 3.2.6.3.

Table of steady-state displacement (continuous pulse) vs. conc. of uric acid in THAM buffer, pH 8.0, flow rate = 0.072 $cm^3 min^{-1}$

Conc. of uric acid/mol dm^{-3}	peak height (units)
0.025×10^{-3}	3.0
0.050×10^{-3}	6.5
0.100×10^{-3}	11.0
0.100×10^{-3}	11.0
0.200×10^{-3}	20.0
0.200×10^{-3}	19.0
0.400×10^{-3}	34.0
0.400×10^{-3}	35.0
0.800×10^{-3}	62.0
0.800×10^{-3}	60.5
1.600×10^{-3}	101.5
1.600×10^{-3}	103.0

$r^2 = 0.990$

FIG. 3.2.6.4. Plot of peak ht. vs. conc of uric acid (0·5 cm^3 pulse), as in table 3.2.6.2.

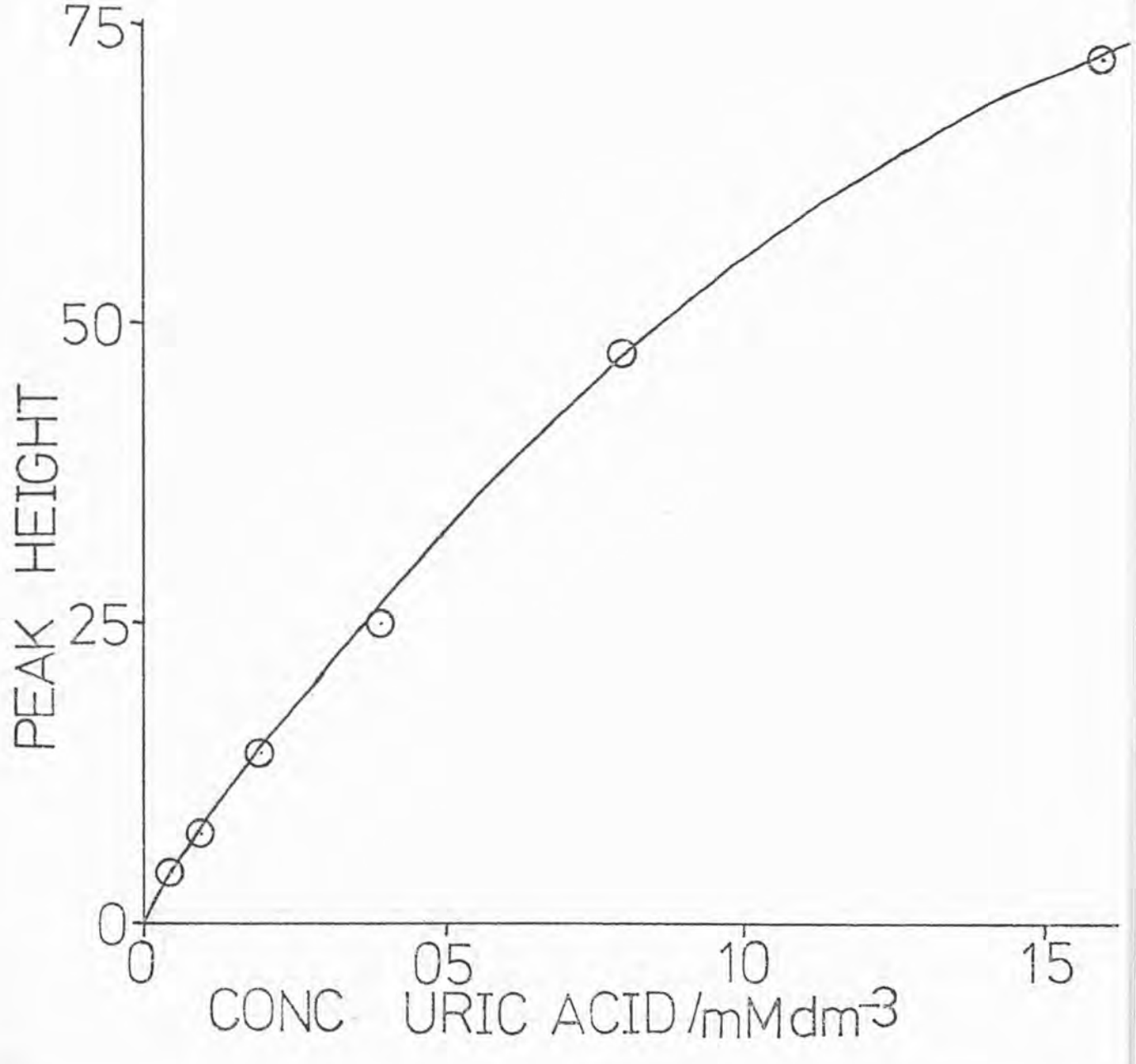
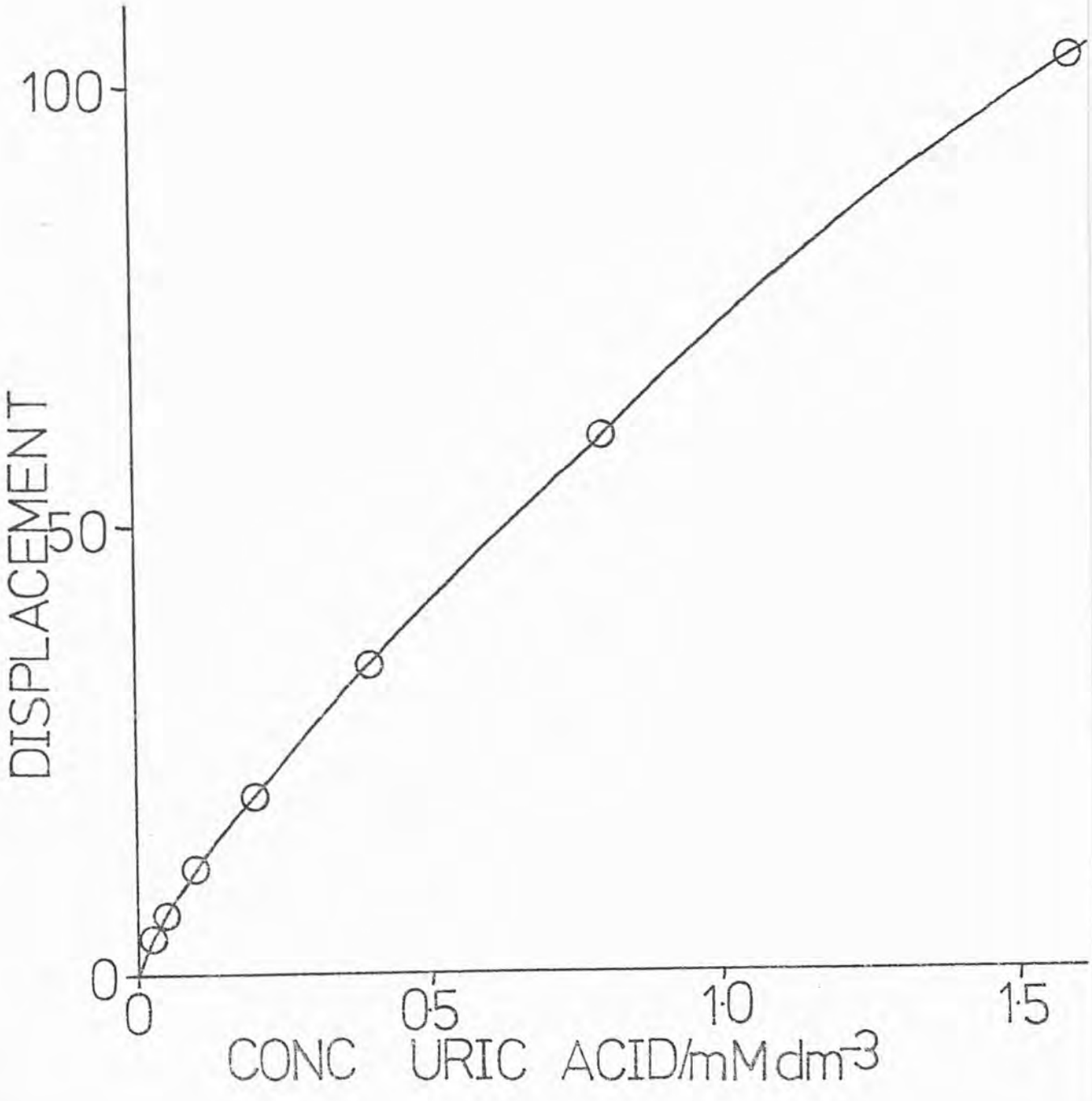


FIG. 3.2.6.5. Plot of steady-state displacement vs. conc. of uric acid, as in table 3.2.6.3.



Section 3.3.

Determination of enzyme substrate using home-made apparatus, with a thermistor as heat sensor

3.3.1. Design, construction and operation of a "semi-adiabatic" flow microcalorimeter using a thermistor as heat sensor.

The application of immobilized enzymes and essentially isothermal (or'heat-leak" type)microcalorimetry in analysis has been described in Chapter 3, Section 2; however, the time required for one measurement (15 min), and the relative complexity and expense of the equipment suggested it might be interesting to look at some alternative designs. With the advent of very small, and extremely sensitive, semi-conductor devices called thermistors, it has become possible to construct very inexpensive measuring systems based on the measurement of a temperature rise by this thermistor, enclosed in an environment made as adiabatic as practical.

Whereas with the LKB 10700-1 assembly, the principle was to conduct any heat produced away from the reaction microcolumn, and over a series of thermopiles, in this work attempts were made to retain the heat produced, to maximize the temperature rise. Similar devices, often termed "enzyme-thermistors" have been used with great success by Mosbach and co-workers (157-161, 168, 170, 175-180) and Bowers and co-workers (164, 166, 167, 169), and it was hoped to use this type of instrumentation to extend their work, and to review systems for which the LKB microcalorimeter was unsuitable. Two such designs are depicted in Figs. 3.3.1.1. and 3.3.1.2. In each case the apparatus was almost completely immersed in a constant temperature water bath (to about 2 cm from the top). Bath temperature was maintained at a nominal 25^{o}C by a variable

power heater, constructed in the laboratory; the circuitry is shown in Fig. 3. 3. 1. 3.

Both pieces of apparatus were constructed at the Laboratory of the Government Chemist, Cornwall House, Stamford Street, London S E 1.

The apparatus illustrated in Fig. 3. 3. 1. 1. illustrates one version of the "enzyme thermistor"- type apparatus used, consisting of four perspex sections, A, B, C and D, each of which was threaded so as to screw together to form a watertight seal, with the aid of a rubber O-ring.

After immersion in the constant temperature water-bath, solution was pumped by a peristaltic pump (LKB 10200 or LKB 4912A) via a threaded connector in B, through a gold plated silver heat exchange coil, situated in the secondary water-bath, A. The purpose of the secondary water bath was to smooth out any short-term temperature variations in the main bath, and its use, in conjunction with the highly efficient heat-exchange coil , makes it unnecessary to keep the solutions thermostatted. From the secondary water bath, solution flowed, via another connector in B, to a length of nylon tubing situated in chamber C, on to which enzymes could be immobilized, if desired, as described in Chapter 2, Section 2. 1. The end of the nylon tube was connected, via a piece of silicone tubing and appropriately sized teflon adaptors, to a glass T-piece, into which a thermistor (R.S. thermistor type GL23, stock no. 151-029, obtained from RS Components Ltd., ca. 2KΩ at 20^{o}C), was bonded with a water-tight seal using epoxy resin. After flowing over the thermistor tip, solution flowed to waste via a piece of tubing leaving the apparatus through a small hole in D.

Thermistor leads (contained in a coaxial piece of silicone tubing), left the apparatus via a threaded, watertight outlet. The thermistor resistance variation was registered as the disbalance potential on a Wheatstone bridge circuit, amplified, and fed to a chart recorder.

The apparatus illustrated in Fig. 3.3.1.2. was constructed entirely of glass. In this case solution was pumped from a secondary water-bath (Sections A and B) from the apparatus described earlier, along a piece of nylon tubing, which entered the apparatus via a watertight suba-seal connection in one of the side arms and eventually connected with the glass T-piece containing the thermistor, all connexions and seals being as previously described. Again, enzyme could be immobilized on to the inside of this section of tubing, if so desired. The thermistor leads and waste solution left the apparatus via another side-arm, protruding just above water-level.

One slight difference in the two is that much of the apparatus illustrated in Fig. 3.3.1.2. is double-walled. Ideally, the glass T-piece and thermistor should never touch the inside of the apparatus, but often (even when some cotton wool lagging was present), this was observed, leading to extra baseline noise as the short-term temperature variations in the main water bath were conducted, via the apparatus wall, to the thermistor. In this respect the double-walled glass apparatus fared better, but the perspex assembly, being considerably less bulky, was more convenient to use.

Problems encountered

The excessively high baseline noise originally encountered could be traced back to two sources, thermal fluctuations in the main water bath, and pulsing from the peristaltic pump.

A=secondary water-bath
a=silver/gold heat exchange coil
b=nylon tube
c=thermistor(set in glass T-piece)

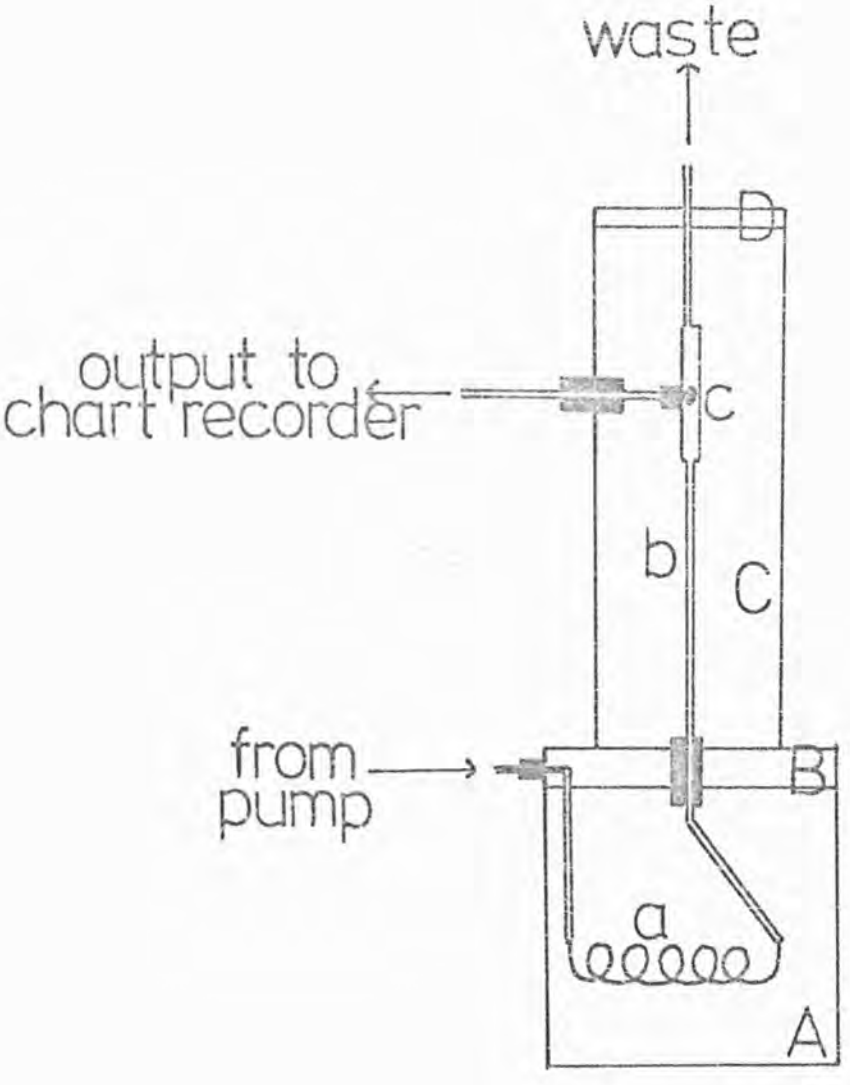


FIG. 3.3.1.1.

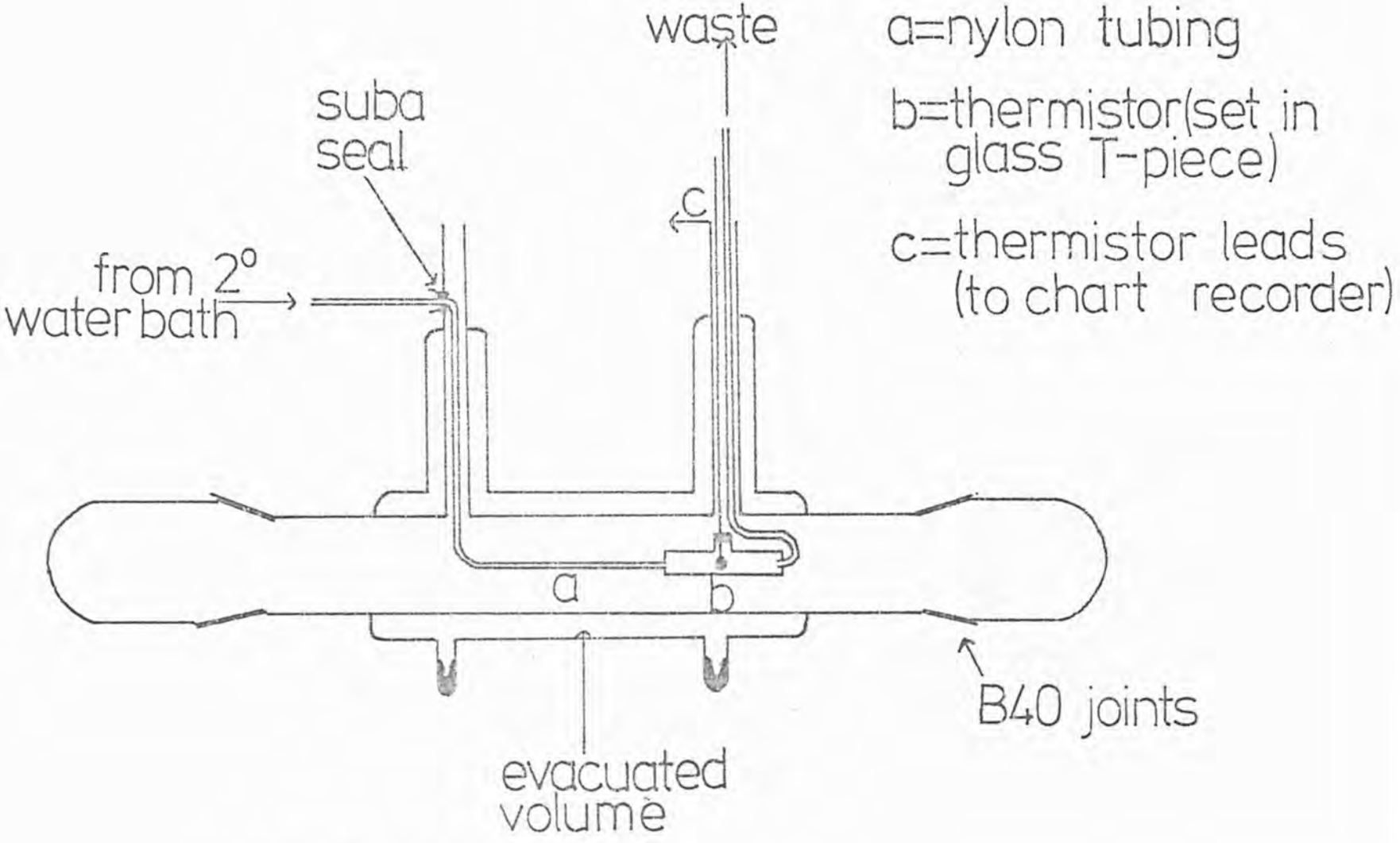
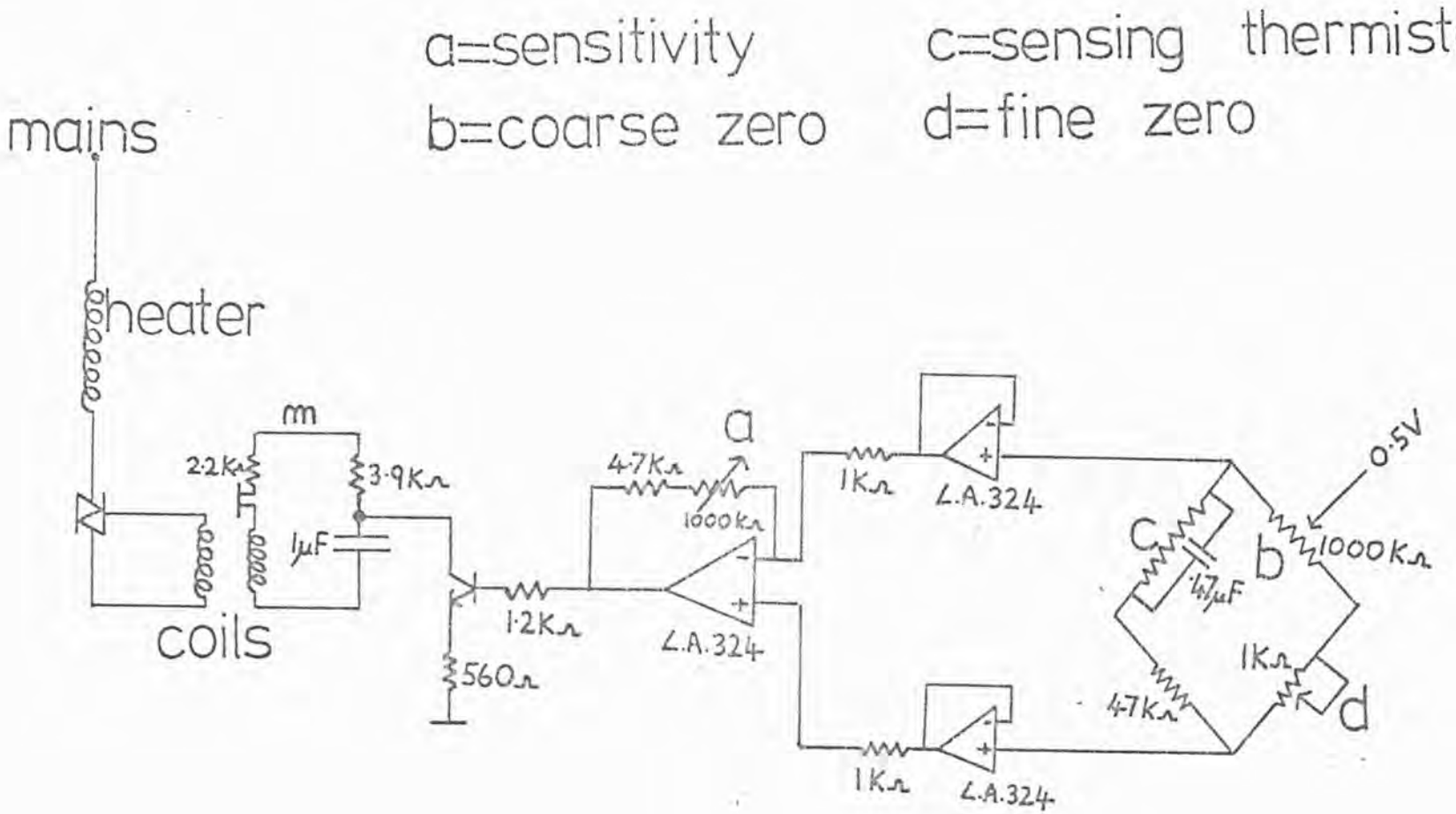

waste
a=nylon tubing
b=thermistor(set in glass T-piece)
c=thermistor leads (to chart recorder)
suba seal
c
from 2° water bath
a
b
B40 joints
evacuated volume

FIG. 3.3.1.2.

FIG 3313 Circuit diagram for variable power heater (water bath constant temperature controller)



a) Thermal fluctuations

Even the incorporation of the secondary water bath into the design of both calorimeters could only improve, not alleviate, the problem. Whenever the main heater cut in, the increase in bath temperature (approx. 0.12°C) was picked up by the thermistor, resulting in a "saw-toothed" baseline. The problem was overcome by incorporating a variable power heater into the main water bath. Although this was sometimes slow to respond to temperature changes, and could not wholly compensate for drastic changes in room temperature (e.g. going from a hot day to a cool evening), changes in temperature were much slower, and, in the short-term at least, the baseline remained reasonably steady.

b) Pulsing noise

This proved to be very difficult to overcome. With the solutions flowing directly over the tip of the heat detector (the thermistor), this method appears much more susceptible to a large baseline noise from pulsing, than in the isothermal LKB assembly. Incorporation of pulse suppressors showed only a marginal improvement, and the answer appears to be an elaborate, non-pulsing peristaltic, or infusion, pump.

3.3.2. Determination of urea using glass-immobilized urease, and a thermistor as the heat-sensing device.

This analytical technique which is very similar to that described by Mosbach and co-workers (157, 168), and by Bowers et al (167), was not studied in depth, as it had already been reported in considerable detail by both groups. The main purpose was to compare the efficiency of the "home-made" apparatus shown in Figs. 3.3.1.1. and 3.3.1.2. with that of other workers, and to the LKB 10700-1 assembly described in Chaper 3, Section 1.1.

Reagents

Urease (Type VII, from jack beans) was obtained from Sigma Chemical Co. U.K.

Urea (99+ % pure) was obtained from B.D.H. Poole, U.K.

All other reagents were obtained as listed in Chapter 2.

Immobilization procedure

Urease (3.6 mg, 250 units), was coupled to glutaraldehyde-activated alkylamine glass (0.2 g, 216.9 $m^2 g^{-1}$ surface area), as described in Chapter 2, Section 1.2.

After immobilization, the glass-bound enzyme was made into a slurry, using the minimum volume of buffer (0.1M phosphate + 0.01M EDTA, at pH 7.0), and as much packed into the T-piece containing the thermistor as possible, by injecting the slurry into the T-piece using a Pasteur pipette.

The glass-immobilized enzyme was confined in the T-piece, in the vicinity of the thermistor tip, by means of double layers of specially cut pieces of filter paper, positioned at the inlet and outlet, as shown in Fig. 3.3.2.1.

500 μ l pulses of urea were introduced into the flowing buffer stream (0.5 $cm^3 min^{-1}$) via the Altex injection valve in the usual manner, and a table and plot of peack height vs. concentration of urea are given in Table 3.3.2.2. and Fig. 3.3.2.3.

The correlation coefficient, r^2, was again calculated using the appropriate programme on a Hewlett-Packard HP65 programmable calculator.

TABLE 3.3.2.2.

Table of peak heights (500 μ l pulse) vs. concentration of urea in 0.1M phosphate (pH 7.0) flow rate = 0.50 cm^3 min^{-1}

conc. of urea/mol dm^{-3}	peak height (units)
0.625×10^{-3}	4.0
1.250×10^{-3}	10.0
1.250×10^{-3}	8.0
1.250×10^{-3}	7.0
2.500×10^{-3}	20.0
2.500×10^{-3}	23.0
5.000×10^{-3}	39.0
5.000×10^{-3}	45.0
5.000×10^{-3}	41.0
10.000×10^{-3}	84.0
10.000×10^{-3}	86.0
20.000×10^{-3}	173.0
20.000×10^{-3}	178.0
25.000×10^{-3}	209.0
25.000×10^{-3}	216.0
40.000×10^{-3}	348.0
40.000×10^{-3}	352.0
50.000×10^{-3}	436.0
50.000×10^{-3}	442.0

r^2 (complete range) = 0.999

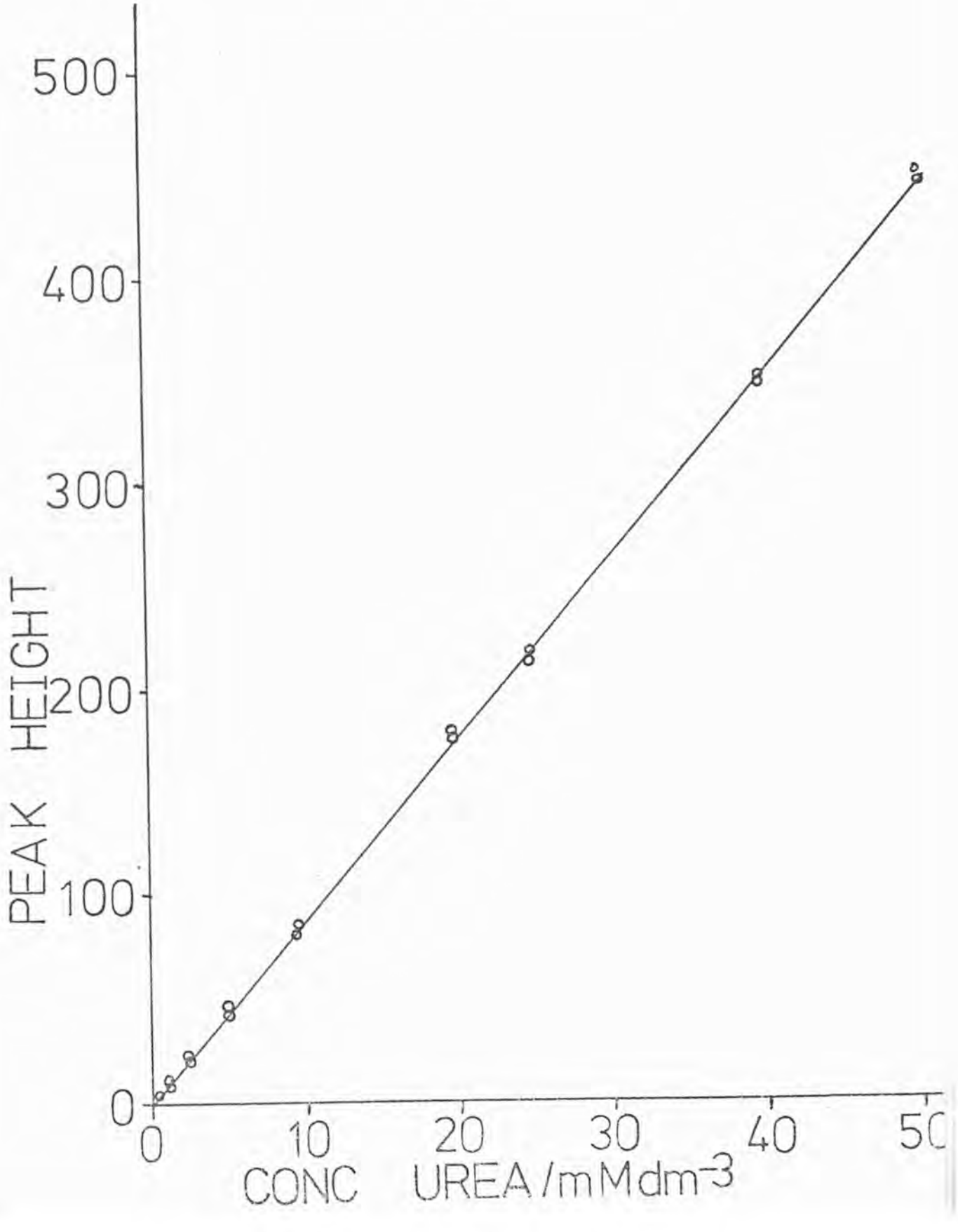
r^2 (up to and including 10×10^{-3}M) = 0.995

r^2 (up to and including 5×10^{-3}M) = 0.984

$0.625\text{mM} \leqslant R \leqslant 50\text{ mM}$

where R = analytically useful range

FIG. 3.3.2.3. Plot of peak ht. vs. conc of urea (0·5cm^3 pulse), as in table 3.3.2.2.



Although over the complete range of urea concentrations studied, the correlation coefficient appears as 0.999, the value is evidently distorted by the lower percentage error apparent at higher urea concentrations. Over the range 0 to 10×10^{-3}M, the correlation coefficient is only 0.995 and over the range 0 to 5×10^{-3}M only 0.984. Inspection of Table 3.3.2.2. shows that at low urea concentrations, the reproducibility is nowhere near the $\pm$ 2% achieved using the LKB assembly.

A large part of this uncertainty is undoubtedly due to the poor quality of baseline achieved in this study, due primarily to the problems already discussed.

One advantage of this method of assay is that, due to the much lower time-constant of the thermistor (compared to the LKB microcalorimeter detected system), 15 analyses per hour could be achieved. Presumably this could be increased still further, at the expense of sensitivity, by using sample volumes less than 500 μ l. The sensitivity obtained was similar to that obtained by Bowers et al (167), but was significantly less than both that obtained by Mosbach and co-workers (157, 168), and the LKB assembly, described in Chapter 3, Section 2.2.

It is felt that the reasons for the poor sensitivity are essentially the same as those that cause the poor reproducibility i.e., problems associated with keeping the water-bath temperature constant, and eliminating the pulsing noise and which prevents higher amplification, rather than poor design (e.g. low adiabaticity) of the apparatus. In any future work, it is strongly recommended that a non-pulsing pump of the highest quality, and a thermostatted water bath capable of providing a constant

temperature environment stable to at least 0.01°C, or better, should be employed.

3.3.3. Determination of urea, using nylon-tube immobilized urease, with a thermistor as heat sensor

Urea analysis in whole blood has tended to present many problems. Its high viscosity and tendency to coagulate has made the use of immobilized enzyme columns, as previously described, difficult. Additionally, the intense absorption of haemoglobin has ruled out many potential techniques based on spectrophotometers, unless exhaustive dialysis of the sample was undertaken to separate out high molecular weight proteins and haemoglobin.

Ammonium-ion sensitive electrodes incorporating urease entrapped with a dialysis membrane have proved unreliable, due to interferences by Na^+ and K^+ (85, 86).

It was hoped that the combination of a thermal detector (a thermistor) with urease bound to the inside of a nylon tube (through which it is known whole blood will pass without coagulation) would provide a method for the determination of blood urea, without addition of reagents, or prior deproteinization.

From the outset, it was obvious that additional problems would be associated with this design of immobilized enzyme reactor. Such problems include the combination of lower enzyme loadings, due to the low surface area of the inside of the nylon tube (typically $6.3 \times 10^{-3} m^2$ per metre of length, compared to $13.3 - 216.9\ m^2\ g^{-1}$ for controlled porosity glass), and the lower mass transfer rates of substrate to the enzyme on the surface of the nylon tube, due to the greater distance that substrate had to diffuse from the bulk solution, compared with finely divided glass.

Combination of these effects required the use of relatively long lengths of enzyme-bound nylon tubing to achieve reasonable extents of reaction. This gave rise to yet another problem associated only with thermal methods of detection, namely heat loss due to incomplete adiabaticity before the temperature rise could be sensed by the thermistor. Consequently a balance had to be struck between these effects, and it appeared that, at the flow rates used (0.5 $cm^3 min^{-1}$), a tube length of about 33 cm gave about optimum results in this study.

Reagents

Urease (Type VII, from jack beans) was obtained from Sigma Chemical Co., U.K.

Urea (99+ % pure), was obtained from B.D.H., Poole, U K.

All other reagents were obtained as listed in Chapter 2.

Immobilization procedure

Three different batches of urease (3.6 mg 250 units) were each coupled to one metre of activated nylon tubing (1mm internal diameter), prepared as described in Chapter 2, Section 2.1.

After immobilization, each enzyme-bound nylon tube was cut into three equal lengths, of ca. 0.33 m each (which preliminary studies had indicated may have been near the optimum length), and a portion of one of the tubes inserted into the inlet tubing immediately preceding the T-piece containing the thermistor, by means of silicone rubber connectors. The others were filled with buffer (0.1M phosphate, + 0.01M EDTA + 0.001M dithiothreitol, pH 7.0), and stored at ca. + 4°C, until required for use.

One by one, each of the tubes were inserted into the apparatus and its performance monitored.

The following generalizations soon became apparent:-

(a) The enzyme activity, as indicated by the peak height for a given concentration of urea, remained constant, within the limits of experimental uncertainty, for each of the 3 lengths cut from the same tube, showing that the enzyme was evenly bound throughout the length of the tube.

(b) The enzyme activities of tubes cut from different one-metre lengths of enzyme-bound nylon tubing were not necessarily the same.

(c) The half-life of the enzyme tubes, as measured by the peak height obtained during passage of a 500 μ l pulse of 32×10^{-3}M urea appeared to be only 24-48 h.at 25^{o}C, but stability whilst kept refrigerated at + 4^{o}C appeared much better.

The problem of the short half-lives of the enzyme-bound tubes could, it was felt, be overcome by immobilization of enzyme to a longer original length of tube (say, 3 or 4 metres), and inserting a new portion of this tube into the apparatus every day.

The problem of the different activities shown by different tubes, despite their apparently identical preparation, suggested that some as yet unforeseen factor exists (e.g. trace contamination of the ether or methylene chloride by water), but no serious attempt was made to investigate this point further.

500 μ l pulses of urea were introduced into the following buffer stream (0.1M phosphate + 0.01M EDTA, at pH 7.0) in the usual manner, and a table and plot of peak heights vs. concentration of urea are given in Table 3.3.3.1 and Fig. 3.3.3.2 respectively, for the most active enzyme tube.

TABLE 3.3.3.1.

Table of peak heights (500 μ l pulse) vs. concentration of urea, using nylon-tube immobilized enzymes (tube length = 0.33 m) is 0.1M phosphate buffer, pH 7.0, flow rate 0.5 $cm^3 min^{-1}$

conc. of urea/mol. dm^{-3}	peak height (units)
4.0×10^{-3}	3.0
4.0×10^{-3}	3.5
4.0×10^{-3}	4.5
8.0×10^{-3}	8 0
8.0×10^{-3}	7.5
8.0×10^{-3}	8.5
10.0×10^{-3}	9.5
10.0×10^{-3}	11.0
10.0×10^{-3}	10.5
16.0×10^{-3}	14.5
16.0×10^{-3}	15.5
20.0×10^{-3}	18.5
20.0×10^{-3}	20.0
32.0×10^{-3}	31.5
32.0×10^{-3}	32.0

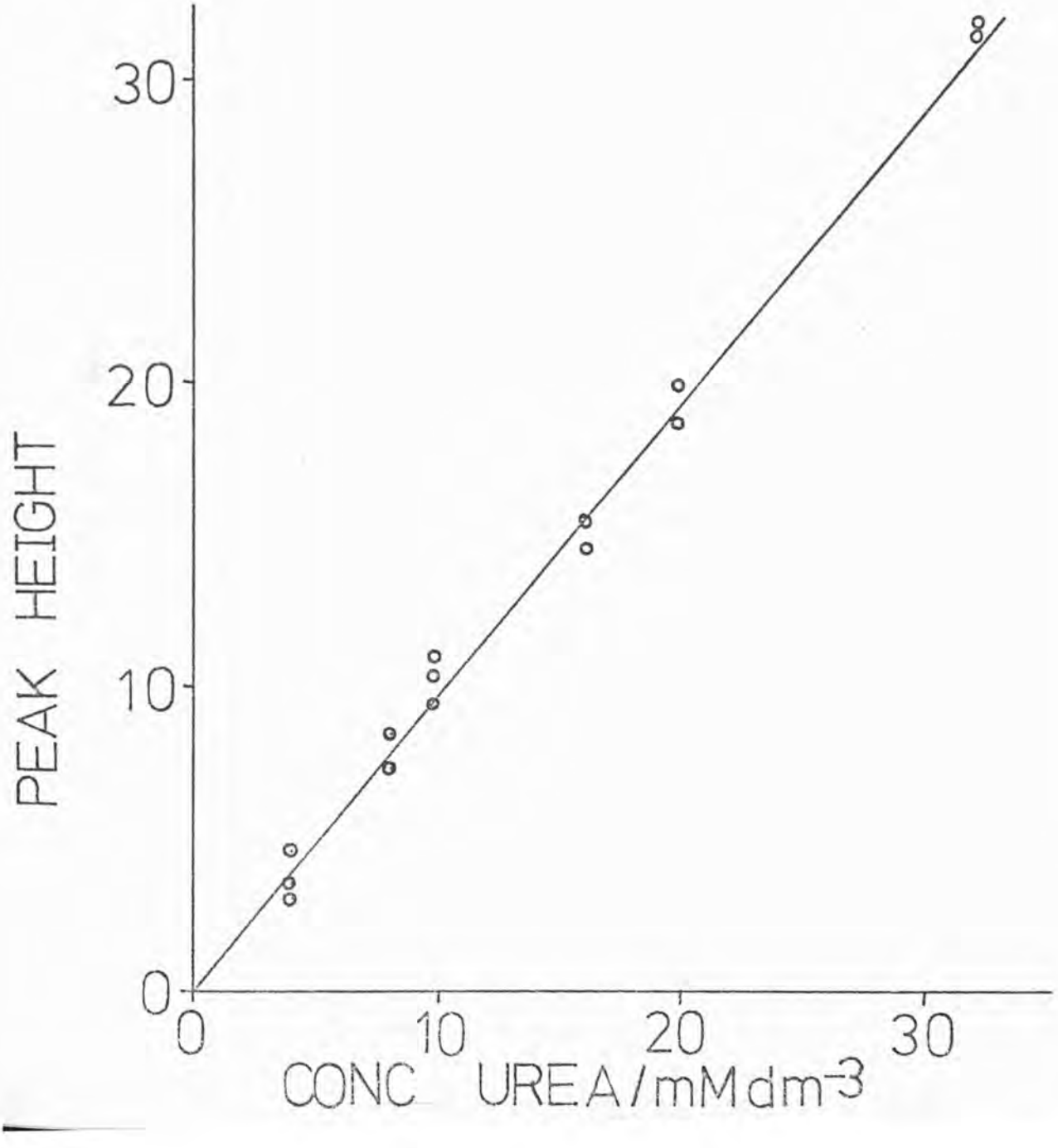
$r^2 = 0.994$

r^2 (up to and including 10×10^{-3}M) = 0.960

$4\text{mM} \leqslant R \leqslant 32\text{mM}$

where R = analytically useful range

FIG. 3.3.3.2. Plot of peak ht. vs. urea conc.(0.5cm^3 pulse), as in table 3.3.3.1.



From the correlation coefficients, especially that for the lower concentration ranges, and from inspection of Table 3.3.3.1., it is again apparent that the precision and reproducibility of results is rather poorer than those obtained using the LKB assembly, and that the sensitivity of the techniques is also disappointing. Reasons for the poor precision and reproducibility have already been discussed in the previous section (3.3.2.), and the explanation of the low sensitivity obtained was also discussed earlier.

The combination of these problems would seem to make this technique of analysis impractical for routine urea determination in whole blood, and such an attempt was not made. However, as the combination of a thermal detector and tube-immobilized enzyme has not yet (June 1980), been reported in the literature, this technique may be of academic interest.

A comparison of the three methods used for urea determinations (LKB assembly, thermistor/glass-immobilized urease, and thermistor/nylon tube immobilized urease is shown in Table 3.3.3.3.

TABLE 3.3.3.3.

Comparison of isothermal (LKB) and adiabatic (thermistor) methods in the analysis of a 0.5 cm^3 pulse of urea, using immobilized urease.

Set-up	Runs per hour	Minimum mass of urea measurable	Minimum conc. of urea measurable	Resolution
LKB 10700 (Isothermal)	4	1.8×10^{-6} g	(60 μ M)	$\pm 0.1 \times 10^{-6}$ g
Thermistor (packed column)	15	18×10^{-6} g	(0.6 mM)	$\pm 1.8 \times 10^{-6}$ g
Thermistor (nylon tube)	15	120×10^{-6} g	(4 mM)	$\pm 12 \times 10^{-6}$ g

3.3.4. Some attempts to produce an enzyme-bound thermistor capable of a rapid and reproducible analysis of substrate, under continuous-flow conditions

Several authors have applied microcalorimetric techniques to the determination of chemical and biochemical species, using a column of finely divided material on to which an enzyme has been immobilized. This technique is limited to solutions of fairly low viscosity; high viscosity solutions either cause unacceptably high pressure drops across the column, or else tend to clog the column (e.g. whole blood).

One possible answer could be to immobilize the enzyme on to the inside of suitable hollow fibres (e.g. nylon tubes), but, for a variety of reasons, this system gives poor sensitivity compared to packed-bed type reactors (see Chapter 3. Section 3.3.)

A possible alternative is to immobilize the enzyme directly on to the sensing device (in this case, a thermistor), and simply incorporate this into the flow-system. With this in mind, several methods have been attempted to immobilize enzymes on thermistors. . [N.T.C. (type F), 2 kΩ thermistors, obtained from I.T.T.].

Although it was realised that very little enzyme could actually be immobilized on to the thermistor tip, due to its extremely low surface area, it was hoped that the very small distance over which the resulting heat would have to be conducted might compensate.

Two papers (162, 163) have appeared in the literature concerned with the determination of substrate using such an enzyme-bound thermistor, but each determination has been in batch reactors,

using discrete samples.

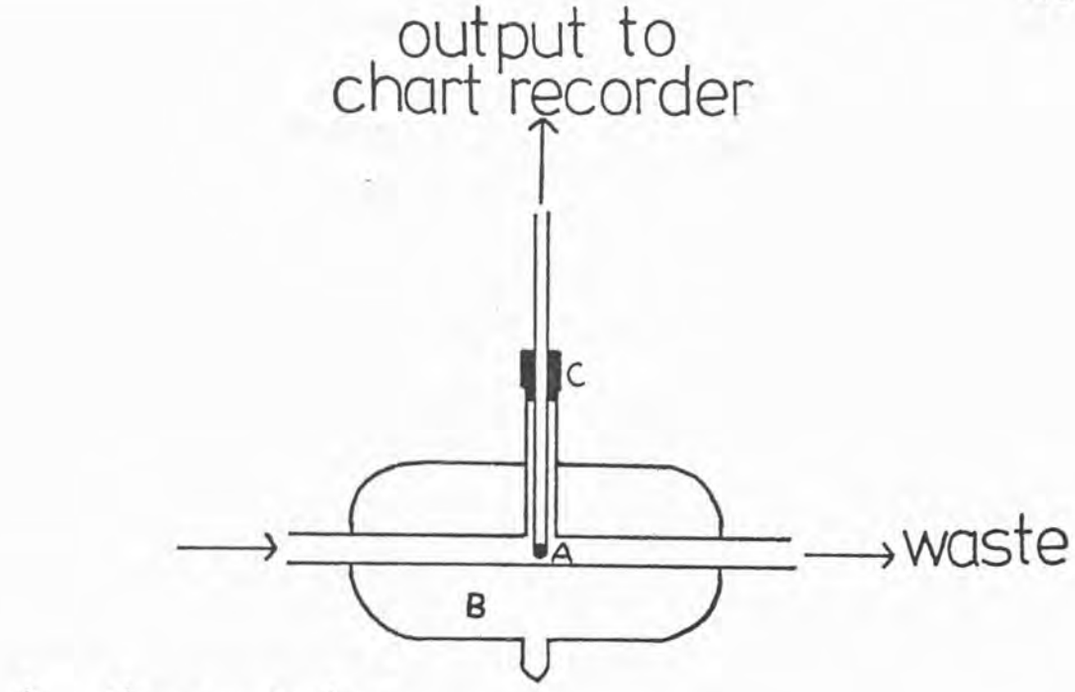
Attempts have been made to adapt both methods, and to devise other methods for substrate analysis using a continuous-flow method.

Apparatus

The schematic representation of the apparatus has been shown in earlier reports, as has the construction of the constant-temperature water-bath.

A modified "semi-adiabatic" flow-cell was used (Fig. 3.3.4.1.), which was much more compact than any previous design. The area around the glass T-piece was surrounded by a glass bulb, which could be evacuated, and sealed off, when required. The enzyme-bound thermistor (which had previously been squeezed through a small hole bored into an appropriately sized suba-seal), could easily be introduced into the flow-stream. The suba-seal formed a water-tight connexion at the top of the T-piece, as shown in Fig. 3.3.4.1. When desired, the apparatus could be used in conjunction with a secondary water bath (Sections A and B of Fig. 3.3.1.1.) to improve thermal equilibration of sample and buffer.

Baseline noise and temperature fluctuations appeared to be approximately the same as in other designs; however, its decreased size made this design much more convenient to use:



A= thermistor
B= evacuated region
C= water-tight suba-seal

Fig. 3.3.4.1.

Methods of enzyme immobilization to the thermistor tip

1. Glass-immobilised trypsin, urease or catalase, prepared as described in Chapter 2, Section 1.2., were stuck to the thermistor tip by use of a suitable adhesive as in (163). Little or no enthalpy change was recorded, even at substrate concentrations up to 50mM, using benzoyl-L-arginine ethyl ester (BAEE), urea and hydrogen peroxide respectively as substrates.

   That some enzyme was still active was shown by the observation of tiny bubbles of (presumably) oxygen, when the catalase/H_2O_2 system was employed. However, no significant enthalpy peak was registered on the chart recorder.

2. Attempts to stick powdered urease directly on to the thermistor tip using either Evostik or UHU glue also

failed to yield an enzyme-bound thermistor capable of generation of any signal at 50mM urea concentrations.

3. An attempt was made to functionalize the glass-coating of the thermistor, by a method identical to that described for the functionalization of controlled porosity glass described in Chapter 2, Section 1.2. This method failed to give evidence of any catalase or urease activity, regardless of whether or not the thermistor had been previously etched with 40% HF for 1 min. Using this method, neither an enthalpy change, nor bubbles of oxygen on the thermistor surface, were observed for the catalase/H_2O_2 system.

4. An attempt was made to prepare a "functionalized glue", by mixing Araldite epoxy adhesive with twice its volume of ethylene diamine, and allowing a portion of the mixture to set on the thermistor surface. Once set, the free amine groups present could be converted to aldehyde groups by reaction with excess glutaraldehyde, and the enzyme coupled to this derivative, by the usual techniques.

   However, as with previous methods, no evidence of enzyme activity could be obtained using the enzymes trypsin, urease or catalase.

5. An attempt was made to "cross-link" the enzyme on to the thermistor, either by placing one drop of a 10 mg cm^{-3} solution of enzyme in 2.5% glutaraldehyde solution at pH 7.0 on to the thermistor tip and allowing to set, or one drop of a solution of 5 mg cm^{-3} of enzyme plus 20 mg cm^{-3} of human serum albumin (HSA) in 2.5% glutaraldehyde solution at pH 7.0 on the thermistor tip, as in (162).

The strength of adhesion of such a drop to the thermistor surface was poor, but appeared to improve slightly if the thermistor had previously been etched with 40% HF for 1 min, presumably due to the higher surface area of the latter.

However, little or no thermal response was observed using 50 mM substrate with the enzymes urease or catalase, although oxygen bubbles were apparent in the case of the latter, indicating some enzyme activity.

6. The enzyme was "set" on to the thermistor by making up a solution of 20 mg cm^{-3} enzyme in 80 mg cm^{-3} gelatin, as previously described in the production of an enzyme electrode (see Chapter 2, Section 2.2.).

The enzyme/gelatin film was found to be more durable if the gelatin was not just simply dissolved in warm water, but also subsequently maintained at 50-60°C for at least 30 min.

The gelatin solution was then cooled to 25°C before addition of the enzyme. The thermistor was dipped two or three times into this solution, which was then allowed to set. Again, using gelatin-set urease or catalase, little or no thermal response was registered using a substrate concentration of 50mM, although bubbles of oxygen were again apparent in the catalase/H_2O_2 system, indicating some enzyme activity. Using the enzyme trypsin, it was found to be impossible to set the gelatin/enzyme solution, presumably due to the fact that trypsin hydrolyses proteins at the carboxylic ends of lysine and arginine. Because gelatin is itself a protein, this hydrolysis affects

its structure, and presumably also its setting characteristics.

Discussion and conclusions

The inability to obtain meaningful results using any method of immobilization of the enzyme is probably due to a combination of the inability to immobilize more than a very small amount of enzyme to the thermistor tip, relative to that which can be immobilized to the very high surface area controlled porosity glass, and to the effects of the diffusion of a large proportion of the heat generated away from the thermistor tip, towards the bulk solution. The "forced convection" of another large proportion of the total amount of heat generated away from the thermistor tip, in the same direction as the flowing stream is another likely factor.

It has been shown that, at least in the case of immobilization of catalase by methods 1, 5 or 6, there is some retention of enzyme activity, as shown by the appearance of oxygen bubbles on the thermistor tip when hydrogen peroxide was passed over the thermistor.

Decreasing the flow rate does not appear to increase dramatically the sensitivity of the method, indicating that the forced convection of the flowing liquid stream is not per se the major cause of insensitivity; however, there appears to be some evidence that use of a "stopped-flow" method, with the appropriate "blank" correction (see earlier report on enzyme electrodes), may produce a slightly more sensitive method, indicating that loss of heat by a forced-convection method may be a contributory factor. Tran-Minh and Vallin (162) recommend fitting the

thermistor with a glass jacket to minimize forced convection in batch analyses. It seems that this might be beneficially adapted to continuous-flow analysis, provided the flow-cell could be adapted to accommodate the considerably more bulky design.

A further contributory factor towards the poor sensitivity of the method could also be the "insulation" of the thermistor by the glue, or gelatin layer, although no experiments were made to determine the effects of an inert layer on the response characteristics of the thermistor.

The combination of the inability of six different immobilization methods to give even reasonable sensitivity, coupled with the fact that, as yet, no account appears in the literature utilizing the combination of an enzyme-bound thermistor with a continuous-flow cell, suggests that the answer (if any) to the problem may lie in the design, and physical characteristics of the system (e.g. inefficient sensing of the total heat produced), rather than in the actual method of attachment of the enzyme to the thermistor.

Section 3.4.

Discussion of the merits of thermal methods in the determination of enzyme substrates using immobilized enzymes

The relative merits of free and immobilized enzymes have already been discussed in detail in Chapter 1, Section 2.5., and in the context of this discussion therefore, the LKB 10700-1, and other heat sensing devices described will simply be regarded as detection systems to be compared with other methodologies, and with other groups of workers using similar techniques.

Undoubtedly, the most sensitive, accurate and reproducible determinations of substrate in this study has been made using

the LKB 10700-1 microcalorimeter assembly, and, as has been shown in each specific case these three factors have always compared favourably with other groups working along similar lines.

The sensitivity obtained on the LKB 10700-1 was more than adequate for accurate determination of all substrates in concentrations commonly encountered; indeed, for many glucose analyses, dilution of the sample would be necessary in order to bring the final concentration down into the linear range.

Determinations carried out using the "home-made" semi-adiabatic microcalorimeters, employing thermistors as heat sensors, were disappointing.

The novelty of the enzyme-bound nylon tubes, in conjunction with thermistors, initially made such a combination attractive, particularly for the assays in viscous fluids; however, poor sensitivity precluded further study.

The sensitivity of the glass-immobilized enzyme packed into the vicinity of the thermistor tip did not reach that obtained by Mosbach and his group; however, because of the similarity of this technique to others previously described, no serious attempt was made to make the rather eleaborate modifications required to obtain greater sensitivity.

Attempts to determine substrates using an enzyme-bound thermistor also looked attractive in view of the general simplicity, and applicability. Despite the fact that no meaningful results could be obtained using this method, this could not have been foretold until the technique was tried.

The advantages of the microcalorimetric technique in assay procedures does not, in general, lie in sensitivity. In this

respect, spectrophotometric procedures are probably more useful, but they do have a special, and in some instances limiting, condition that either the substrate or product should contain a chromogenic group. Thermal methods have, moreover, other advantages as well over the more commonly used spectrophotometric technique, in that there is no need for optical clarity or freedom from suspended matter etc., and that few, if any, species will cause interference.

CHAPTER 4

Determination of enzyme substrates using immobilized enzymes in conjunction with non-thermal detection systems.

The literature cites many references utilizing the combination of an electrode, and immobilized enzyme, e.g. a liquid enzyme layer electrode has been reported (71), requiring diffusion of the substrate through the membrane, followed by reaction with the enzyme. A major problem of such designs was the slow response time imposed by the physical design of the detector. Another drawback to an immobilized enzyme/pH electrode is the fact that most enzyme reactions are not linear over a broad pH range, and so the useful range of application is limited. The object of the work was to construct a simple, substrate-sensitive electrode, which could be easily prepared from readily available materials, and to investigate its characteristics with respect to factors such as pH, enzyme-loading, capacity of buffer solution, substrate concentration, addition of cofactors (where appropriate), method of analysis, stability, speed of analysis, etc. Although there was not time for an exhaustive survey of all relevant parameters, four electrodes were constructed, using two different enzymes, two different immobilization methods, and relatively high and low enzyme loadings on the electrode. A model has been proposed to account qualitatively for the shape of the pH/concentration plot obtained.

4.1.1. Determination of glucose and urea using glucose oxidase/catalase, and urease bound electrodes.

Reagents

Glucose oxidase (Type VII, from Aspergillus niger), catalase (from bovine liver), urease (Type VII, from jack beans)

gelatin (Type III 225 bloom from calf skin), and human serum albumin (fraction V), were obtained from Sigma Chemical Co. U.K. Urea (99+ % pure) was obtained from B.D.H., Poole, U.K. Glucose and glycine (puriss grades), were obtained from Koch-Light Laboratories, Colnbrook, Bucks.

Immobilization methods and procedures

Four enzyme electrodes were constructed (designated electrodes I - IV), by immobilization of the appropriate enzyme(s) to a 33 116D 200 Standard Combination pH electrode (obtained from E.I.L., Chertsey, Surrey, U.K.), by the methods described in Chapter 2. Section 2.2.
The particulars of each electrode are listed below:-

Electrode I

This was a glucose sensitive electrode obtained by entrapping glucose oxidase (Sigma, Type VII, 4 mg, 800 units) in the vicinity of the electrode surface using the gelatin entrapping technique, as described in Chapter 2, Section 2.2 (a).

Electrode II

This was also a glucose sensitive electrode made by the same technique as electrode I, except that catalase (10 mg. 18500 units), and a greater activity of glucose oxidase than in electrode I (20 mg, 4000 units) were used.

Electrode III

This was also a glucose sensitive electrode, but in this case glucose oxidase (4 mg, 800 units) and catalase (2 mg, 37000 units) were immobilized by cross-linking on to the electrode surface with glutaraldehyde, in the presence of an excess of inert protein

(human serum albumin, 20 mg), as described in Chapter 2, Section 2.2 (b).

Electrode IV

This was a urea sensitive electrode, obtained by entrapping urease (Sigma, type VII, 3.6 mg. 250 units) in the vicinity of the electrode surface using the gelatin-entrapping method described in Chapter 2, Section 2.2.

Measurement of pH change

The enzyme electrode was connected to a pH-meter (EIL 7050 laboratory pH/millivoltmeter), and introduced into a home-made flow cell (free volume in the presence of the electrode, approx. 2 cm^3), constructed as shown in Fig. 4.1.1.1., and described in part c). Buffer was pumped into, and drawn out of, the flow cell by a two-channel, Masterflex perstaltic pump, operating at a nominal flow rate of about 4.2 cm^3 min^{-1}; no attempt was made to optimize the flow rate.

The active electrode was stabilized in the appropriate flowing buffer solution (for appropriate buffer, see results section). One of two methods was then used to measure the change in pH when the immobilized enzyme reacted with the substrate in the vicinity of the enzyme electrode.

Either, as with the high enzyme loading, substrate solution was introduced into the flow steam for a known time. The change in pH whilst the substrate solution flowed over the electrode was then recorded, as a function of time, by means of a chart recorder connected to the output of the pH meter.

Alternatively, as in the case of low enzyme loadings, where the reaction rate was much slower (and hence only small pH changes

would be produced whilst the substrate solution flowed over the enzyme electrode), it was found to be advantageous to wait until a steady-state had been reached, and then to stop the flow completely, leaving the enzyme electrode in contact with only a small volume of substrate solution. Again, the change in pH as a function of time could be monitored. This method was found to produce a much larger pH change than would be obtained by using a continuous flow method, as the hydrogen ions produced (as with glucose/glucose oxidase) or removed (as with urea/urease) would remain in the vicinity of the electrode, and not be mostly swept away by the incoming solution. This method possessed another advantage in that the "wash" time, between runs, appeared to be less than for the continuous-flow method (using the same substrate/buffer concentration), particularly when there was little or no variation in the substrate concentration between consecutive analyses.

The following observations were made:-

a. analysis time decreased with decreasing time of pulse duration (continuous flow method) or incubation (stopped-flow method).
b. analysis time decreased with increasing buffer capacity.
c. analysis time increased with increasing substrate concentration.
d. analysis time was generally less using stopped-flow methods, than continuous flow methods (a, b and c being constant).
e. presumably, analysis time would vary inversely with the "dead-volume" of the flow cell. However, as only one flow-cell was used, this was not demonstrated experimentally.

The range of analysis times varied from about 30 h^{-1} (one min incubation, stopped-flow, 100 mM buffer, 1 mM urea) to about 3 h^{-1} (3 min continuous flow, 2 mM buffer, 100 mM glucose).

Construction of flow-cell

The external diameter of the glass electrode appeared to be about 11×10^{-3}m.

In order to minimize the "dead-volume" of the cell, it was constructed out of 12×10^{-3}m internal diameter silicone (20×10^{-3}m ext diam) tubing, with one end given a water-tight seal by insertion of a rubber bung of appropriate size. The inlet was as low as possible in the tube, and the outlet at such a height that the solution in the flow-cell just covered the active area of the electrode (experimentally 14×10^{-3}m). Inlet and outlet tubing were connected to the flow-cell by means of teflon nipples inserted through holes bored into the cell in the appropriate places:

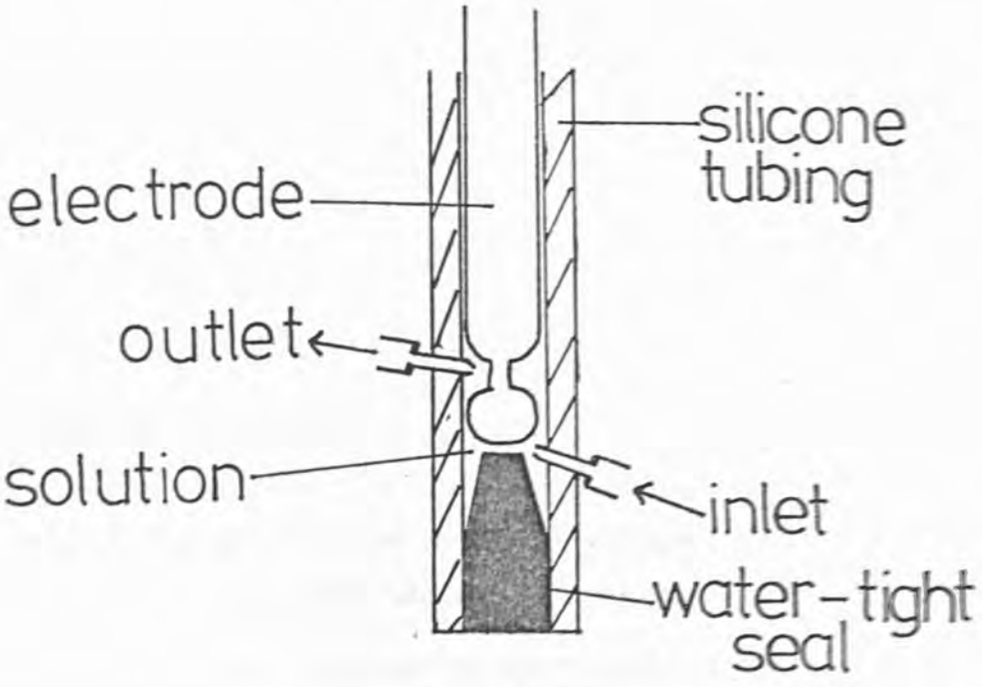


Fig. 4.1.1.1.

Using a high buffering capacity will tend to:

a. decrease the absolute sensitivity
b. decrease baseline noise
c. decrease "blank" for stopped-flow method
d. give an approximately linear plot of concentration vs. ΔpH, particularly in the case of urea
e. quench any small pH difference between buffer plus sample solution
f. give quicker analysis time (as pH change in cell will be "quenched" rather than just "washed out")
g. enzyme activity will remain more constant, due to the smaller pH change.

Often, particularly for urea, b, c, d, e, f and g will outweigh a, although for standard solutions of glucose, which appear to have no effect on the pH of the buffer solution, there may be little difference. Using the continuous flow method of analysis for low concentrations of glucose, it may even prove advantageous to use a low buffer capacity although, in this case, analysis time is also rather long, and extrapolation of the baseline was often necessary. This was so as, to avoid unduly long analysis times, the next run was often carried out before the original peak had returned to the baseline.

Stability of electrodes

The urea electrode (IV) appeared to retain 90% of its activity over a period of two weeks, when readings given by 10mM urea were compared, under identical conditions.

For 1 mM urea, this stability appeared quantitative. Longer-term stability studies were impracticable, due to the limited period of time available for the study.

Results and discussion

It has already been mentioned that, using low enzyme loadings, a stopped-flow method of analysis resulted in greater sensitivity, and hence this was the only method used for electrodes I, III and IV.

Using an electrode with high enzyme loading (i.e. II), the stopped-flow method proved impracticable, due to the long time taken to reach a steady-state, the longer analysis time needed, and also to the fact that, at high glucose concentrations (i.e. conc. glucose $\gg$ conc. 0_2 dissolved in the buffer), most of the oxygen would have been already removed from the bulk solution in reaching a steady-state. Thus, using 20mM glucose, the change in pH upon reaching a steady-state in the flow-through mode was more than the change in pH using the subsequent stopped-flow method.

Additionally, for the urea electrode, where the addition of urea to the buffer solution could be shown to alter the pH *per se*, the stopped-flow method would have to be used, regardless of the enzyme loading on the electrode. This would equally well apply to any sample whose constituents were unknown and, hence, could not be relied upon not to affect the pH of the buffer solution. Throughout these series of experiments, the effect of buffer concentration was critical.

Although, looking at Fig. 4.1.1.2 it is readily apparent that the absolute change in pH decreases with increasing buffer concentrations (for a given substrate concentration); this does not necessarily mean that it is advantageous to use a low buffer capacity solution, as often reported in the literature (71).

Glucose electrode (I) showed poorer stability, decreasing by about 10% per day when used continuously for analysis. On days when it was not used for analysis (e.g. at weekends), the stability appeared to be constant. This rapid de-activation was ascribed to de-activation of the glucose oxidase by H_2O_2 (a by-product of the reaction). Therefore, for subsequent electrodes (II) and (III), catalase was co-immobilized with glucose oxidase, to decompose the hydrogen peroxide so formed. These electrodes showed quantitative stability over two days, but time precluded further studies.

For the glucose electrode (II), the addition of H_2O_2 to the substrate solution greatly increased the linear range of the plot (Fig. 4.1.1.7.) due to the fact that the oxygen supply in the reaction solution would be continually replenished by decomposition of H_2O_2 to water and oxygen by the immobilized catalase, as shown in Fig. 4.1.1.7. However, addition of 100×10^{-3} M H_2O_2 to the bulk buffer solution completely destroyed enzyme activity during the course of one afternoon, presumably because the catalase could not decompose all the peroxide.

It appeared that the increase in the linear range of the glucose electrodes by addition of excess hydrogen peroxide could not be carried too far (e.g. for routine analysis of ca. 100 mM glucose), or poor stability of the enzyme electrode would occur.

The human serum albumin (HSA) cross-linked glucose electrode (III) showed appreciably poorer sensitivity than the corresponding gelatin-immobilized glucose electrode (I), presumably due to the fact that a large proportion of the enzyme had been inactivated by

the cross-linking process (Fig. 4. 1. 1. 4.).

Shapes of plots shown in Figs. 4. 1. 1. 2 to 4. 1. 1. 9.

Plots of ΔpH vs. concentration of glucose using electrodes (III) and (II) typically reached a plateau around 2. 5 - 5 mmol dm^{-3}, due to oxygen depletion in the buffer solution (Figs. 4. 1. 1. 4 to 4. 1. 1. 5), unless an excess of hydrogen peroxide were employed (Fig. 4. 1. 1. 7).

In the presence of excess oxygen, formed in situ by the decomposition of added hydrogen peroxide by the immobilized catalase, plots of $\log_{10}$[glucose] vs. -ΔpH were linear over an extended range (5 x 10^{-3}M glucose to 80 x 10^{-3}M glucose), as would be predicted from the Nernst equation (Fig. 4. 1. 1. 9). The slight non-linearity of the plot obtained using 80 x 10^{-3}M H_2O_2 (Fig. 4. 1. 1. 9), was probably due to deactivation of some of the enzyme by the excess of hydrogen peroxide, and a subsequent plot using only 20 x 10^{-3}M H_2O_2 produced a good straight-line fit.

However, prolonged use of even 20 x 10^{-3} H_2O_2 in the buffer resulted in a slow deactivation of the enzyme as shown by a decrease in the gradient of the calibration plot, and it was concluded that glucose determinations over extended concentration ranges by this technique would present difficulties.

The enzyme system most convenient to study over a large range of concentrations was the urea/urease system, where no secondary substrates are needed. Theoretically, the response to increasing concentrations of urea should be proportional to log (concentration). Since one mole of urea removes two moles of hydrogen ions from solution, according to the stoichiometry of the enzymic reaction, and pH is a logarithmic function of hydrogen

FIG. 4.1.1.2. Plot of ΔpH v.s. urea conc, in varying concs of phosphate buffers, originally at pH=7·0

×=100mM phosphate buffer
⊙= 25 " " "
+= 10 " " "
⊡= 2 " " "

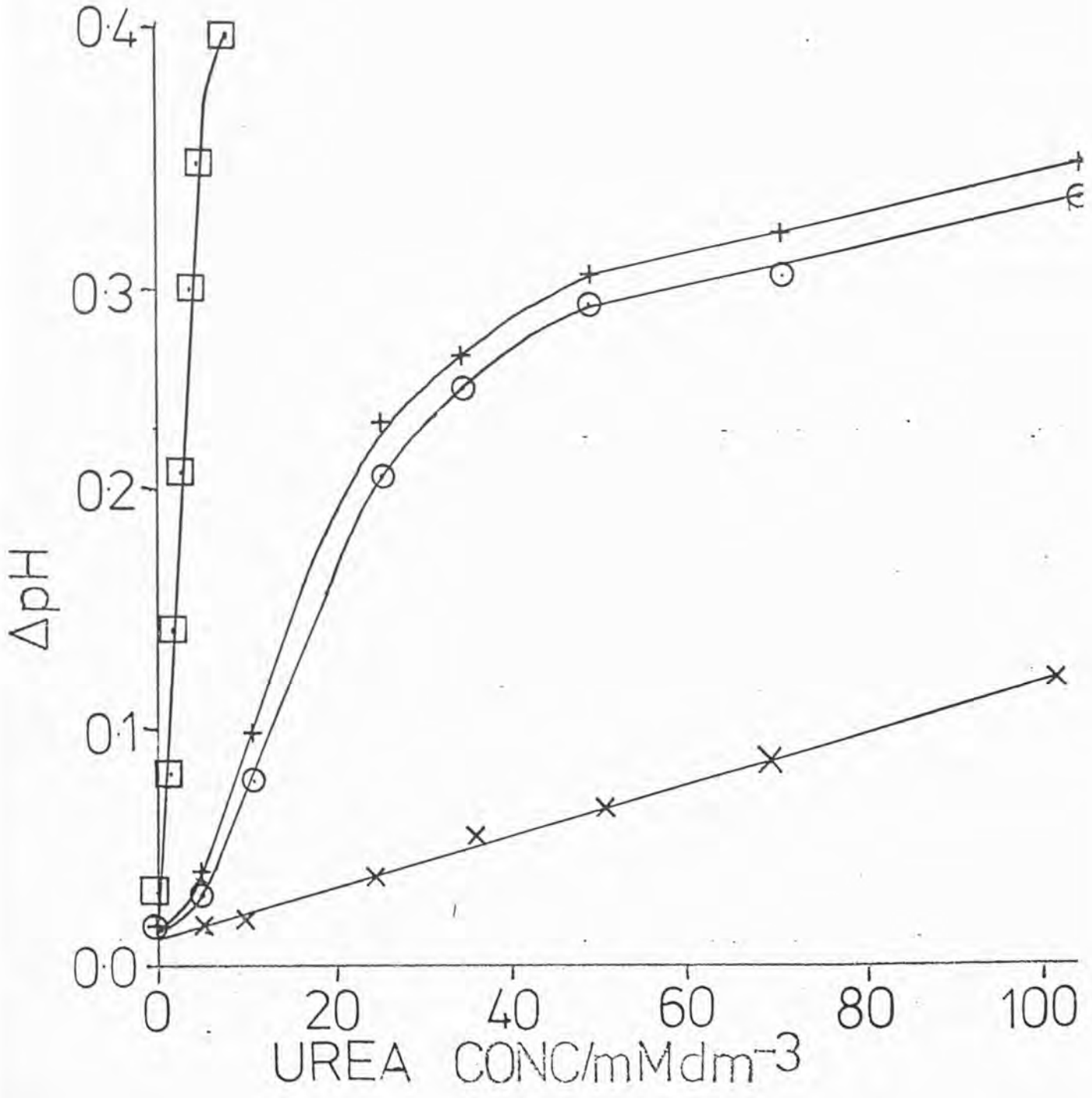


FIG. 4.1.1.3. Plot of ΔpH v.s. conc of urea, in various concs. of phosphate buffer, originally at pH=7.0 (bottom left corner of 4.1.1.2.)

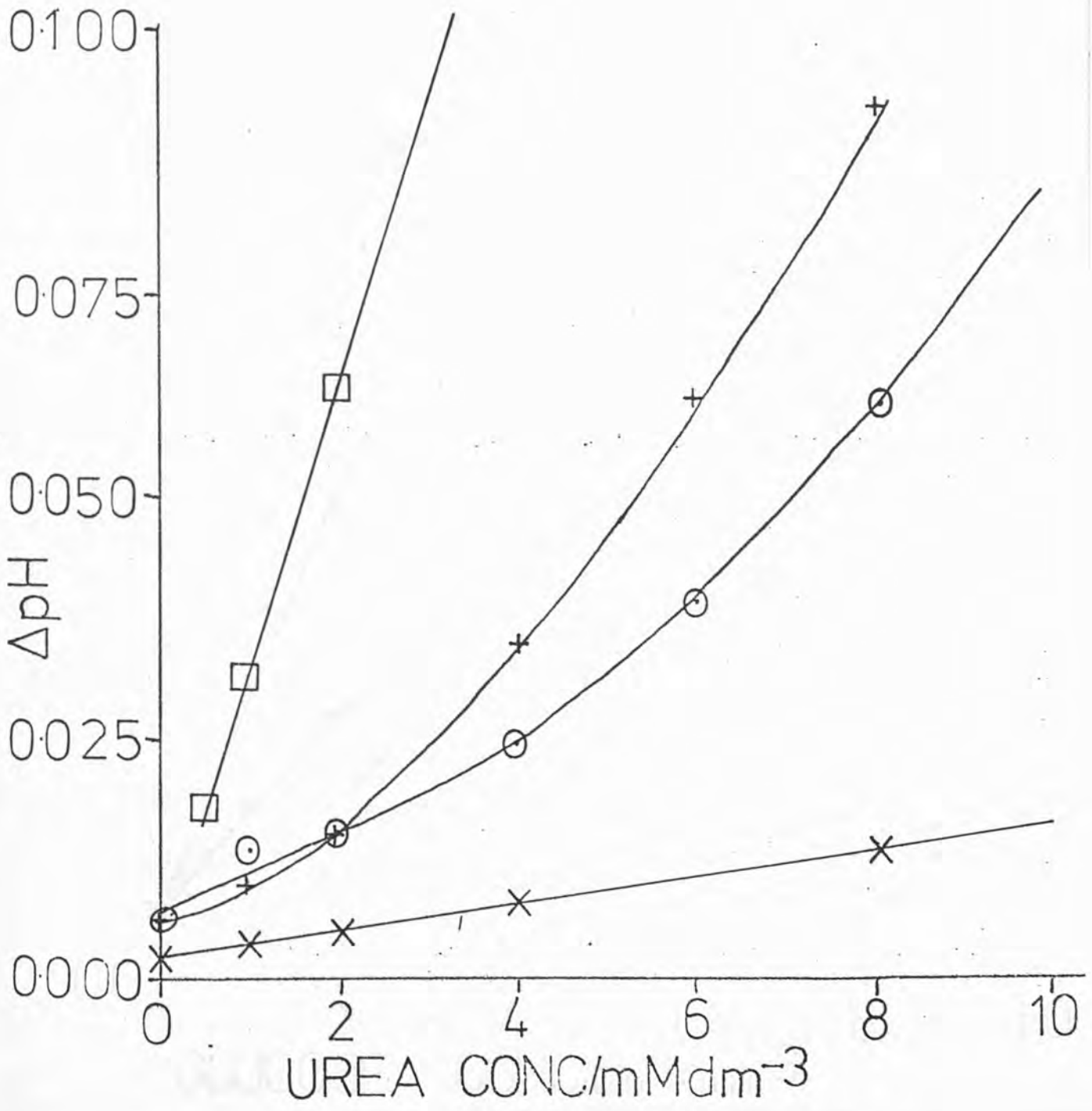


FIG. 4.1.1.4. Plot of -ΔpH vs. glucose conc. using the stopped-flow technique (3 min incubation) in 1mM NaH_2PO_4

×=electrode III (H.S.A. cross-linked)

o= " I (gelatin-entrapped)

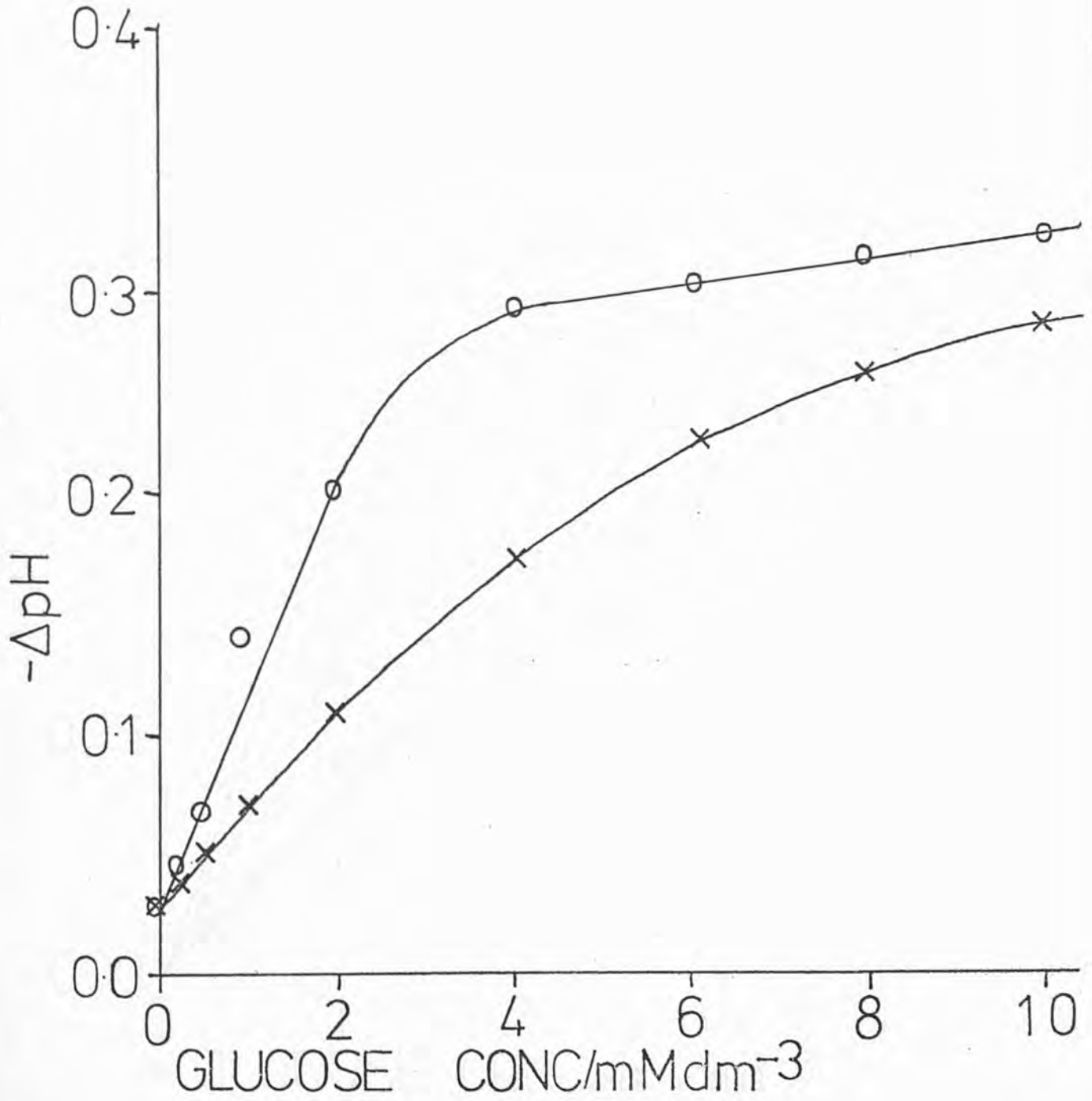


FIG. 4.1.1.5 Plot of -ΔpH v.s. conc of glucose (3 min pulse) using electrode II in various concentrations of phosphate/citrate buffer, originally at pH=5·1

×=10mM buffer
⊙= 5mM ,,
+= 2mM ,,

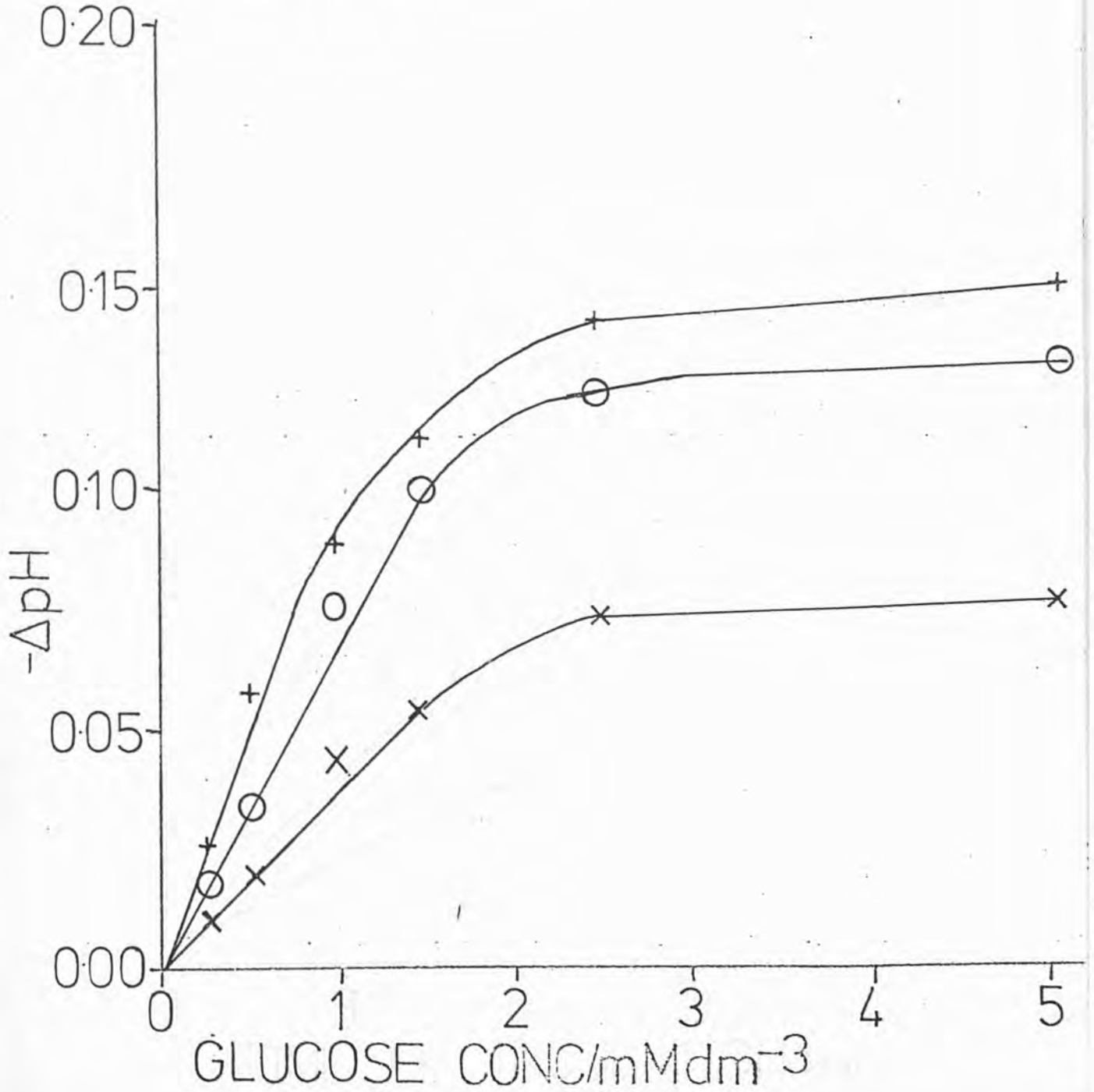


FIG. 4.1.16. Plot of $-\Delta pH$ v.s. time of incubation of glucose soln (stopped-flow method), in 1mM NaH_2PO_4, using electrode I

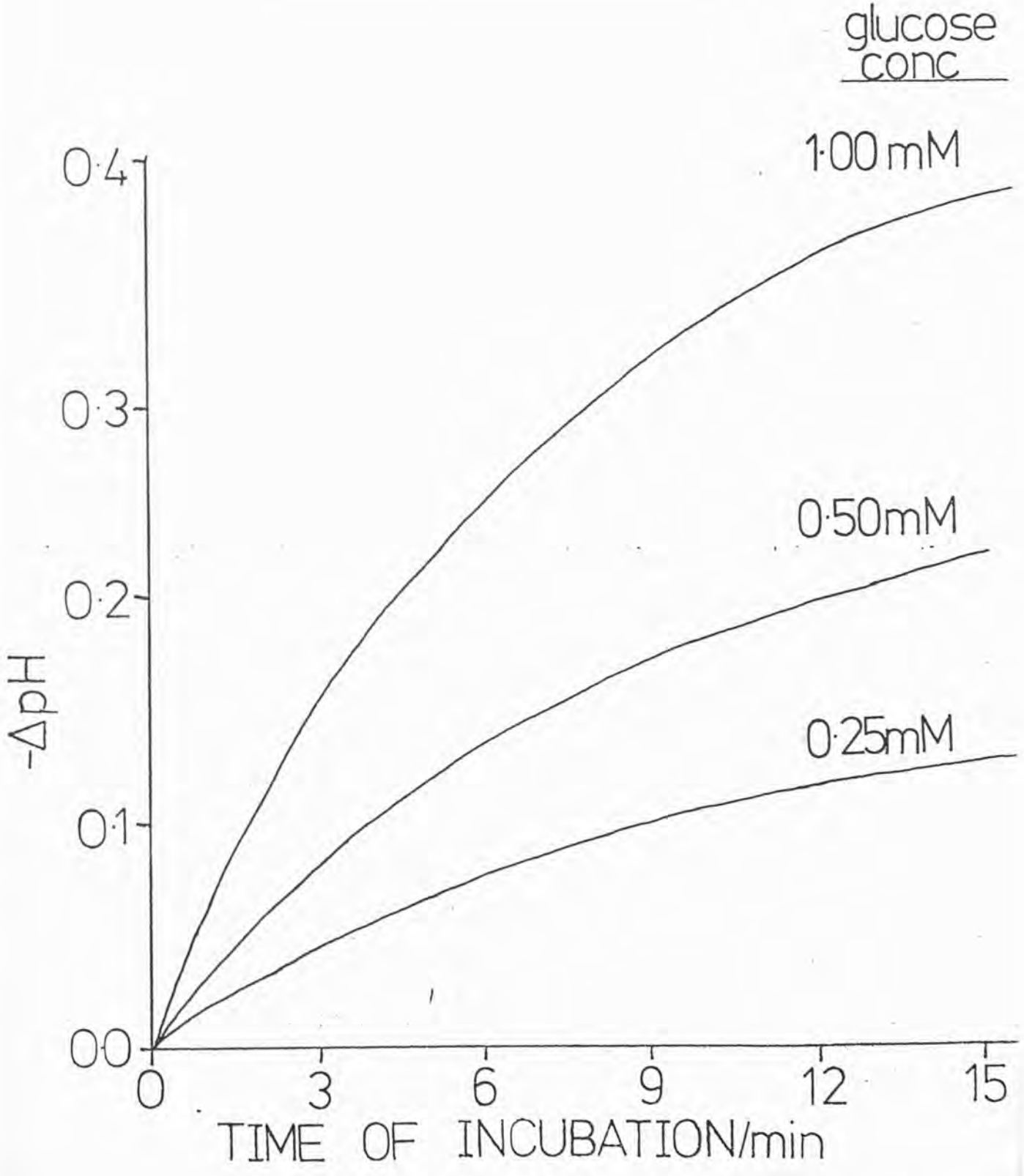
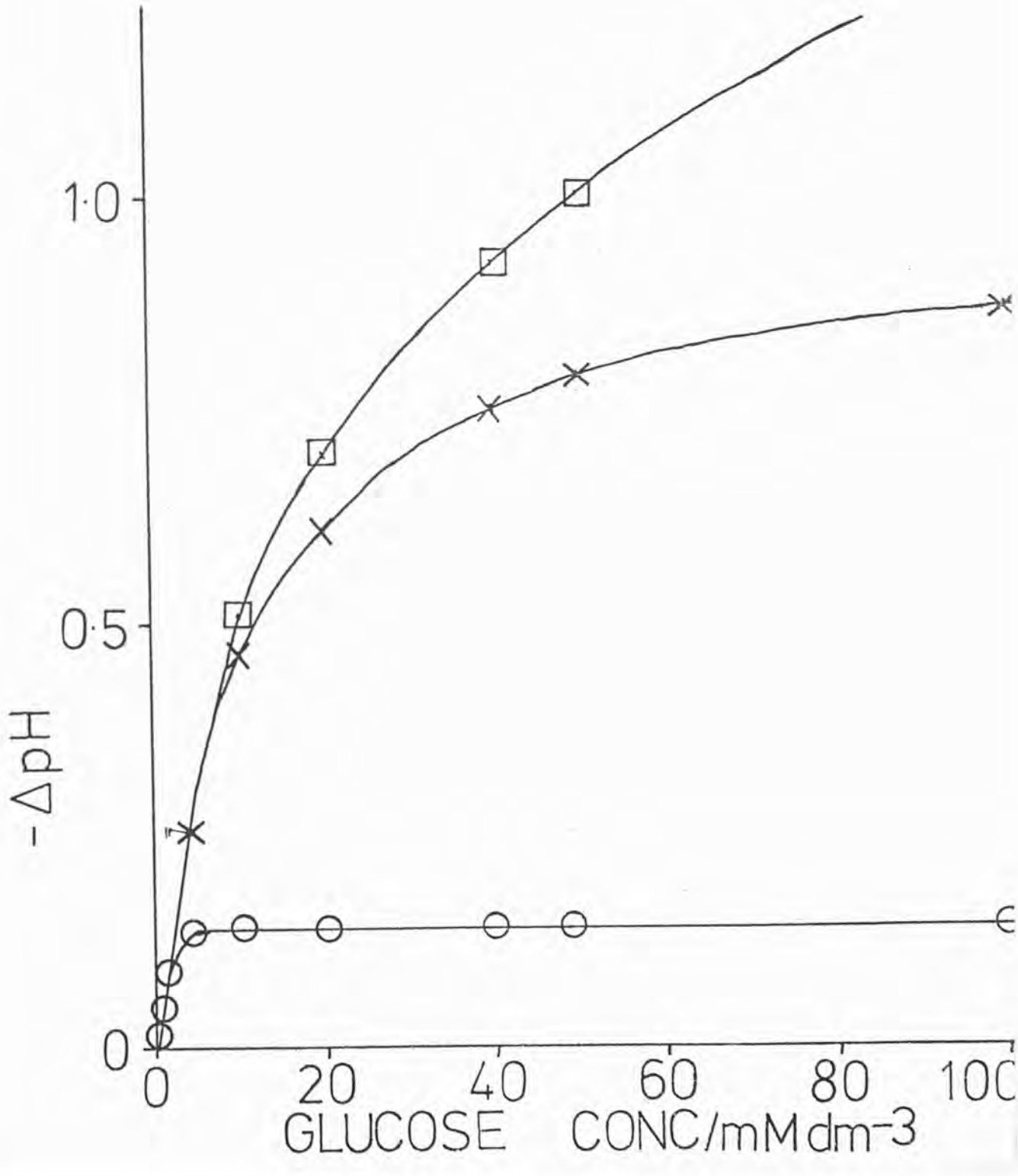


FIG 4.1.7 Effect of addition of H_2O_2 on glucose oxidation ($-\Delta pH$) in 5mM phosphate buffer, initial pH 5·10

□ H_2O_2 = 80mM
× " 20 "
○ " 0 "



ion concentration, according to the Nernst equation, the sensitivity of the electrode should decline with increasing concentrations of urea, and although at higher concentrations of substrate (w.r.t. buffer), this was observed, where the substrate concentration was much less than the buffer concentration, the plot appeared to be "super-Nernstian" - i.e. there must be something which makes the electrode more sensitive to decreasing hydrogen ion concentration when the urea concentration increases. (Fig 4.I.1.2.).

One possibility is that this increased sensitivity is a consequence of the decrease in buffer capacity which is found in the phosphate buffer system as the system moves away from the pKa value (6.8) of the $H_2PO_4^- \rightleftharpoons HPO_4^{2-} + H^+$ couple, which will counteract the Nernstian behaviour.

That this is a reasonable explanation is shown in Fig. 4.I.1.8, where the change in pH given by a fixed concentration of urea is plotted as a function of the pH of the buffer solution. Free urease has a maximum activity at pH 6.75-7.0, a similar value to the maximum buffer capacity of the phosphate buffer system. However, the apparent activity of the immobilized urease appears to be at a minimum at around pH 6.7, doubtless due to the fact that, at this pH, the buffer capacity is a maximum, hence giving the least change in pH for a given amount of urea hydrolysed, despite the fact that the intrinsic activity of urease is near maximum.

Hence, as the system moves away from pH 6.7 (to either side), the sensitivity of the electrode will increase, due to the decrease in the capacity of the buffer. In summary, it is suggested that the enzymatic reaction within the vicinity of the electrode (an unstirred and "stagnant" area) results in a local increase in pH

FIG. 4.1.1.8. Plot of apparent activity of immobilized urease (ΔpH) vs pH of buffer (10mM urea in 0·1M phosphate)

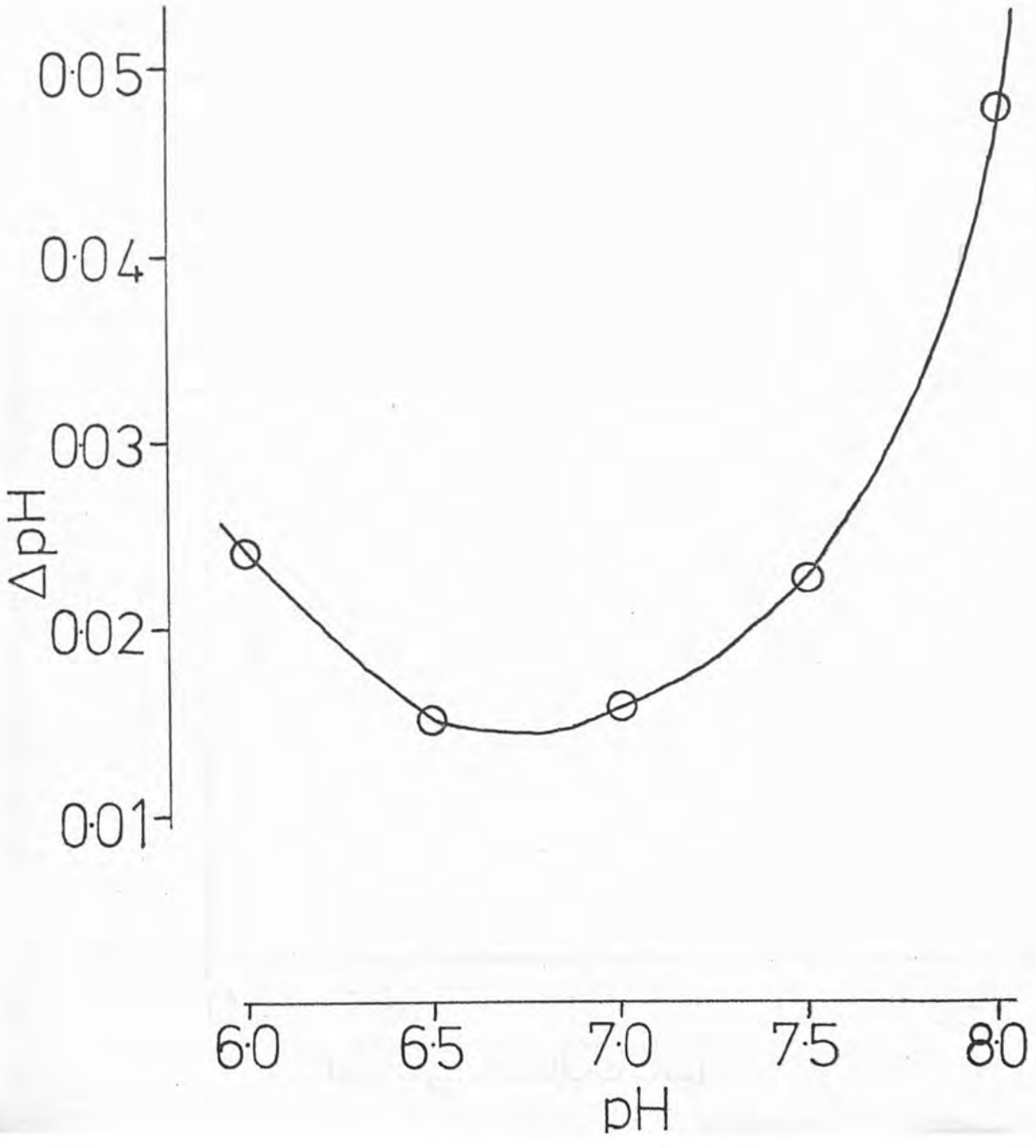
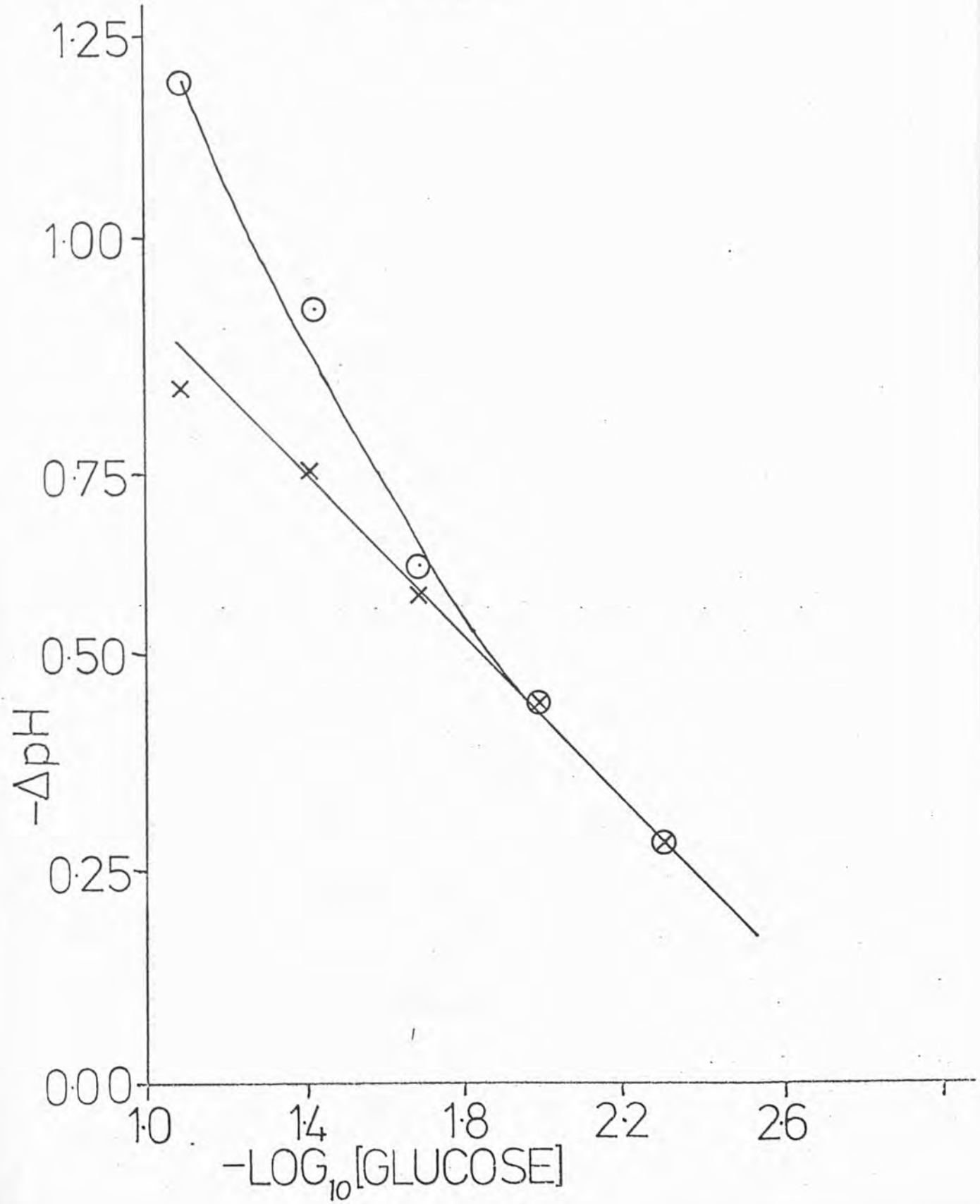


FIG. 4.1.19. Plot of $-\Delta pH$ v.s. $\log_{10}$ [conc of glucose] with added H_2O_2

×=+20mM H_2O_2

⊙=+80mM ''



which decreases the buffer capacity, and hence increases the sensitivity of the electrode to decreasing hydrogen ion concentrations. This more than counterbalances the decreasing sensitivity predicted by the Nernst equation, giving rise to this "super-Nernstian" response. Similar plots have been obtained by Enfors and Nilsson (103), in the determination of penicillins in phospate buffers.

That there appears also to be a "blank" reaction when using the stopped-flow method, even in the absence of any substrate, is probably explained by the buffering capacity of the immobilized protein (enzyme + gelatin or HSA), slightly altering the pH of the solution, in the microenvironment of the electrode.

4.2.1. Determination of glucose using nylon-tube immobilized enzymes in conjunction with an Autoanalyzer II and spectrophotometer.

The system chosen for investigation was the hexokinase catalyzed phosphorylation of glucose by ATP (Adenosine 5'-Triphosphate) followed by the oxidation of the resulting glucose 6-phosphate by $NADP^+$ (β-Nicotinamide Adenine Dinucleotide Phosphate) in the presence of glucose 6-phosphate dehydrogenase (GPD), with the resulting NADPH absorbance being measured at 340 nm. The reaction scheme is shown in Fig. 4.2.1.1.:-

$$\text{glucose} + \text{ATP} \xrightleftharpoons{\text{hexokinase}} \text{glucose 6-phosphate} + \text{ADP}$$

$$\text{glucose 6-phosphate} + \text{NADP}^+ \xrightleftharpoons{\text{GPD}} \text{6-phosphogluconolactone} + \text{NADPH (absorbance measured at 340 nm)}$$

Fig. 4.2.1.1.

The above scheme has been shown by Slein (201), to be a highly specific assay for plasma or serum D-glucose, (in conjunction with a spectrophotometric detection system), and can be easily adapted to detect other polysaccharides which can be enzymically broken down to D-glucose. It will be discussed later why this reaction scheme would not be specific for D-glucose if a thermal detection system were employed.

Widdowson and Penton (202) have shown how this assay can be applied to the automated determination of glucose in the Technicon Autoanalyzer II, and Morris et al (32) have demonstrated the feasibility of using co-immobilized nylon tube derivatives of hexokinase and GPD incorporated into the above system.

Reagents

Hexokinase and glucose 6-phosphate dehydrogenase were obtained as the mixed enzymes from Sigma Chemical Co. U.K. ATP (disodium salt), and $NADP^+$ (disodium salt) were obtained from Boehringer Mannheim Ltd., Lewes, Sussex, U.K. All other reagents were obtained as stated in Chapter 2.

Immobilization procedure

Hexokinase (175 units) and glucose 6-phosphate dehydrogenase (90 units) were coupled to activated nylon tubing (2 m), as described in Chapter 2, Section 2.1.

Experimental details

All glucose determinations were carried out using the flow system shown in Fig. 4.2.1.2.

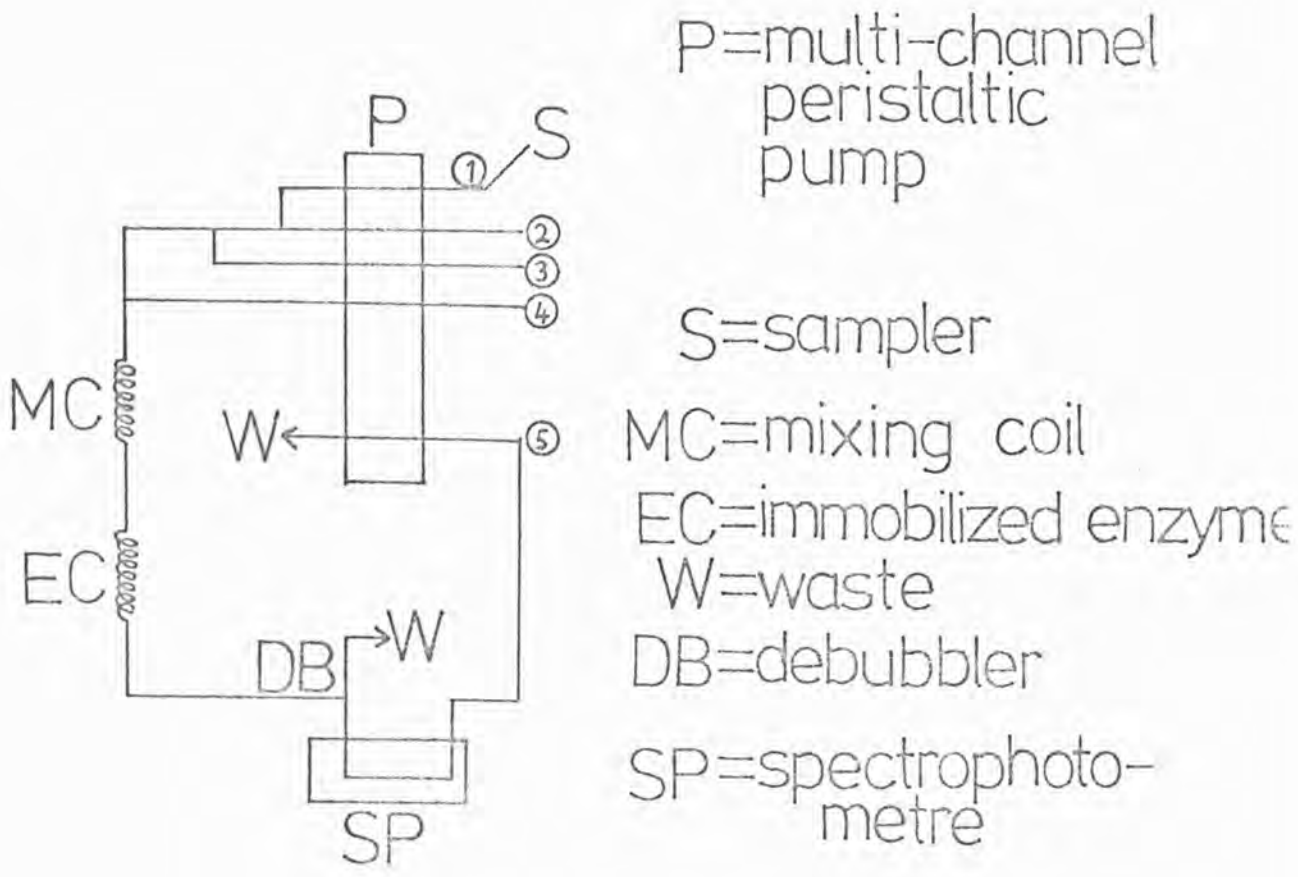


Fig. 4.2.1.2.

Through lines 1, 2, 3 and 4 were pumped: sample (0.16 $cm^3 min^{-1}$); 0.1M THAM + 8×10^{-3}M $MgCl_2$ + 0.25M NaCl (pH 7.6, 1.20 $cm^3 min^{-1}$); air (0.32 $cm^3 min^{-1}$); 1.0×10^{-3}M $NADP^+$ + 1.7×10^{-3}M ATP (0.32 $cm^3 min^{-1}$) respectively. The sampler (S) and pump (P) were standard Technicon equipment, and absorbance was measured using a Beckmann DBGT spectrophotometer fitted with a flow-through cuvette of 1 cm light path.

Table 4.2.1.3. and Fig. 4.2.1.4. show tables of results obtained, and the plots of these results, respectively, when standard glucose solutions, in varying sample to wash ratios, were passed through the nylon tubing (2 m) contining the co-immobilized hexokinase and GPD, inserted at position EC in the flow system shown in Fig. 4.2.1.2, demonstrating that this method of assay can be used for the determination of glucose in the range 1 - 10 mM.

TABLE 4.2.1.3.

Table of peak heights (Δ Absorbance, 340 nm) vs. conc. of glucose (in sample and in enzyme bound tubing) obtained in the spectrophotometric determination of glucose, using various sample:wash ratios.

Conc. of glucose in sample/ mol dm^{-3}	Conc. of glucose in reaction coil/ mol dm^{-3}	Peak heights (Δ absorbance at 340 nm) 2:1 sample:wash ratio (20 samples per hour)	1:1 sample:wash ratio (10 samples per hour)	1:2 sample:wash ratio (10 samples per hour)
1.0×10^{-3}	0.095×10^{-3}	0.045	0.055	0.050
1.0×10^{-3}	0.095×10^{-3}	0.040	0.065	0.055
2.0×10^{-3}	0.190×10^{-3}	0.080	0.120	0.100
2.0×10^{-3}	0.190×10^{-3}	0.080	0.110	0.105
4.0×10^{-3}	0.381×10^{-3}	0.170	0.225	0.195
4.0×10^{-3}	0.381×10^{-3}	0.160	0.225	0.210
6.0×10^{-3}	0.571×10^{-3}	0.245	0.330	0.320
6.0×10^{-3}	0.571×10^{-3}	0.235	0.340	0.310
8.0×10^{-3}	0.762×10^{-3}	0.315	0.415	0.390
8.0×10^{-3}	0.762×10^{-3}	0.315	0.405	0.380
10.0×10^{-3}	0.952×10^{-3}	0.370	0.450	0.425
10.0×10^{-3}	0.952×10^{-3}	0.365	0.460	0.415
		$r^2 = 0.995$	$r^2 = 0.997$	$r^2 = 0.973$
		r^2 (1×10^{-3}M to 6×10^{-3}M)=0.0997	r^2 (1×10^{-3}M to 6×10^{-3}M)=0.998	r^2 (1×10^{-3}M to 6×10^{-3}M)=0.997

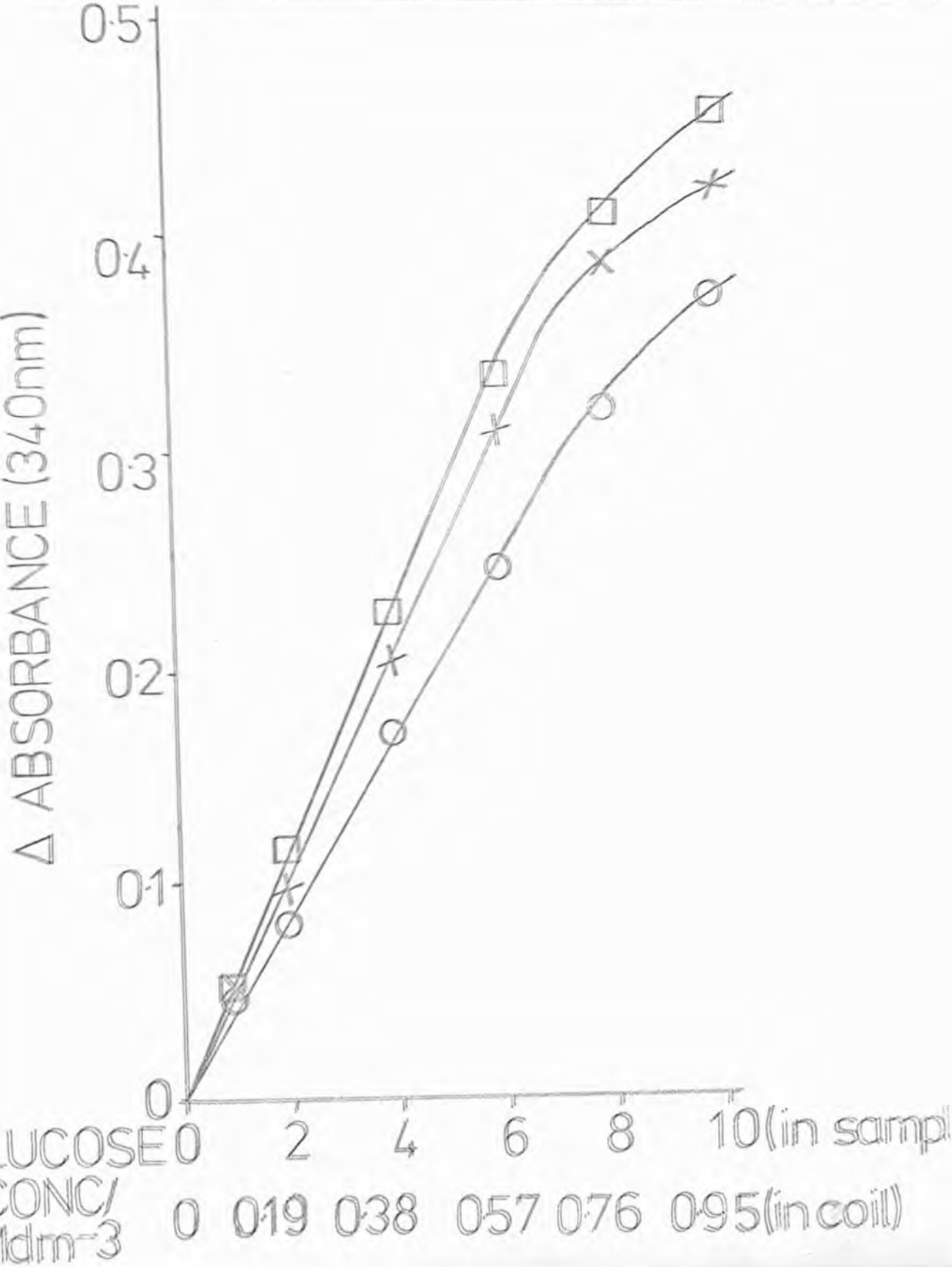
$1 \times 10^{-3}M \ll R \ll 10 \times 10^{-3}$ where R = analytically useful range.

FIG. 4.2.14. Plot of Δabs(340nm) v.s. glucose conc, as in table 4.2.13.

□ 1:1 sample : wash ratio, 10 samples h^{-1}

× 1:2 " : " " 10 " "

o 2:1 " : " " 20 " "



As expected, maximum sensitivity (i.e. flat-topped peak), was obtained with the maximum sampling time used (3 min.), in the case of the 1:1 sample to wash ratio, at a sampling rate of 10 per hour. Longer sampling times would not be expected to increase the sensitivity further. Maximum sample throughput consistent with reasonable sensitivity was obtained using a 2:1 sample to wash ratio, with 20 samples per hour. However, in this case the sensitivity was greatly reduced (see Table 4.2.1.3.) due to the combination of shorter sampling time (2 min.), and also because of insufficient washing to return the signal recorder to the original baseline between each peak.

Intermediate between the extremes appears the 1:2 sample wash ratio (10 samples per hour), where the shorter sampling time (2 min.), was somewhat compensated by the longer wash time, allowing the signal to return to the original baseline between samples, therefore maximizing the peak height under the sampling conditions of the experiments.

Correlation coefficients obtained using these results appeared generally poorer than those obtained using thermal means, even at relatively low glucose concentrations.

This was generally ascribed to the fact that the mixing coil (MC) and enzyme coil (EC) were not thermostatted, as no suitable apparatus was available for this at the time these experiments were carried out. However, it did serve to underline the advantages of using the packed bed type of reactor used in the LKB assembly over the nylon tube reactor used in this type of assay, in that, as well as achieving quantitative end-points (and hence maximum sensitivity), end-point type assays are free from interferences often experienced in kinetic ("non-100%") type enzyme assays, such as minor temperature variations which

could affect the reproducibility.

The stability of the nylon tube supported enzymes at room temperature was rather disappointing, with the half-life appearing to be less than three days, although when stored refrigerated at + 4°C the tubes did, as expected, exhibit better stability.

However, provided frequent calibration was carried out, say, by including a known standard solution of glucose periodically as a sample, the tubes were useful for glucose determinations for a period of three or four days.

As mentioned earlier in this section, although the particular enzyme system is specific for the determination of D-glucose when used in conjunction with a spectrophotometric detection system, it would not be specific if a thermal detector were used, and hence would appear to present one specific example of an advantage of the inherently more specific spectrophotometric detector over the less specific, but more general, thermal detectors.

The reason for this lies in the relative lack of specificity of the enzyme hexokinase, which catalyses the phosphorylation of D-fructose, D-mannose, D-glucosamine(204) and 2-deoxy-D-glucose (205), as well as D-glucose. The combination of the relatively specific spectrophotometric detection system, and the highly specific enzyme glucose 6-phosphate dehydrogenase (GPD) can compensate for this lack of specificity provided that no products of the hexokinase catalysed reactions absorb at around 340 nm. However, in the case of the completely non-specific thermal detection system, a highly specific enzyme system is always required, as specificity is here completely dependent upon the enzymes.

4.3. Discussion of the merits of the other techniques (described in Chapter 4).

Considering the relative crudity involved in the construction of the enzyme electrodes described in Chapter 4, Section 1.1., their sensitivity compares well with those reported by Mosbach and co-workers (71).

Reproducibility of these electrodes in the determination of identical samples of substrate appeared reasonable ($\pm$ 5%), but could be improved by making up solutions in 0.1M NaCl, to keep the ionic strength constant, particularly where low concentration buffers were used. In every case, reproducibility and precision were improved with increasing buffer concentration, although the absolute change in pH was decreased. Another reason for reproducibility being lower than in the LKB/packed bed techniques is that, being a kinetic type assay, minor variations in temperature would be expected to affect both the rate of the enzyme reaction and the rate of diffusion (mass transfer) of the substrate to the surface of the enzyme. The shape of the plots obtained in Figs. 4.1.1.2. to 4.1.1.9. has already been discussed in detail. That no plot appeared to have an extended linear range (except [urea] vs. ΔpH, in 100 mM phosphate buffer, pH 7.0, Fig. 4.1.1.2., and log [glucose] vs. ΔpH in 5mM phosphate buffer + excess H_2O_2 pH 5.1, Fig. 4.1.1.9.), is undoubtedly a serious limitation to this as an anlytical technique, particularly in the case of glucose, where employment of the excess of hydrogen peroxide necessary to achieve a linear plot over extended periods of time seriously affected the stability of the enzyme electrode.

Another obvious drawback to the use of a pH detector to monitor immobilized enzyme catalyzed reactions in continuous flow

methods, particularly in complex biological fluids, is that the sudden presence of any species which will alter the pH of the fluid will, per se, produce a response from the detector. This can be, to a certain extent, overcome by use of relatively high concentration buffers, or, provided that the pH is not altered to such an extent that it affects the rate of the enzyme-catalyzed reaction, the use of stopped-flow methods, as described in Chapter 4, Section 1.1.

Throughout the history of enzyme chemistry, meticulous attention has been paid to factors which influence rates of reaction (e.g. temperature, pH, ionic strength etc.). However, little attention has been taken over the type of transducer used to monitor the enzymic reaction. In particular the plot of apparent urease activity vs. pH of phosphate buffer (Fig. 4.1.1.8) provides an excellent example of how the apparent enzyme activity, under identical conditions may vary according to the nature of the transducer used to monitor enzyme activity. Had it been possible to monitor, for example, the rate of change of urea concentration spectrophotometrically, it would undoubtedly have been found that immobilized urease exhibited maximum activity around pH 7.0, instead of the apparently near minimum activity observed in these experiments.

The spectrophotometric determination of glucose using nylon-tube-immobilized hexokinase and glucose 6-phosphate dehydrogenase described in Chapter 4, Section 2.1., illustrates well the principles involved in the incorporation of nylon-tube-immobilized enzymes in the Technicon Autoanalyzer assembly. This is, in fact, the basis of the Catalinks^{R} now marketed by Miles Laboratories Ltd., Stoke Poges, Bucks. U.K.

Sensitivity, accuracy and precision obtained were all considerably better than in the corresponding thermal technique using nylon tube immobilized enzymes with a thermistor as sensor, and could probably be improved still further by thermostatting the mixing and enzyme coils.

In fact, spectrophotometric techniques appear far more suitable than thermal techniques for combination with this type of immobilized enzyme, due to imperfect adiabaticity preventing the use of long lengths of tubing to achieve high percentage conversions when using thermal detectors.

CHAPTER 5

A study and determination of immobilized enzyme inhibitors.

5.1. A thermal investigation of the inhibition of the cholinesterase system, using an LKB 10700-1 calorimeter, with immobilized enzyme packing, and analytical determinations of some of the stronger inhibitors.

Introduction

Cholinesterases have been amongst the most studied of enzyme systems, playing a vital role in the nervous system of animals in general.

Two cholinesterase (Ch. E.) enzymes have been recognised (E.C 3.1.1.7. and E.C.3.1.1.8.) which have been referred to by a variety of names, but will here be named after their optimum substrates, acetylcholine and butyrylcholine, i.e. acetylcholinesterase (Ac Ch. E.) and butyrylcholinesterase (Bu. Ch. E.), respectively.

Each of these enzymes has been found in a great number of animals, enzymes from each species having slightly different properties (e.g. w.r.t. molecular wt. and inhibition characteristics).

A great number of Ch. E. assay procedures have been described, based on the measurement of acetic acid or other moiety released during hydrolysis, or on the direct measurement of acetylcholine (Ac. Ch.) remaining after hydrolysis. Amongst the more common methods are manometric (measuring the volume of CO_2 released from a bicarbonate solution after reacting with the acid released during the hydrolysis of Ac. Ch. or other choline esters); potentiometric (measuring pH change); colorimetric etc.

In the area of calorimetric analysis, the enzymatic activity after treatment with inhibitors has been confined to the analysis of the

stronger organophosphorous pesticides (206, 207), and a few miscellaneous alkaloids (208), using soluble enzymes; however, little work appears to have been carried out using immobilized cholinesterases, despite the potential advantages of immobilization.

Apart from the well-publicized, irreversible organophosphorous compounds, cholinesterases are also inhibited, reversibly and competitively, by many other compounds which usually contain a quaternary nitrogen atom, or at least a nitrogen atom capable of acquiring a positive charge by protonation (i.e. a basic nitrogen atom), or an ester-like group. The stronger inhibitors are often also highly methylated. Molecules of this overall structure can compete successfully with choline-type substrates, and, accordingly, a number of quaternary ammonium compounds, carbamates and alkaloids inhibit cholinesterases, or one or both types, to a greater or lesser extent. Indeed, some alkaloids owe their pharmacological activity to an inhibitory mechanism (e.g. eserine (209)). Experimentally, it is found that drugs effective in Parkinsonism (including Parkinson's disease and similar nervous disorders) inhibit Bu. Ch. E. much more strongly than Ac. Ch. E., whilst powerful neuromuscular blocking agents inhibit Ac. Ch. E. much more powerfully than Bu. Ch. E. (210).

Consequently, apart from a purely analytical view point, any method which offers a quick comparison of inhibitor strengths may provide a useful screen for initial testing of new drugs, as well as being academically interesting

This chapter is concerned with the comparison of the inhibitor strengths of several reversible inhibitors of cholinesterases on both glass-immobilized acetylcholinesterase (from electric eel), and butyrylcholinesterase (from horse serum) and of analytical

determination of the stronger inhibitors, at varying pHs, and enzymatic activity.

In all cases, cost was considered uppermost, as was availability. Consequently, it was not feasible to use Ac. Ch. E. and Bu. Ch. E. from the same animal source (the cheapest sources were used).

Although hundreds of compounds which reportedly inhibit the cholinesterase system appear in the literature, two criteria were placed upon all potential inhibitors:

1. They must have been readily available from Sigma Chemical Company
2. They must have been reasonably cheaply available (not more than approximately £2 per gramme)

Emphasis was placed as much on evaluation of the technique as on actual experimental results. Where possible, results were compared to the same (calorimetric) technique, using the soluble enzymes.

Reagents

Distilled water was used throughout.

All inhibitors were obtained from Sigma, except $Me_4N^+ Br^-$ and $Et_4N^+ Br^-$ (BDH) and $(n\text{-}Pr)_4N^+ Br^-$ and $(n\text{-}Bu)_4N^+ Br^-$ which were obtained from Eastman Chemical Company.

Acetylcholinesterase (Type III) and Butyrylcholinesterase (Type IV-S) were also obtained from Sigma Chemical Co., U.K.

Imadazole (reagent grade) and THAM (Analar) were obtained from BDH. Where advised, compounds were stored desiccated at 4^oC. Substrates Actylcholinechloride and Butyrylcholineiodide were obtained from Sigma, and stored desiccated at 4^oC.

Butyrylcholine iodide from Koch-Light was a yellow powder, dissolving in buffer to give a yellow solution, which gradually decolourised leaving a slight brown precipitate. Results

obtained using this appeared identical to those obtained using the Sigma reagent.

Immobilization procedures and experimental details.

Acetylcholinesterase (E.C. 3.1.1.7., 1.36 mg, 500 units), and butyrylcholinesterase (E.C. 3.1.1.8., 20 mg, 426 units) were each immobilized to controlled porosity glass (0.5 g, 13.3 $m^2 g^{-1}$ surface area), as described in Chapter 2, Section 1.2. After immobilization the microcolumn of the microcalorimeter was packed approximately one quarter full of the glass-bound enzyme, confined in the microcolumn by the usual means, and buffer pumped through the microcolumn at a rate of 0.43 $cm^3 min^{-1}$ until thermal equilibrium was established, as evidenced by a horizontal baseline. Each series of runs was initiated by running through buffer only until a steady baseline was obtained. Buffer + substrate was then run, until another steady-state was reached. Provided the enzyme was totally saturated, which could easily be demonstrated by increasing the substrate concentration still further, and observing no increase in displacement, the steady-state displacement obtained was a measure only of the enzyme activity in the column (i.e. the reaction was zero order w.r.t. substrate). By comparison of this steady-state displacement with that obtained using the excess of enzyme, and low substrate concentration (when 100% reaction was assumed), one could obtain an "apparent" enzyme activity of the immobilized enzyme column, in terms of international units (I.U.) [one I.U. will react with 1 μ mole of substrate per min]. This is not necessarily the same as the true enzyme activity, but is that which the microcalorimeter actually senses, and would be the true enzyme activity modified by factors such as diffusion of product to enzyme, and substrate and/or product inhibition, etc.

The flow rate used throughout these experiments was 0.43 cm^3 min^{-1}.

A 10^{-2}M solution of inhibitor in buffer/substrate solution was made up by weighing 10^{-4} moles of inhibitor into the lid of 25 cm^3 sample bottle (using a milligram balance), to an accuracy of $\pm$ 0.2 mg (using the vernier scale). For an inhibitor of molecular wt 200 Daltons, this represents $\pm$ 1%, but most inhibitors had molecular weights well in excess of 200. This was dissolved in 10.00 $\pm$ 0.02 cm^3 of buffer/substrate solution by placing the lid plus contents over the 25 cm^3 sample bottle containing the 10.00 cm^3 buffer, pushing the top down until it formed a seal with the sample buttle, and then shaking to dissolve, to give 10 cm^3 of a 10^{-2}M solution of inhibitor in buffer/substrate solution. This solution was then pumped through the microcolumn until another steady-state was obtained (experimentally found to be approximately 20 min), and from the depression of the substrate steady-state, the % inhibition could be calculated, as shown in Fig. 5.1.1.1.

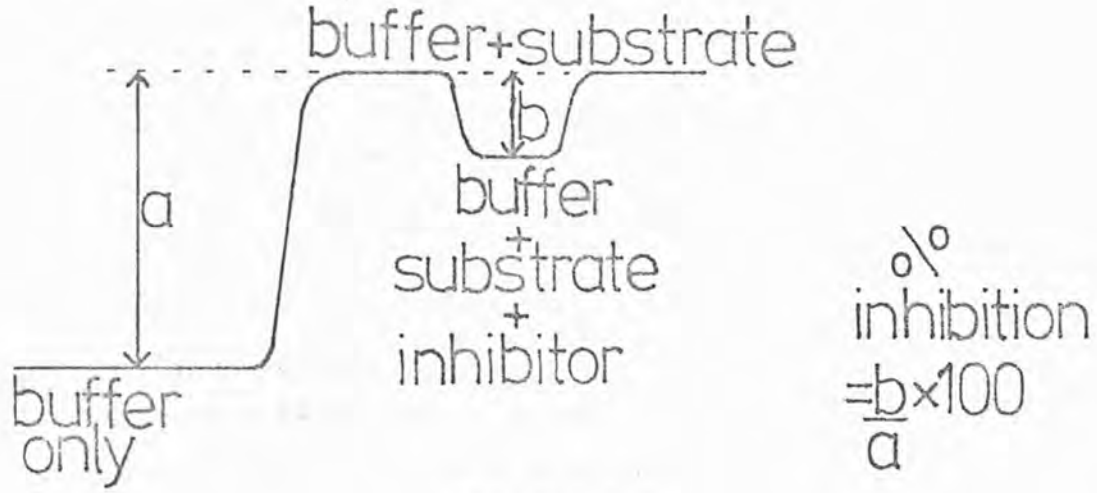


Fig. 5.1.1.1.

Attempts were made to repeat these experiments by using an $0.5\ cm^3$ pulse of substrate solution, followed by a $0.5\ cm^3$ of substrate/inhibitor solution as shown in Fig. 5.1.1.2.

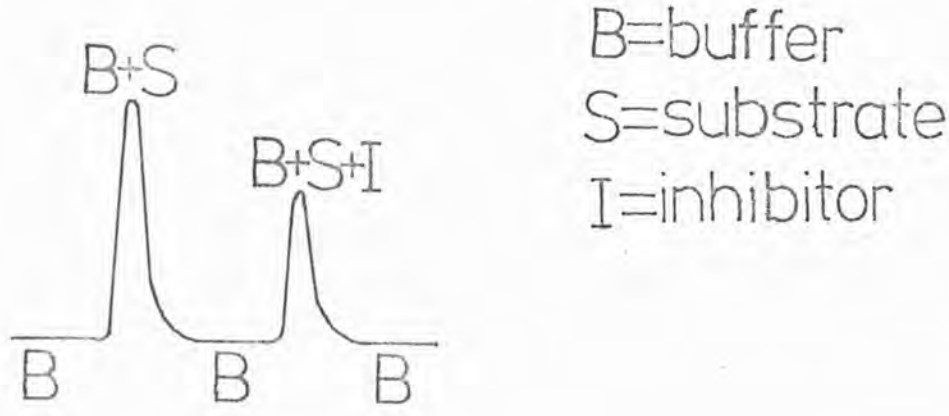


Fig. 5.1.1.2.

However, results using this method gave less reproducible results, as well as lower sensitivity, presumably because a steady-state was never reached, and subsequent runs were carried out using the scheme depicted in Fig. 5.1.1.1. Where lower concentrations of inhibitor were required, they were prepared by appropriate dilution of the 10^{-2}M inhibitor solution with buffer/substrate solution.

Although it was possible to obtain rough measurements by injecting one inhibitor solution directly after another, more reproducible results were obtained by following the 20 min inhibitor injection with 20 min pulse of buffer/substrate solution, as the previous inhibitor then did not interfere with that following, and it was also easier to compensate for any steady-state drift, caused by changes in room temperature, or by slow, non-enzymatic hydrolysis of the substrate by the buffer solution.

All light sensitive solutions (i.e. coloured solutions), were always freshly prepared, and stored in sample bottles covered by aluminium foil.

The concentration of substrate used was originally kept at 50 mM. This was found to be adequate for the low-activity columns, at pH 7.0 or 7.8. For the higher-activity columns, 100 mM substrate solution was needed to give zero order kinetics, as it was for runs at pH 8.5, where non-enzymatic hydrolysis by the alkaline buffer solution necessitated the use of excess substrate.

Results

For acetylcholinesterase two columns of varying apparent enzyme activities were used.

1. With excess substrate, Col. I gave approx. 80 units steady-state deflection on 30 uV scale
2. With excess substrate, Col II gave approx. 32 units steady-state deflection on 300 uV scale
3. From the steady-state analysis runs, it was known that 100 unit deflection on 10 uV scale was given by a 1.5 mM solution of acetylcholine chloride.

All the above results were for THAM buffer, pH 7.8 and a flow rate of $= 0.43\ cm^3\ min^{-1}$

$\therefore$ activity of column (in u mole min^{-1}) $= 1.5 \times 10^3 \times \frac{0.43}{1000} \times \frac{defl.}{100}$

$\therefore$ activity of Col. I 1.55 I.U.

activity of Col. II 6.2 I.U.

For Butyrylcholinesterase, only one column was used which, by an analogous calculation, was shown to have an activity of

2.3.I.U.

The percentage inhibition given by a 10^{-2}M solution of the following inhibitors, on the enzymes acetylcholinesterase and butyrylcholinesterase, immobilized on controlled porosity glass at pH 7.0, 7.8, 8.5, is given in Table 5.1.1.3., and that given by a 10^{-3}M solution of some of the stronger inhibitors is listed in Table 5.1.1.4. Tables of percentage inhibition vs. conc. of a few of the strongest inhibitors are given in Tables 5.1.1.5 and 5.1.1.6.

TABLE 5.1.1.3.

Table of % inhibition of a 10^{-2}M solution of inhibitor on glass immobilized acetylcholinesterase and butyrylcholinesterase at pH 7.0, 7.8, 8.5.

( ) Parentheses indicate which column was used in acetylcholinesterase runs
* For key to structure characteristics, see appendix 3, and for complete structures, see appendix 4.
** Light sensitive

Compound	Structure characteristics*	% inhibition (10^{-2}M) Acetylcholinesterase						% inhibition (10^{-2}M) Butyrylcholinesterase		
		pH 7.0		pH 7.8		pH 8.5		pH 7.0	pH 7.8	pH 8.5
		(I)	(II)	(I)	(II)	(I)	(II)			
Antazoline PO_4	E, G, I		26 24		22 26		27 25	75	100	95
Antipyrene	G, J		≤5		≤5		≤5	50	60 57	42
Arecoline HCl	G, J		≤5		≤5		≤5	14	14	12
Atropine SO_4	G, I, J		≤5	6 8 10	7		≤5	35	41	13
Atropine Methyl NO_3	F, I, J		≤5		≤5		≤5	34	42	21
Azosulphamide** (satd. soln.)	H, I, J		≤5		≤5		≤5	≤5	≤5	≤5
Barbituric Acid	H, J		≤5		≤5		≤5	≤5	≤5	≤5

Table 5.1.1.3. - continued.

Compound	Structure characteristics*	% inhibition (10^{-2}M) Ac.Ch.E. pH 7.0 (I)	pH 7.0 (II)	pH 7.8 (I)	pH 7.8 (II)	pH 8.5 (I)	pH 8.5 (II)	% inhibition (10^{-2}M) Bu.Ch.E. pH 7.0	pH 7.8	pH 8.5
p-Aminobenzoic Acid	H, I, J		<5		<5		<5	<5	<5	<5
d-Biotin (satd. soln.)	H, J		<5		<5		<5	<5	<5	<5
Caffeine	B, G, J		56	54 55 55 51 49	68		53	9	10	10
Diphenhydramine HCl	G, I		18		13		11	100	92	65
Eserine SO_4 **	A, G, I, J	100		100		100		100	100	100
Fluoride	-		95 91 93	38 46 42	42	17 20	17	100	71	21
Folic Acid	D, E, H, I, J		<5		<5		<5	8	12	<5
Glycine	H, J		<5		<5		<5	<5	<5	<5
Hexamethonium Bromide	F		<5		<5		<5	8	10	8
Histamine HCl	B, H		<5		<5		<5	10	13	8

Table 5.1.1.3. - continued

Compound	Structure characteristics*	% inhibition (10^{-2}M) Ac.Ch.E. pH 7.0 (I)	pH 7.0 (II)	pH 7.8 (I)	pH 7.8 (II)	pH 8.5 (I)	pH 8.5 (II)	% inhibition (10^{-2}M) Bu.Ch.E. pH 7.0	pH 7.8	pH 8.5
Imidazole	B		<5		<5		<5	<5	<5	<5
Morphine (satd. soln.)	G, I		<5		<5		<5	<5	<5	<5
Methylene Blue	C, G, I	100		100		100		97	98	95
Naphazoline HCl	B, I	32	28	29	29	29	28	95	100	98
Nicotine SO_4**	A, C		10	12			9	28	32	25
Niacinamide (Nicotinamide)	C, J		<5		<5		<5	<5	<5	<5
D-Pantothenic Acid (Ca. salt)	J		<5		<5		<5	<5	<5	<5
Phenazine Methosulphate**	E, F, I		86 90	87	95		85	100	100	100
Phosphate	-		<5		<5		<5	<5	<5	<5
Pilocarpine NO_3	B, G, J		24	34 40 34	36		18	26	26 23	20
Procaine HCl	G, H, I, J		8	18 16 14 17	14		8	90	83 83	79

Table 5.1.1.3. - continued

Compound	Structure characteristics *	% inhibition (10^{-2}M) Ac.Ch.E. pH 7.0	pH 7.8	pH 8.5	% inhibition (10^{-2}M) Bu.Ch.E. pH 7.0	pH 7.8	pH 8.5
		(I) (II)	(I) (II)	(I) (II)			
Putrescence 2HCl	H	≤5	≤5	≤5	≤5	≤5	≤5
Pyridine-2-aldoxime** Methiodide	C, F	19 21	23 22	18 20	36	42	37
Pyridoxal HCl	C	9	24 24 24 26 26	24 24 24	13	21	20
Pyridoxine HCl	C	≤5	≤5	≤5	12	13	7
Quinacrine 2HCl **	C, G, H, I	60 65	62 66	63 60	100	100	91
Quinine SO_4	C, G, I	10	11 16 15	12	86	97	80
Riboflavine ** (satd. soln.)	D, I, J	≤5	≤5	≤5	≤5	≤5	≤5
(-)Scopolamine HBr	G, I, J	≤5	≤5	≤5	20	17	9
L(-)Sparteine	G	20 21	23 22	20 23	81	93	77
Sulphanilamide	H, I	≤5	≤5 ≤5	≤5	≤5	≤5	≤5
THAM	H	≤5	-	-	≤5	-	-
Thiamine HCl	D, F, H	21 20	28 36 32 32	23	18	24 26	32

Table 5.1.1.3. - continued

Compound	Structure characteristics*	% inhibition (10^{-2}M) Ac.Ch.E. pH 7.0	pH 7.8	pH 8.5	% inhibition (10^{-2}M) Bu.Ch.E. pH 7.0	pH 7.8	pH 8.5
		(I) (II)	(I) (II)	(I) (II)			
Tolazoline HCl	B, I	40 37	40 45	33 35	80	97 96	85
Trimethylphenyl Ammonium I	B, I	19	29 26	17 17	18	23	19
Tetramethyl Ammonium Br	F	<5 <5	<5 <5	<5 <5	<5	<5	<5
Tetraethyl Ammonium Br	F	<5 <5	<5 <5	<5 <5	<5	<5	<5
Tetra (n-Propyl) Ammonium Br	F	37 37 35 35	45 48 49	36 38	27	34	19
Tetra (n-Butyl) Ammonium Br	F	22 26	25 23	26 23	40	46	38
Citric Acid	-	-	-	<5	-	-	14
Ascorbic Acid	-	<5	<5	<5	<5	<5	<5
Maleate	-	<5	<5	<5	<5	<5	<5

TABLE 5.1.1.4

Inhibition of glass-immobilized butyrylcholinesterase by a 10^{-3} M solution of some of the stronger inhibitors

	Structure characteristics	Bu. Ch. E.		
		pH 7.0	pH 7.8	pH 8.5
Antazoline PO_4	B, G, I		66	
Diphenhydramine HCl	G, I	74	37	17
Naphazoline HCl	B, I	81	72, 72	66
Procaine HCl	G, H, I, J	18	30, 30	25
Phenazine methosulphate	E, F, I	93	85	78
Quinine SO_4	C, G, I	18, 17	51	39
Quinacrine, 2 HCl	C, G, H, I	50	69	61
L(-)Sparteine SP. 4	G	24	27	25
Tolazoline HCl	B, I	41	44	38

TABLE 5.1.1.5

Inhibition vs. conc. of inhibitor for glass immobilized butyrulcholinesterase

a)

conc./mol dm^{-3}	% inhibition	
	phenazine Me SO_4	naphazoline
10^{-2}	100	100
10^{-3}	93	80
5×10^{-4}	82	64
2.5×10^{-4}	68	48
1.25×10^{-4}	54	34
6.3×10^{-5}	39	23
3.1×10^{-5}	25	16
1.6×10^{-5}	17	10
8×10^{-6}	10	4
4×10^{-6}	3	0
min. mass detectable	10 µg (as phen. $MeSO_4$)	15 µg (as naphazoline)

b)

conc./mol dm^{-3}	% inhibition by a 30 min pulse of eserine	
10^{-2}	100	
10^{-3}	100	
1.6×10^{-4}	100	
8.0×10^{-5}	89	
4.0×10^{-5}	62	min. mass
2.0×10^{-5}	35	detectable $\simeq$ 3.5 µg
1.2×10^{-5}	22	
6×10^{-6}	12	
1×10^{-6}	6	
5×10^{-7}	2	
1×10^{-7}	0	

TABLE 5.1.1.6

Inhibition of glass-immobilized acetylcholinesterase by caffeine.

conc./mol dm^{-3}	% inhibition
4×10^{-2}	76
2×10^{-2}	64
1×10^{-2}	51, 54
5×10^{-3}	38
2.5×10^{-3}	32
1.25×10^{-3}	23
6.2×10^{-4}	14
3.1×10^{-4}	9
min. mass detectable	500 μg

Using buffer at pH 7.0, or below, F^- could be detected down to concentrations of 1×10^{-5}M. However, due to the extreme sensitivity of fluoride inhibition to pH, absolute inhibition values were only reproducible if the same buffer/substrate solutions were used for each of the runs (minimum mass detectable 21.5 μg of F^-).

A 30 min pulse of the pesticides malathion (saturated solution, 455 ppm), or dimethoate (10^{-2}M), failed to produce any noticeable inhibition of either glass immobilized enzyme.

Discussion of results

Throughout these series of experiments, the assumption has been made that soluble and immobilized enzymes will be inhibited by the same inhibitors, and by the same mechanisms. Although the absolute degree of inhibition for a given concentration of inhibitor may be greater, or lesser, on going from a soluble enzyme to the immobilized enzyme, the order should be the same, if this method is to be of any use in screening for potential inhibitors of enzymes.

A limited study of the inhibition of alkaloids on horse-serum-butyrylcholinesterase has been carried out, using soluble enzymes, and a thermistor as heat sensor by Grime and Tan (208) to study the inhibition of eserine, quinine, procaine, atropine, morphine, codeine, pilocarpine, thiamine and caffeine at varying concentrations.

In this work, 10^{-2}M was the concentration most used. Codeine was unobtainable and morphine was not soluble enough in buffer to give a 10^{-2} M solution.

A comparison of results is given below:

% Inhibition of soluble β^{208} and immobilized horse serum Bu.Ch.E. by some inhibitors (10^{-2}M)

10^{-2}M	sol. Bu. Ch. E. β^{208} (pH 8.0)	Immobilized Bu.Ch.E. (pH 7.8)
Eserine	100	100
Quinine	100	97
Procaine	95	83
Atropine	70	41
Thiamine	65	25
Pilocarpine	55	24
Caffeine	55	10

FIG. 5.11.7 Plot of % inhibition vs. p[I], for phenazine methosulphate(x-x) and naphazoline(o-o) on glass-immobilized BuChE

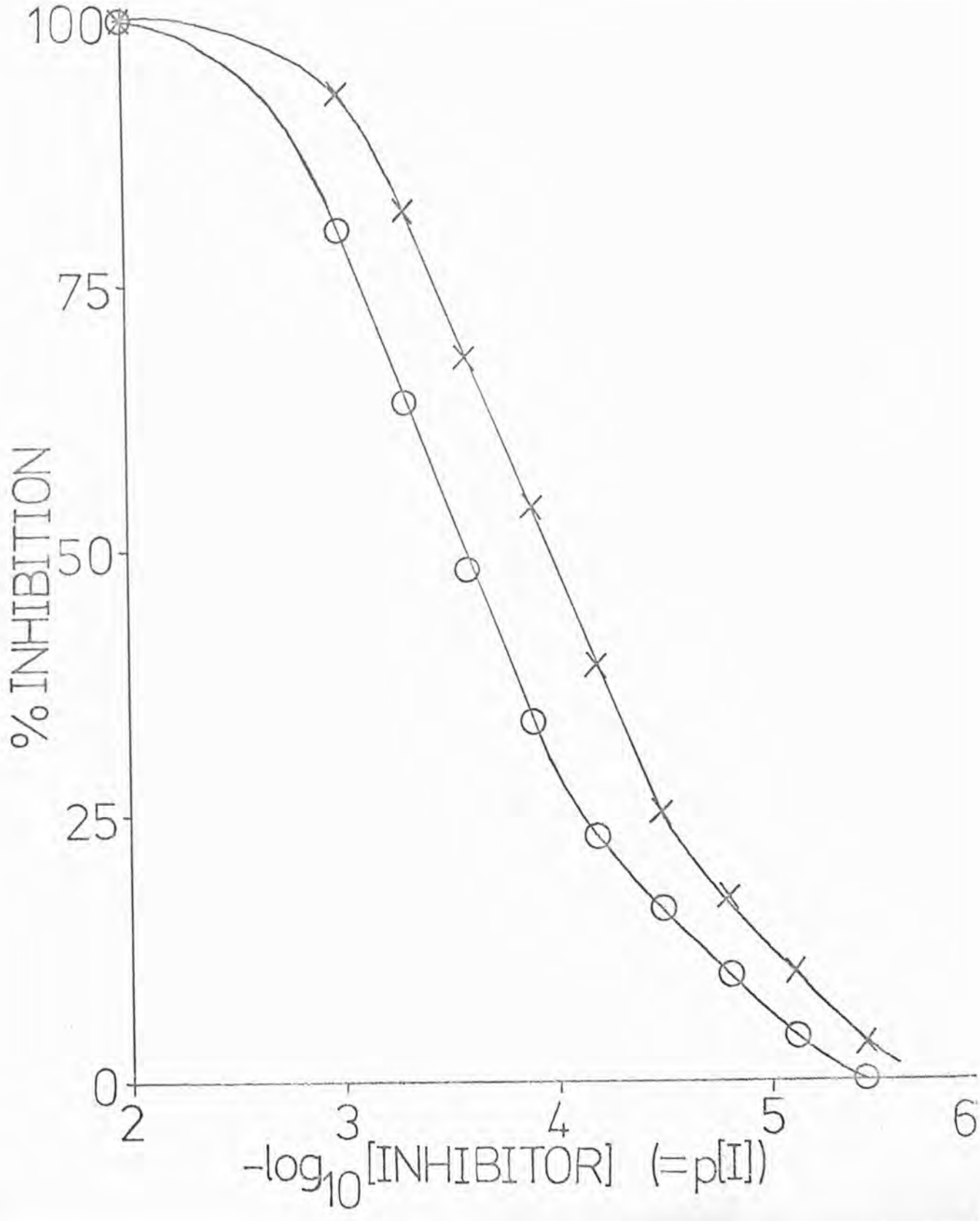


FIG 5.1.8 Plot of % inhibition of glass-immobilized BuChE by eserine

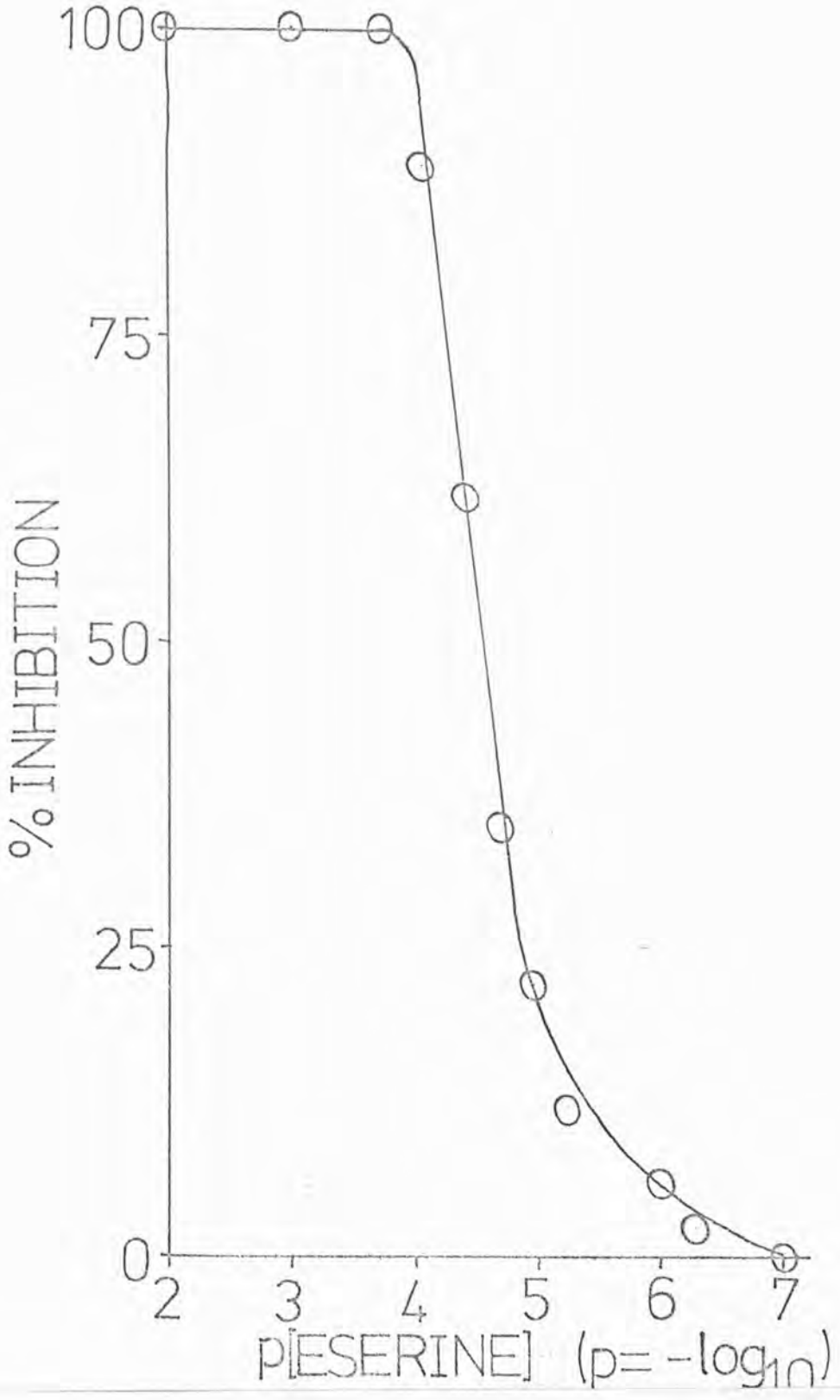
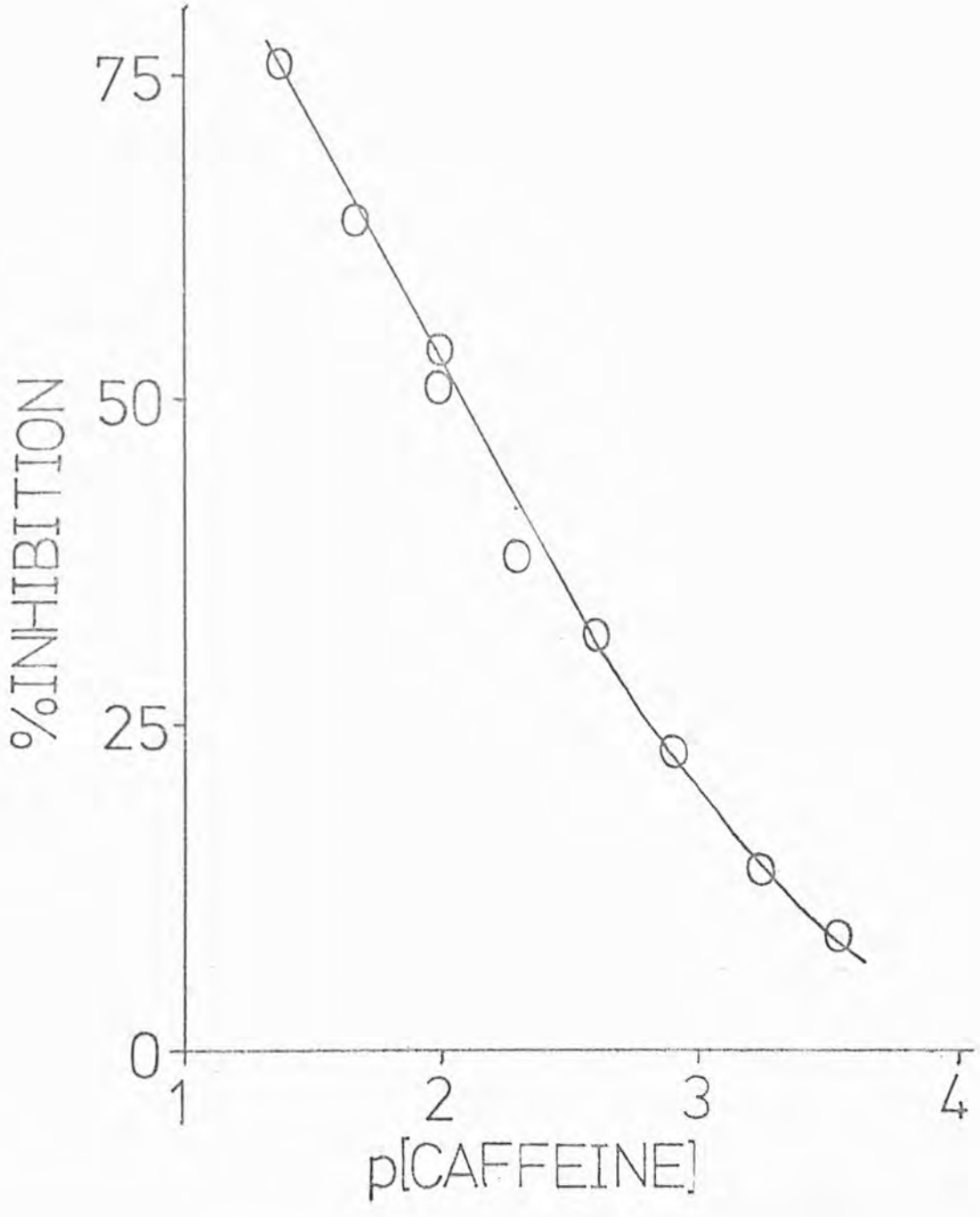


FIG. 5.1.1.9. Plot of %inhibition of glass-immobilized AcChE by caffeine



Grime and Tan (208) quote the inhibitory power of the alkaloids as follows:

eserine $\gg$ quinine $>$ procaine $>$ atropine $>$ thiamine $>$ pilocarpine $>$ caffeine.

This trend is reflected exactly in this work, thus justifying this method as useful for the comparison of inhibitory power.

Although many compounds have been reported in the literature to inhibit the cholinesterase system, quantitative data is scarce, and even those results that are quoted will probably not stand up to comparison, because of the different methods, and conditions, used to determine inhibition. Most investigations classify inhibitors simply as strong, weak or non-inhibitors.

The probable reason for the lack of comparable, quantitative data in the literature for the reversible inhibitors of the cholinesterase system is due to either the length of time taken (including equilibration time) for one run, difficulty in using exactly the same activity of enzyme per run, and the expense of using soluble enzymes, or a combination of all three. This method, although less sensitive than those using soluble enzymes, circumvents all these difficulties, and should therefore prove potentially useful. However, it should be stressed that for irreversible inhibitors, many of the advantages of using immobilized enzymes (e.g. re-usability) would be lost, unless it could be shown to be possible to reactivate the immobilized enzyme (e.g. by pyridine-2-aldoxime methiodide, which is known to reactivate the soluble enzyme when inhibited by certain organophosphorous nerve gases or pesticides).

Another weakness of such a technique is its relative lack of sensitivity to weaker inhibitors (even when compared to the inhibition of the soluble enzyme). Such insensitivity has been exposed by the inability of this technique to detect either of the weakly inhibiting organophosphorous pesticides Malathian (saturated solution, 455 ppm) or dimethoate (10^{-2}M).

The actual degree of inhibition obtained appeared largely independent of the activity of enzyme immobilized (provided that it was kept saturated with substrate), although the high enzyme activities did permit lower amplications to be used, thereby improving baseline qualities.

Choice of buffer and pH

Although a large proportion of the inhibition studies were carried out at pH 7.8 in0.1M THAM HCl buffer, studies made at higher (pH 8.5) and lower (pH 7.0) values did present some problems.

At pH 8.5 (THAM HCl buffer) great difficulty was experienced in keeping the immobilized enzyme saturated with substrate, due to a rapid, non-enzymatic hydrolysis of both choline esters (Ac.Ch and Bu.Ch) under the mildly alkaline conditions, and it is this effect (i.e. incomplete substrate saturation of the immobilized enzyme) that is believed to be mainly responsible for the apparently slightly lower inhibitory power of many compounds at pH 8.5.

It should be stressed that the buffer used (250 mM THAM) may inhibit the enzyme appreciably (10^{-2}M THAM, using imidazole buffer, appears to produce a very small inhibition) but this should not matter appreciably as the inhibition will be constant, and only a loss of sensitivity will occur, due to the inhibitor having to compete with THAM, as well as with the substrate, for the active

site.

Using an "oxygen" base buffer offers no advantage whatsoever. Phosphate and citrate also inhibit slightly at 10^{-2} M concentration, although inhibition using citrate could only be carried out at pH 8.5, due to an unacceptable reduction in the buffer capacity of the system when citric acid was added to THAM buffer at pH 7.8. Maleate buffer, for which there was no evidence of inhibition at 10^{-2}M, only gave a low signal, due to its having a low enthalpy of protonation.

Runs at pH 7.0 were carried out in 250 mM imidazole buffer, due to the poor buffer capacity of THAM at pH 7.0, with some of the more acidic inhibitors. The signal given with imidazole as buffer was less than with THAM, due probably either to the fact that the enthalpy of protonation of imidazole is less than that of THAM, that imidazole at 250 mM inhibits the enzyme (having a similar structural characteristic to some of the more powerful inhibitors, although little inhibition appears at 10^{-2}M) or that the enzyme is not as active as pH 7.0 as at pH 7.8, or a combination of all three.

As mentioned earlier, it appeared that the degree of inhibition of each inhibitor varied with pH, as a rule of thumb, being greater at pH 7.8 than at either pH 7.0 or 8.5, and this was ascribed largely to inhibition by the buffer solution (pH 7.0) and non-zero order kinetics (pH 8.5), and so it is probably not fair to compare inhibition with pH change too rigorously.

However, the striking case of the fluoride ion cannot be explained by these hypotheses alone. Invariably, at pH 8.5, 10^{-2}M fluoride inhibited slightly, but at pH 7.0 it inhibited very strongly indeed, 8 cm^3 of a 10^{-5}M solution being easily detectable. It seems,

therefore, reasonable to assume inhibition by HF, rather than F^-. Other notable features were the fact that neither NMe_4^+ or NEt_4^+ inhibit either enzyme noticeably, whereas $N(n\text{-}Pr)_4^+$ and $N(n\text{-}Bu)_4^+$ inhibit both fairly strongly, (see Fig. 5.1.1.10). This was very surprising in view of the fact that the substrate has the formula

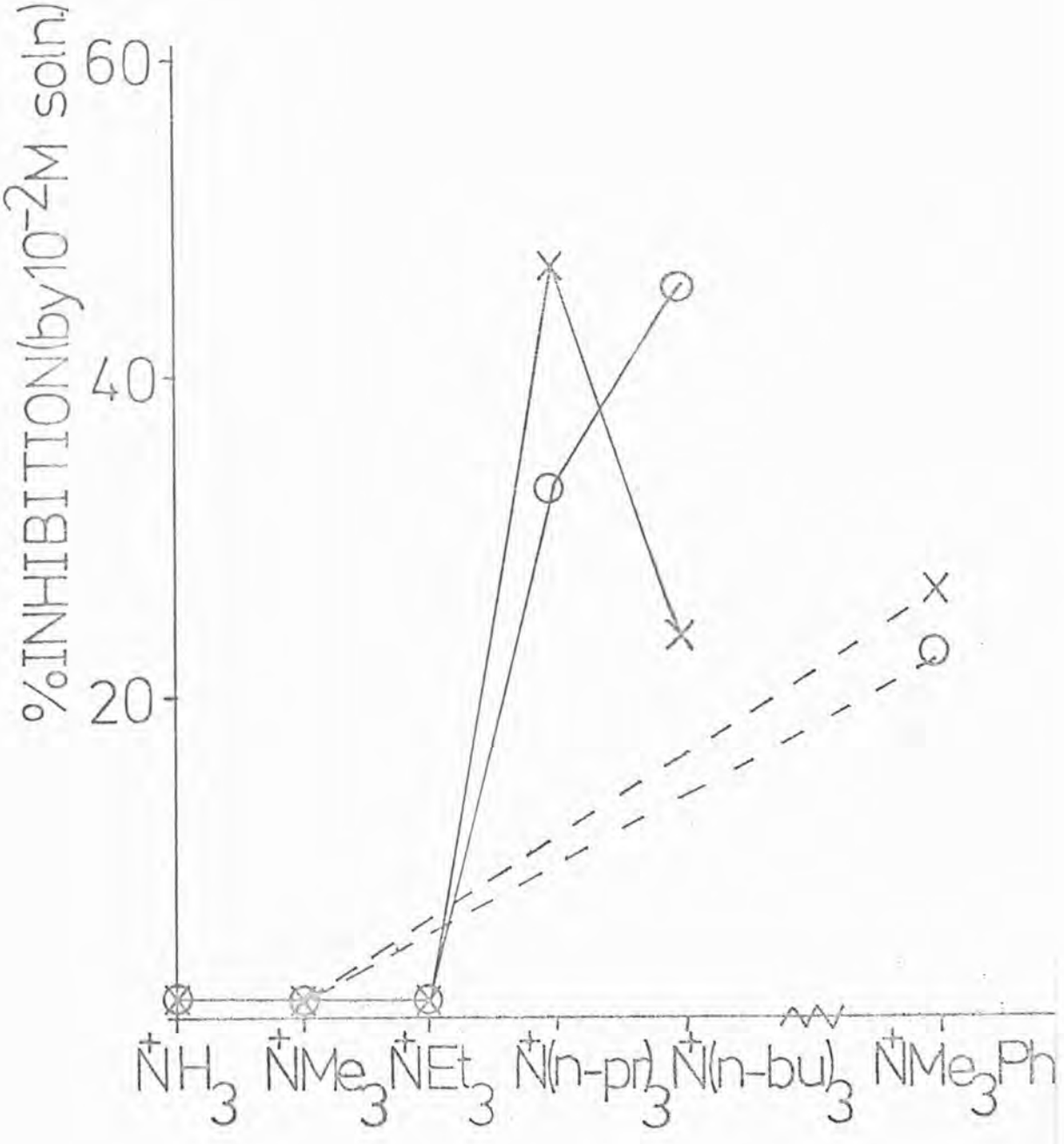
$$Me - \underset{Me}{\overset{Me}{N^+}} - (CH_2)_2 - O - CO \cdot \text{or} \quad \overset{CH_3}{\underset{C_3H_7}{}}$$

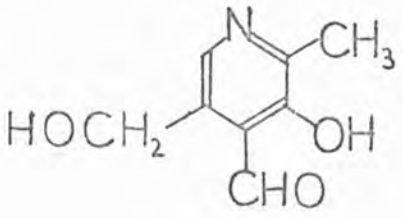
- so one would empirically have expected strongest inhibition from NMe_4^+, by virtue of the fact it most resembled choline. The observation that increasing the chain lengths of the alkyl groups in the tetra-substituted ammonium ions leads to an increase in inhibition given by equal concentrations of these ions (up to tetra n-propyl for Ac.Ch.E. or up to tetra n-butyl for Bu.Ch.E.), and that replacement of one methyl group in the tetramethyl ammonium ion by one phenyl group led to an increase in inhibition in both cases, is taken as evidence that hydrophobic, as well as ionic interactions, play an important part in the binding of inhibitors to the anionic site (see Fig. 5.1.1.11.).

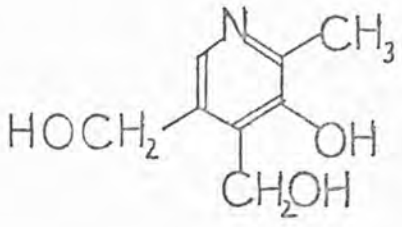
The relative insolubility of tetra n-pentyl ammonium salts in the aqueous buffer used (0.1M THAM, 0.01M EDTA, pH 7.8), precluded investigation of tetra-substituted ammonium ions of alkyl chain length greater than 4 carbon atoms.

Another interesting case was the comparison of pyridoxal with pyridoxine

FIG. 5.1.1.10. Illustration of the influence of carbon chain length on the inhibition of glass-immobilized AcChE (x–x) and BuChE (o–o) by a series of substituted ammonium ions.



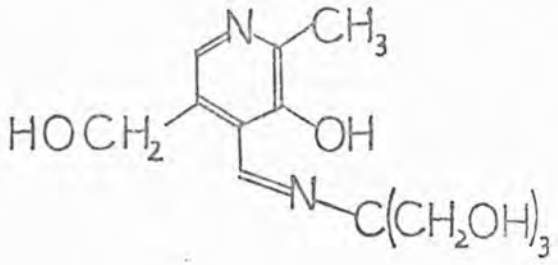




Pyridoxal Pyridoxine

Pyridoxine was invariably a fairly weak inhibitor of both enzymes. Pyridoxal was a much stronger inhibitor, particularly in alkaline THAM buffers, when its solution turned yellow.

It seemed that a large part of the extra inhibition of pyridoxal may have been due to the formation of a Schiff base complex between pyridoxal and THAM which might have been a stronger inhibitor than pyridoxal itself.



Pyridoxal/THAM compound

It was also noticeable that, on average, glass immobilized butyrulcholinesterase appeared to be inhibited more strongly than acetylcholinerase, particularly when benzene rings (structural group I) were present.

e.g. antazoline, antipyrene, atropine, atropine methyl sulphate, diphenhydramine, naphazoline, procaine, quinacrine, quinine, (-)scopolamine, L (-) sparteine and tolazoline inhibit Bu.Ch.E. much more strongly than Ac.Ch.E.

Only caffeine appeared to inhibit Ac.Ch.E. much more strongly than Bu.Ch.E.

For very strong inhibitors, and especially at low concentrations (e.g. 10^{-6} Meserine), a steady-state did not appear to be reached for a very long time, presumably due to the extreme stability of the enzyme-inhibitor complex, and a simple progressive inhibition was seen. Consequently, in the analysis of eserine, a 30 min pulse was always injected, and the percentage inhibition taken at the end of this pulse, regardless of whether or not a steady-state had been reached.

Plots of % inhibition vs $-\log_{10}$[conc. of inhibitor] produced curves with a sigmoid shape, which appear often when soluble enzyme inhibition is studied. Analytically, the linear range is the most useful region of these curves and is usually in the range 25%-80% of total inhibition. However, because the cholinesterase system appeared to be inhibited by so many compounds, in the analytical determination of any particular inhibitor, care would have to be taken to ensure the solution was free from all other inhibitors of comparable strength, which would interfere. As the method relied solely on the inhibiting capacity of the solution used, this method would be expected to be most effective for the

strongest inhibitors.

In this study, eserine was undoubtedly the strongest inhibitor found, but some nerve gases and other organophosphorous compounds are known to inhibit cholinesterases at least 100 times more powerfully than eserine; using this method, concentrations in the range of $10^{-9} \longrightarrow 10^{-10}$ M should be able to be determined with ease.

Various attempts have been made to correlate structure with inhibitory power for organophosphorous inhibitors, but little appears to have been done with the reversible alkaloid inhibitors. For molecules with a structure similar to choline esters (i.e. positively charged, or basic nitrogen atom, with an ester-like group - e.g. procaine), it would be easy to envisage inhibition occurring. However, it seems that all that is really necessary is either a positive nitrogen atom (e.g. $(n\text{-}Pr)_4N^+$, or one that can become positive by protonation, in order to effect a fair degree of inhibition.

A simplified picture of the active site in cholinesterases is as follows:

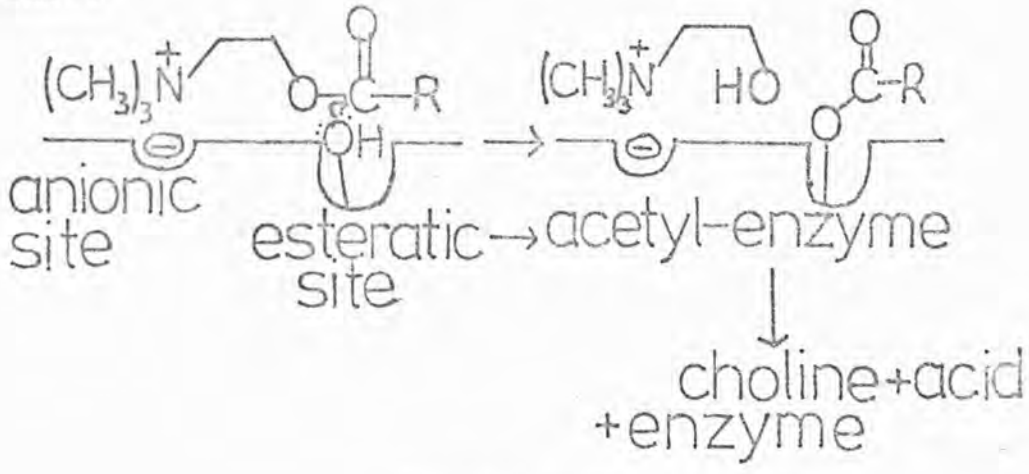


Fig. 5.1.1.11.

In order to react, a potential substrate molecule must be "held" by the electrostatic attraction between the anionic site and a positive charged atom (nitrogen) whilst its ester group is attacked (hydrolysed), by the esteratic site. In principle, groups which can block either of these sites will inhibit.

As most of the inhibitors studied have either a positive, or a basic, nitrogen atom, these should, in principle, be capable of inhibiting cholinesterases, the strength of inhibition presumably being determined by the strength of attraction (i.e. the "fit") between the positive centre in the inhibitor and the anionic site.

The inhibition of Ch. E. by NaF is reported to be of the uncompetitive type, with fluoride adding not only to the free enzyme, but also to the enzyme-substrate complex (blocking acetylation of the enzyme) and to the acetyl enzyme (blocking de-acetylation).

In conclusion, it would appear that this method may be of some interest in the study of reversible inhibitors of enzymes. Because of the different microenvironments of soluble, and of immobilized enzymes, absolute quantitative data (e.g. pI_{50} of inhibitors) will differ. These series of experiments demonstrate that, in a series of inhibitors, the relative inhibitor strengths do not appear to be altered.

Despite the lower sensitivity of immobilized enzymes, over soluble enzymes, the ease of the method, and the relatively short analysis time, make this an interesting method for studying series of inhibitors, and in the screening for, and analysis of, stronger inhibitors.

Chapter 5. Section 2.

Trace determination of some heavy metal ion inhibitors of immobilized urease, using the LKB 10700-1 microcalorimeter as a monitor of enzyme activity.

The inhibitory effect of many heavy metal ions upon enzymes in solutions is a well-known phenomenon (212). However, little advantage appears to have been taken of this effect when the enzyme is coupled to an insoluble matrix, despite the potential advantages of immobilization. Baldridge and Jespersen (213) stress the fact that, in the analytical determinations of inhibitors of soluble enzymes, identical activities of enzyme must be used in each case. This often presents difficulties, and hence leads to irreproducibility, especially when enzymes of low stability are used.

The use of immobilized enzymes would appear to help overcome this problem, as well as enabling more efficient use of the enzyme, provided a suitable step to remove the inhibiting species, and regenerate full enzyme activity after each inhibition could be found.

The urea/urease system was chosen for this study, principally because metal ions known to inhibit the enzyme in solution (principally Hg^{2+}, Ag^{+} and Cu^{2+}) had already been determined by their inhibitory effect on soluble urease (213, 214). Attempts to adapt this process to inhibitor determinations using immobilized urease are described in this section using a thermal (LKB 10700-1) detection system.

In contrast to the type of inhibition in the previous section, inhibition of urease by heavy metal ions is of the non-competitive type, and hence inhibition would be expected to be proportional

to inhibitor concentrations, and independent of substrate concentration.

Reagents

Mercury(II) chloride(Analar), silver nitrate (99.8% pure), copper(II) chloride(Analar), cadmium sulphate (lab. reagent, cobalt(II) chloride (lab. reagent), nickel bromide (lab. reagent), lead nitrate (99% pure), manganese(II) sulphate (lab. reagent), EDTA (Analar) and urea (99% + pure) were obtained from B.D.H. Chemicals Ltd. U.K.

Potassium iodide (99.5%) was obtained from May and Baker Ltd., Dagenham, Essex. U.K.

L-cysteine (pure) was obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks. U.K.

Urease (Type C-3, from jack beans) was obtained from Sigma Chemical Co. U.K.

All other reagents were obtained as stated in Chapter 2.

Immobilization procedure

Urease (3.6 mg, 250 units) was immobilized on to controlled porosity glass (0.2 g, 216.9 $m^2 g^{-1}$ surface area), as described in Chapter 3, Section 2.2. The apparent solubility of this type of urease in the buffer solution used to dissolve it before immobilization was often found to be low, but could be readily overcome by addition of a trace of 2-mercapto-ethanol or dithiothreitol to the enzyme.

Experimental procedure and results

After immobilization, the microcolumn of the LKB 10700-1 microcalorimeter was packed approximately half-full of the immobilized enzyme, in the usual manner, and equilibrated by passing the working buffer for these experiments (0.1M maleate, pH 6.5 obtained by adjusting the pH of a 0.1M solution of maleic acid

to pH 6.5 with potassium hydroxide). All details of the experimental set-up are as described in Chapter 3, Section 2.1. Maleate was chosen as buffer, because of the insolubility of many of the metal ions used in this study in phosphate buffer.

When thermal equilibration was complete, urea solutions of increasing concentration in the working buffer were passed over the immobilized enzyme (flow rate 0.43 cm^3 min^{-1}), until the flat-topped peaks obtained showed no significant increase with increasing urea concentration, whereupon the heat signal recorded would reflect only the activity of the immobilized enzyme. After establishment of the "zero order" steady-state displacement, (termed 100% enzyme activity) a 10 min pulse of a known concentration of metal ion, in the buffer/urea solution was passed over the column, and, from the enzyme activity remaining after this 10 min pulse, the % inhibition caused by the known concentration of the metal ion could be easily calculated. The 10 min pulse of metal ion was followed by a 10 min pulse of freshly prepared 0.25M L-cysteine dissolved in the buffer/urea solution, to remove the bound metal ions, and also to reduce any disulphide bridges formed by oxidation of thiol groups whilst the metal had been bound. The observations of Mattiasson et al (211) that 0.3M I^-, plus 0.01M EDTA will also reactivate metal ion inhibited urease (see discussion later in this section), was verified in this study.

Following the passage of reactivator, the buffer/urea solution was again passed over the immobilized enzyme, until a steady-state was again established, and the system was ready for passage of another pulse of inhibitor.

A typical trace is shown in Fig. 5.2.1.1.

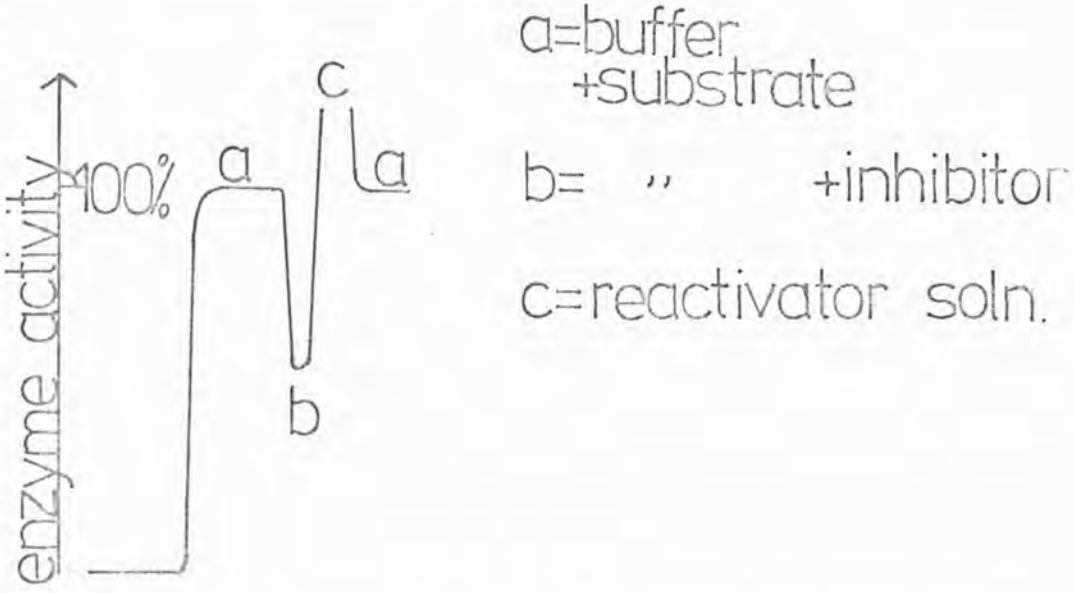


Fig. 5.2.1.1.

The apparent increase of enzyme activity to >100% during the passage of (c) is doubtless due to an extra dilution enthalpy change because of the mismatch of pH and ionic strength of solution (a) or (b) and (c). A 10 min pulse of re-activating solution (c) was generally sufficient to restore full enzyme activity, particularly with Hg^{2+}, although occasionally, using Ag^{+} or Cu^{2+} as the inhibiting ion, difficulty was experienced in regaining full enzyme activity, due to some unidentified effect.

Using mercury as the inhibiting ion, concentrations in the range $10^{-3}M \longrightarrow 10^{-5}M$ could be determined, with reasonable reproducibility ($\pm$ 5%). Nonetheless, for accurate work a calibration with an accurately known concentration of Hg^{2+} of a similar concentration is recommended, to help compensate

for factors that could interfere such as trace contamination from the tubing leading to the reaction microcolumn by the re-activator, thus effectively reducing the concentration of the inhibiting metal ion reaching the immobilized enzyme. That this was a factor influencing inhibition could be demonstrated by the observation that the first inhibition of immobilized enzyme (where no reactivator had been introduced into the system) was often greater than expected from subsequent calibrations.

A table and plot of % inhibition vs. conc. of Hg^{2+} + Cu^{2+} are given in Table 5.2.1.2. and Fig. 5.2.1.3. respectively. Similar studies were made on Ag^{+} but this appeared to show greater irreproducibility.

The relative inhibitor strengths of some metal ions on glass-immobilized urease are shown in Table 5.2.1.4., where % inhibitions given by identical concentrations of metal ions are given.

TABLE 5.2.1.2.

Table of inhibition vs. conc. of Hg^{2+} using glass immobilized urease

conc./mol dm^{-3}	% inhibition (Hg^{2+})		% inhibition (Cu^{2+})	
		mean		mean
1.0×10^{-5}	2, 1.5, 2	1.8	-	
3.0×10^{-5}	5, 4, 4	4.3	-	
1.0×10^{-4}	17, 15, 17	16.3	4, 3, 3	3.3
2.0×10^{-4}	30, 28, 31	29.7	7, 7, 6	6.7
3.0×10^{-4}	43, 40, 46	43.0	10, 9.5, 11	10.2
5.0×10^{-4}	57, 53, 59	56.3	16, 14, 18	16.0
7.5×10^{-4}	72, 67, 75	71.3	28, 25, 30.5	27.8
1.0×10^{-3}	85, 79, 86	83.3	37, 35.5, 37	36.5

FIG. 5.21.3. Plot of inhibition of glass-immobilized urease by Hg^{++} (o-o) and by Cu^{++} (x-x)

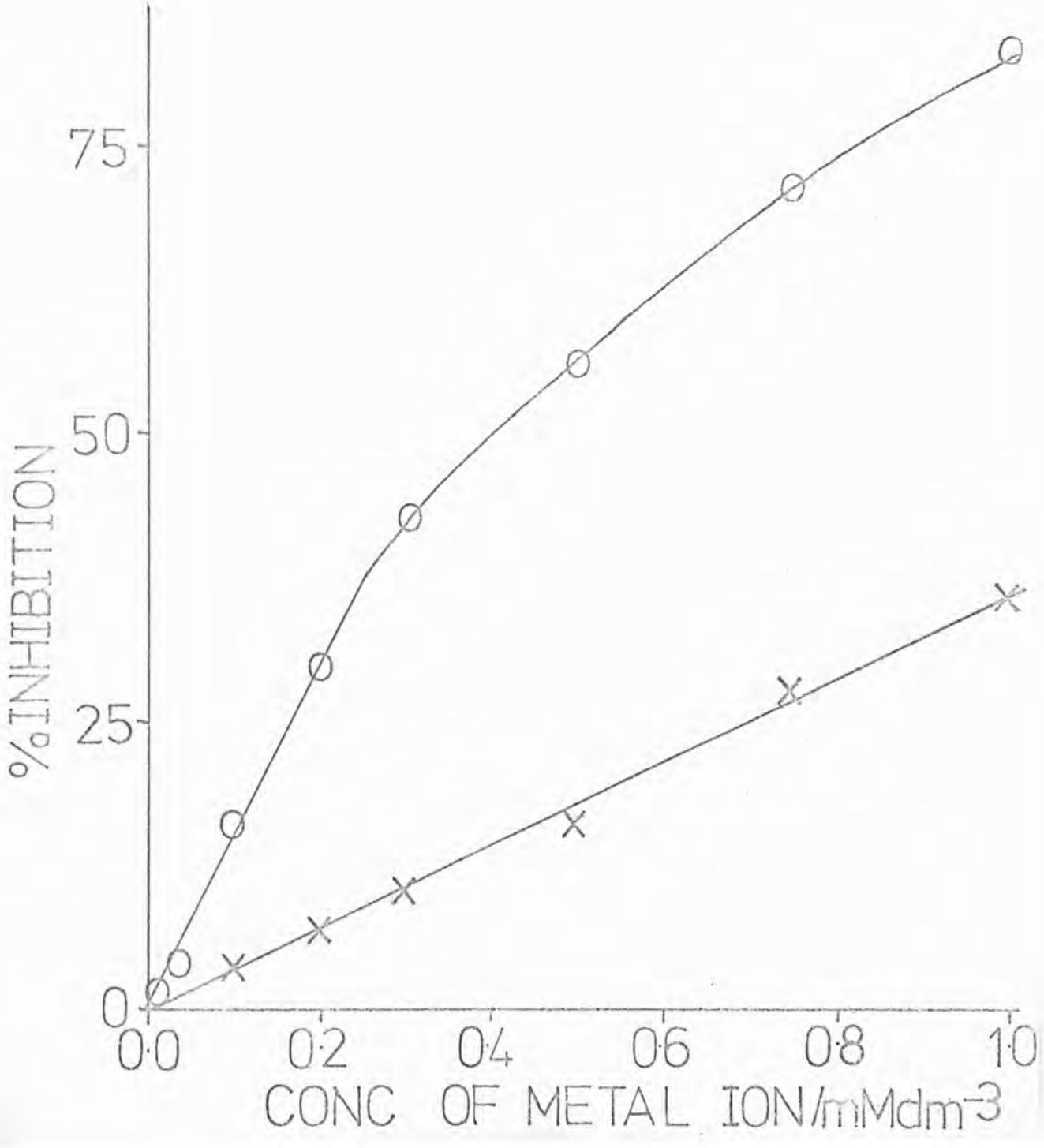


TABLE 5.2.1.4.

Table of % inhibition for a known concentration of metal-ion inhibitor

metal ion	conc./mol dm^{-3}	% inhibition
Hg^{2+}	10^{-4}	17, 18
Ag^{+}	10^{-4}	14, 13
Hg^{2+}	10^{-3}	85
Ag^{+}	10^{-3}	70
Cu^{2+}	10^{-3}	38, 36
Pb^{2+}	10^{-3}	0
Cd^{2+}	10^{-3}	8
Co^{2+}	10^{-3}	7
Mn^{2+}	10^{-3}	6
Ni^{2+}	10^{-3}	0

Discussion of results

During the course of this study, Mattiasson et al (211) published the results of a study similar to that reported here, using a thermistor heat sensor. However, there are a number of differences worthy of comment.

Firstly, the concentration of inhibitor required to achieve 50% inhibition are, in each of the cases for Ag^{+}, Hg^{2+} and Cu^{2+}, approximately an order of magnitude higher that those reported by Mattiasson. Despite repeated attempts to optimize conditions to achieve greater inhibition, the percentage inhibition obtained in this study did not compare with Mattiassons. The reason for this is not apparent.

Another difference in the two studies is that Mattiasson et al found Ag^{+} to be a slightly stronger inhibitor than Hg^{2+}, whereas in this study, the order was found to be reversed though the difference is small.

Both studies agree in the fact that the concentration of copper required to inhibit the immobilized urease by 50% is approximately 5-6 times greater than the concentration of Hg^{2+}, and that iodide can be used to re-activate the inhibited enzyme. In the opinion of the author, cysteine (freshly prepared) is superior even to iodide. Mattiasson et al appear only to have studied the inhibition by Ag^{+}, Hg^{2+} and Cu^{2+} ions, but in this study the inhibition by 10^{-3}M Pb^{2+}, Cd^{2+}, Co^{2+}, Mn^{2+} and Ni^{2+} has also been measured (Table 5.2.1.4.), the order appearing to be the same as that obtained by Toren and Burger (214), who used a pH-stat detection system to monitor the rate of reaction of urease in solution, in the presence of inhibitors. There is one exception,

viz., that the order of silver and mercury were again reversed. The apparent reversal of the order of inhibitory power of the Hg^{2+} and Ag^{+} ions used in this study is in some ways worrying, and the possibility of some photo-oxidation of silver ions must be considered.

Also worthy of note is the non-linearity of the plot of inhibition vs. conc. of Hg^{2+} (Fig. 5.2.1.3.), at inhibition $\geqslant$60 - 70%, which may suggest either that an equilibrium is established between heavy metal ions being bound to, and washed off the immobilized enzyme by the aqueous buffer solution, or that some of the immobilized enzyme is bound in such a way that binding of the metal ions to the regulatory sites (believed to be sulphydryl groups) is sterically or conformationally hindered. The failure of buffer alone (in the absence of metal-ion inhibitors) to restore activity to the metal-inhibited immobilized enzyme favours the latter explanation.

5.3. Discussion of the merits of immobilized enzyme inhibition as an analytical technique

It appears from the results of both cholinesterase and urease presented in this chapter (where competitive, and non-competitive inhibitors have, respectively, been studied), that this approach to inhibitor analysis is successful, at least for solutions known to contain only one inhibitor. However, a major disadvantage of this type of technique is the loss of much of the specificity normally associated with enzymes, and the interferences that may be present by trace contamination of other metal ions, or complexing agents or other species which must be controlled or eliminated.

For immobilized urease, and a crude solution containing more than one species of inhibiting metal ion, it might be possible to differentiate between metal ions by stepwise regeneration of the immobilized enzyme column with different regenerating agents. Mattiasson et al (211), claim to have specifically removed inhibiting Ag^{+} in the presence of inhibiting Hg^{2+}, by use of imidazole buffer, but attempts to repeat this in this study, using mixtures of Hg^{2+} and Ag^{+}, of known concentrations, yielded poor results. Alternatively, it may be possible, by choice of a suitable complexing agent selectively to mask the majority of interfering ions.

However, the metal ions of most concern environmentally today (e.g. Ag^{+}, Hg^{2+}, Cu^{2+}, Cd^{2+}) appear, with the exception of Pb^{2+}, to be the most effective inhibitors of enzymes (particularly urease), and hence, determination of a "total effective inhibitor concentration", obtained without discrimination as to how much inhibition is actually being effected by each ion, may be a useful screening test for possible pollution or contamination studies.

Whilst sensitivity compared well with that obtained in determinations of substrates (Chapter 3, Section 2), reproducibility and precision were generally poorer, variations between identical samples were frequently of the order of $\pm$ 5% and occasionally $\pm$ 10%.

CHAPTER 6

Determination of enzyme activators.

6.1. Reactivation of immobilized apo-enzymes by trace quantities of metal ions.

Many enzymes require the presence of a particular metal ion (e.g. K^+ Ca^{2+}, Mg^{2+}, Mn^{2+}) for activity. When such enzymes are investigated in vitro, however, few of the activation effects are sufficiently sensitive and specific to have analytical potential. Only one appears to have been proposed as an analytical method, vis, the activation of isocitrate dehydrogenase by Mg^{2+}. A method for determination of magnesium in blood serum is based on this effect (215).

Reports in the literature indicate that greater selectivity and sensitivity may be achieved by utilizing the re-activation of apo-enzymes. Metalloenzymes have specific metal ions located in their active centres, and such ions play essential roles in determining enzyme activity; removal of this prosthetic metal ion, e.g. by dialysis against a suitable chelatine agent, renders the enzyme in the form of a metal-deficient inactive apoenzyme. This may be subsequently reactivated by the re-introduction of the metal ion which has been removed. Such activation processes have been claimed to be sensitive and highly selective, and various groups of workers have used such principles to determine nanogram quantities of zinc (216-219).

Stone and Townshend (220) have extended this technique to copper determinations using immobilized apopolyphenol oxidase. However, their work was in no other way similar to that described later in this chapter, in that immobilization was only

attempted to facilitate handling and recovery of the enzyme, all determinations of copper being done by a batch process, the enzyme activity after incubation with copper(II) ions being monitored using an oxygen electrode. Attempts are described in this chapter to determine trace (nanogram quantities), of metal ions, based on the reactivation of the immobilized apoenzyme, using the LKB 10700-1 to monitor enzyme activity.

6.1.1. Determination of copper utilizing its reactivation of glass-immobilized apopolyphenol oxidase.

The reactivation of apoenzymes in solution by trace quantities of metal ions has been shown to provide very sensitive techniques for determination of certain ions (216-219).

Several workers have reported that apopolyphenol oxidase from plant (221, 222) and animal (223-225) sources was only reactivated by copper, and that the native enzyme was not susceptible to inhibition or activation by other metals (226).

Attempts have been made to remove the prosthetic metal ion (copper) in glass-immobilized apopolyphenol oxidase by choice of a suitably strong complexing ligand, and then to re-activate the apoenzyme with traces of copper(II) ions, using the LKB10700-1 microcalorimeter assembly (described in Chapter 3, Section-1.1) to monitor the activity of the immobilized enzyme.

Reagents

Polyphenol oxidase (E.C. 1.14.18.1, type III, from mushrooms) was obtained from Sigma Chemical Co., U.K. $CuCl_2.2H_2O$ (analar), catechol (lab. reagent), and potassium cyanide (lab. reagent) were obtained from B.D.H. Chemicals Ltd., U.K. All other reagents were as stated in Chapter 2.

All glassware was kept thoroughly clean. Containers were washed with a detergent (DECON 90 or TEEPOL) followed by 0.1M EDTA, and then washed thoroughly with de-ionized water prior to use.

Immobilization procedure.

Polyphenol oxidase (6.25 mg, 25000 units) was coupled to glutaraldehyde activated alkylamine glass (0.5 g, 13.3 $m^2 g^{-1}$ surface area), as described in Chapter 2 Section 1.2, and packed into the LKB microcalorimeter in the usual manner.

Experimental details and results.

After thermal equilibration was complete enzyme activity was verified by observation of a heat peak upon passage of a pulse of 0.1M catechol (the substrate) in 0.1M maleic acid buffer, adjusted to pH 6.5 with potassium hydroxide.

Attempts to remove the metal ion by passing an EDTA/buffer solution over the immobilized enzyme failed, even with 0.1M concentrations of EDTA. Eventually it was found that circulating a 2M solution of KCN over the enzyme (pH 7.0, in maleate buffer) for 12-24 h reduced the activity of the enzyme to an acceptably low level (activity determined by using 0.1M catechol as substrate). Passing a 2M KCN buffer over the enzyme for 72 h removed all traces of enzyme activity. The enzyme could then be re-activated by passing traces (e.g. 10^{-7} g) of a copper (II) solution, the activity of the enzyme after treatment with each sample of copper being plotted as a function of either the concentration or of the mass of Cu in the re-activating solution. Throughout the series of experiments a flow rate as low as was consistent with reasonable analysis times was used (0.08 cm^3/min), because uptake of metal ion by apoenzyme is not instantaneous, and appears to require some 'incubation' time before maximum activity is reached.

Interestingly, copper in the native enzyme exists as Cu(I), but the apoenzyme will bind copper(II), the protein subsequently reducing this to Cu(I), as demonstrated by the disappearance of a Cu^{2+} EPR signal with time (227).

2M cyanide was re-circulated over the enzyme using the set-up schematically shown in Fig.6.1.1.1.

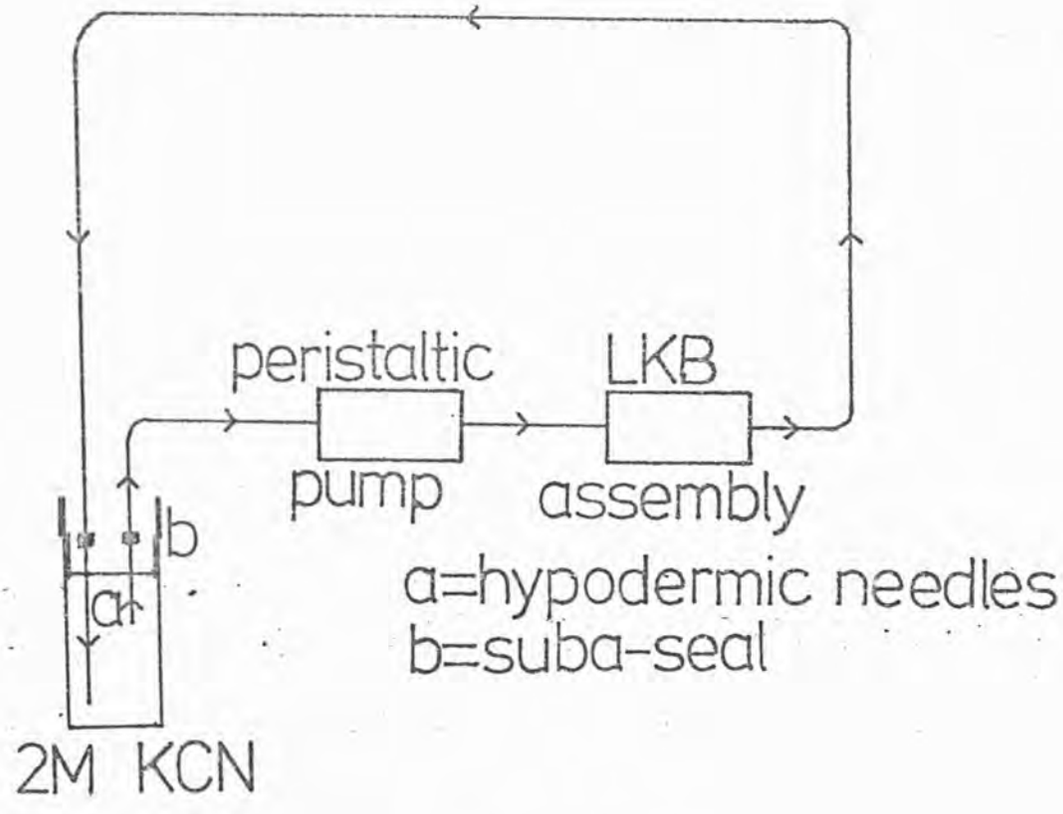
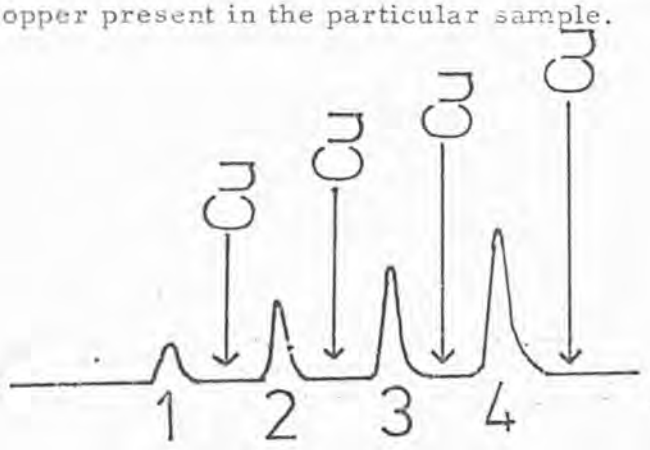


Fig. 6.1.1.1.

After recirculation, the assembly was washed with 0.1M maleate buffer (pH 6.5), for 2 h to remove all traces of cyanide from the apparatus and equilibration tubing. Trace of copper were determined by the following methods:-

(a) The residual enzyme activity was checked using a 0.5 cm^3 pulse of catechol solution (0.1M). A 0.5 cm^3 aliquot of Cu(II) solution of known concentration was injected, followed by another 0.5cm^3 aliquot of 0.1M catechol solution, the catechol solution being made up in the maleate buffer (exact concentration did not seem to matter as long as the reaction was zero order with respect to substrate (i.e. enzyme was 'saturated'). The alternate copper/substrate injections were repeated, and peak area, (corresponding to enzyme activity) was plotted against total mass of copper passed. Alternatively, differences between the two successive peak areas could be plotted against the mass of copper present in the particular sample.



1=residual enzyme activity
Cu=passage of Cu^{++} ions over enzyme
2,3,4=activity after Cu^{++} injections

Fig. 6.1.1.2.

It was hoped that any given mass of Cu^{2+} injected would give a reproducible activation of the enzyme, (as shown in Fig. 6.1.1.2.); however, this was not found. Although there was absolutely no doubt that Cu^{2+} did indeed reactivate the apoenzyme, this reactivation was not reproducible, and was also found to be critically dependent on the time elapsing between the injection of copper and the subsequent catechol injection; the closer the catechol injection after a low concentration of Cu^{2+}, the larger the peak area obtained. For higher concentrations of Cu^{2+}, even this did not necessarily seem to hold true.

Consequently it was decided to try a steady-state method to determine copper. This had the advantage that it could be continuously monitoring enzyme activity, whereas during the "heat-pulse" type experiments, enzyme activity could only be monitored during the passage of the pulse of catechol.

After flushing through with maleate buffer, as previously described, a solution of catechol of high enough concentration to give zero order kinetics was passed over the enzyme, until a steady-state was reached, followed by a pulse of copper solution of known concentration, made up in the same substrate/buffer solution. This was also passed over the enzyme, until yet another steady-state was reached (experimentally about 30 min), followed by the buffer-substrate solution alone. A typical chart-recorder trace is as shown in Fig. 6.1.1.3.

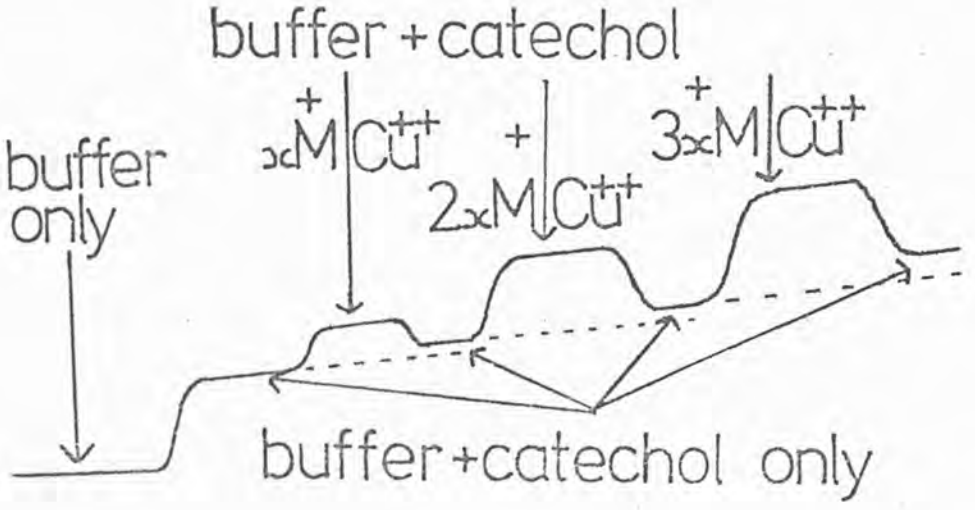


Fig. 6.1.1.3.

At low concentrations of copper(II), steady-state displacement and concentration of Cu^{2+} were approximately linearly related. However, at higher concentrations of Cu^{2+}, steady-state displacement was less than expected, and at still higher concentrations inhibition (negative displacement), was observed.

There are several interesting features to this trace that are worthy of comment.

First, it could be seen that the general slope always corresponded to increasing enzyme activity, and this was attributed to traces of Cu^{2+} ion in the distilled water, or to contamination of the solution vessels with copper ions. This is consistent with the observation that this gradual "residual" reactivation was not reproducible.

Upon Cu^{2+} ions reaching the enzyme the expected rapid reactivation over a period of some 15-20 min could be seen. However, the enzyme activity then appeared to level off in an apparent steady-state manner, whilst copper solution was still passing over the enzyme. When only buffer + substrate was then passed over the enzyme, the enzyme activity fell off again, but not quite to the level expected by extrapolation of the original baseline of buffer + substrate.

It was tentatively assumed that, as copper originally reached the apo-enzyme, a relatively large proportion of this copper was bound, causing an initial increase in enzyme activity, but for some reason as yet unknown, most of this was only bound loosely and could be easily washed off again, so whilst copper was still present in the bulk solution, an equilibrium was established in 15-20 min. and enzyme activity did not increase after this. However, when no copper was present in the bulk solution, this loosely bound copper was rapidly washed off, and hence the enzyme activity decreased. That it did not quite go back to its originally expected level could be explained by the fact that a little of the copper was firmly bound to the enzyme, and was hence retained. Little work appears to have been reported on the mechanism of reactivation of apopolyphenol oxidase by the Cu^{2+}. However, Kertesz et al (227) propose a scheme based on EPR evidence whereby fast binding of Cu^{2+} ions is followed by a much slower reduction of part of the bound copper to Cu^{+} (the active species), yielding the active holoenzyme, although they also admit that the data is not unambiguous, and possibly subject to different interpretations. It would seem from the experiments previously described that the initial binding process may not be as simple as has been previously been assumed, and that copper initially bound to the re-activated

apoenzyme is not bound in exactly the same way, or as strongly, as copper in the natural enzyme, although it must have been bound in such a way so as to be at least partially catalytically active. More work is needed before the mechanism of reactivation, particularly the binding stage, is fully understood.

At higher concentrations of copper, steady-state displacement was not as large as expected, probably due either to inhibition of the enzyme by excess Cu^{2+} (as definitely shown at still higher Cu^{2+} levels), or by the fact that the enzyme was active enough for oxygen depletion in the bulk solution to become the rate determining step (see earlier reports on "oxidase" type enzymes in Chapter 3. Section 2.5).

Finally, the effect of reactivation by some other metal ions on the apoenzyme was investigated.

Co^{2+} was found not to activate at all

Mn^{2+} and Zn^{2+} activated very slightly (10% of equiv. Cu reactivation)

Fe^{2+} and Ni^{2+} activated slightly (20% of equiv. Cu reactivation)

and surprisingly, Ag^{+}, which may have been expected to inhibit, activated strongly (comparable to Cu^{2+} activation).

} at concentration highest conc. of Cu^{2+} in analytically useful range.

However, this system (using an "oxidase" type enzyme), does suffer some disadvantages. As has previously been shown, with oxidase-type enzymes, linearity is only observed with substrate concentrations up to about 5 mM, due to oxygen depletion of the buffer.

When enzyme activities are being determined, the linear region would only be expected to extend up to an enzyme activity high enough to reduce the concentration of substrate by about 2.5 mM

for the same reason.

As a consequence of this, the number of runs capable of being carried out by gradual reactivation of the enzyme, before the enzyme had to be de-activated again, was limited, the exact number depending on the amount of re-activities achieved in each run.

Whilst for one particular series of runs, reactivation and concentration of copper seemed to be linearly dependent, once the enzyme was de-activated again the same concentration of copper injected for the same time, would not necessarily produce the same amount of re-activation as in the previous series of runs, and hence re-calibration would be necessary for each set of runs.

The accuracy of this method (as shown by the reproducibility of results in one series of runs) does not approach the $\pm 2\%$ generally obtained in substrate analysis, frequently being of the order $\pm$ 10%, providing at best only fair accuracy. However, the very low concentrations of copper detectable still make this an interesting method.

Some results are tabulated in Tables 6.1.1.4, 6.1.1.5 and 6.1.1.6, and shown graphically in Fig. 6.1.1.7.

Table 6.1.1.6 illustrates well the discrepancies found between different series of runs, and also the trend generally observed, that re-activation tended to decrease with increasing number of cyanide treatments.

It may be worthy of note that the three sets of runs carried out in Table 6.1.1.6 were not the only series of runs conducted, nor were they necessarily successive series of runs, but were only those in which comparable conditions and concentrations were used. The listing is chronological.

Table 6.1.1.4.

Steady-state displacement vs. conc. of Cu^{II} in a 30 min ($2.4\ cm^3$) injection of $CuCl_2.2H_2O$ solution in one particular set of runs.

Conc. Cu^{II}/mol dm^{-3}	Mass of Cu^{II} in 30 min ($2.4\ cm^3$) pulse/gram	Displacement (1mM on 10 μ VFSD
3.7×10^{-6}	90×10^{-9}	2
7.4×10^{-6}	180×10^{-9}	3.5
18×10^{-6}	450×10^{-9}	8
37×10^{-6}	900×10^{-9}	14
74×10^{-6}	1.8×10^{-6}	22
180×10^{-6}	4.5×10^{-6}	10

Table 6.1.1.5.

Comparison of the steady-state displacements produced by successive injections of a 30 min (2.4 cm^3) pulse of 18 μ M $CuCl_2.2H_2O$ solution.

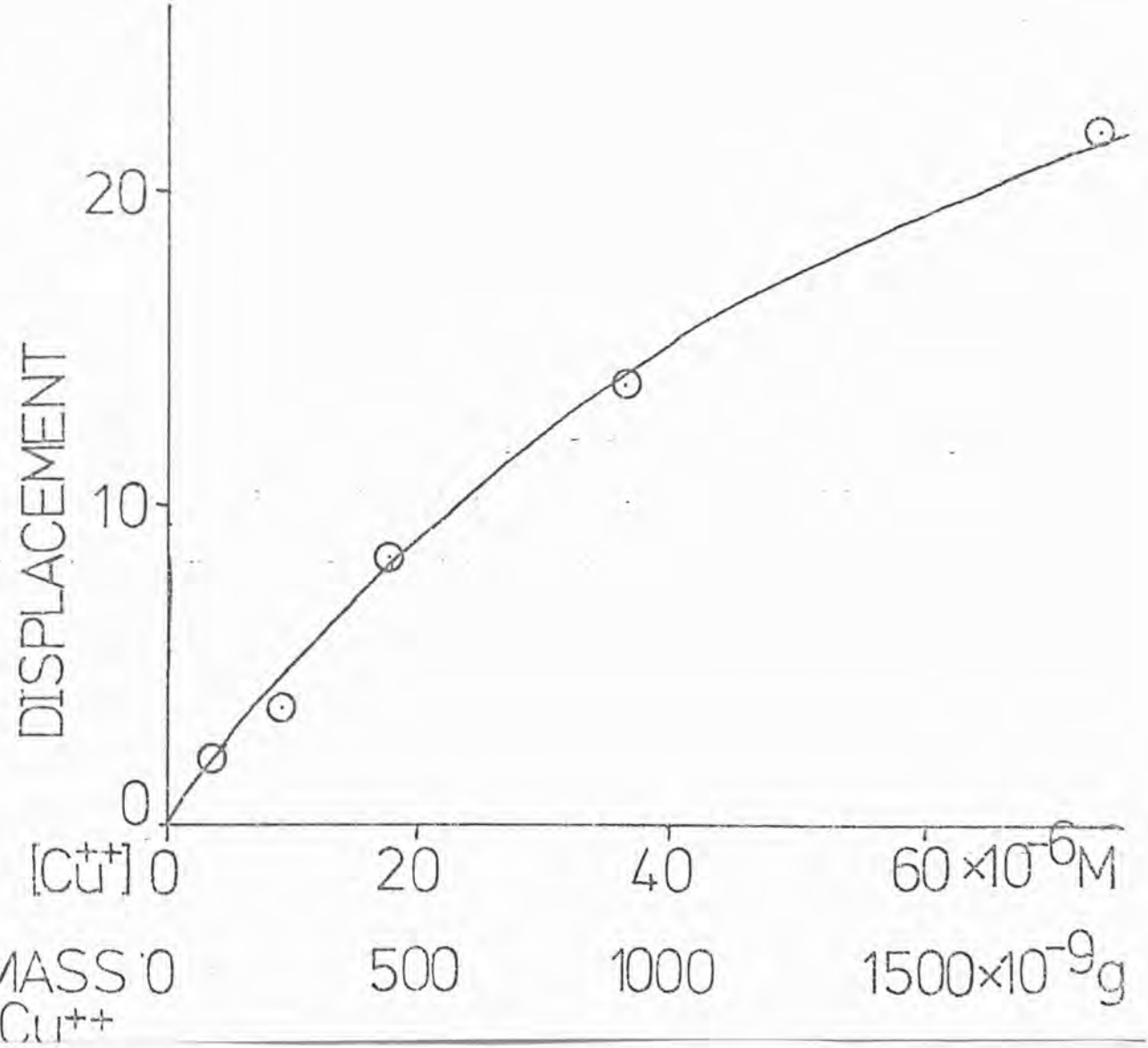
Conc. Cu^{2+} /mol dm^{-3}	Mass Cu^{2+} /ng	Displacement/ mm
18	450	21
18	450	18
18	450	17
18	450	21

Table 6.1.1.6.

Comparison of the steady-state displacements produced by injection of a 30 min (2.4 cm^3) pulse of 18 μ M $CuCl_2.2H_2O$ solution. (450 ng Cu^{II}) for different runs in the chronological order in which the various runs were carried out.

Conc. Cu^{2+} / mol dm^{-3}	Mass Cu^{2+} /ng	Displacement/mM	Mean displacement/mm
1. 18	450	21, 18, 17, 21	19
2. 18	450	15, 18	16.5
3. 18	450	8	8

FIG. 6.1.1.7 Plot of re-activation of glass-immobilized apo-polyphenol oxidase by Cu^{+} ions



6.1.2. Reactivation of apo-alkaline phosphatase by Zn^{2+} ions.

Copper analysis by means of the re-activation of immobilized apopolyphenol oxidase was shown in the previous section to be subject to many difficulties, including the need to use concentrated cyanide solutions over extended periods of time to remove the prosthetic metal ions from the immobilized enzyme and the limited linear range of the activity vs.copper conc. plot, due to the dual-substrate nature of the oxidase type enzyme (see Chapter 3. Section 2.5 and Chapter 6. Section 1.1.).

Calf intestinal alkaline phosphatase (E.C. 3.1.3.1) has been shown to contain zinc as the prosthetic metal ion (228), which can be easily removed by other chelating agents (e.g. EDTA), (229, 230), leaving an apoenzyme with no activity.

Townshend and Vaughan (216, 217) have demonstrated that minute quantities of Zn^{2+} can be determined by the reactivation of apo-alkaline phosphatase but no attempt appears to have been made to extend this technique to the immobilized enzyme. Hence it was decided to investigate the suitability of using immobilized alkaline phosphatase for the determination of zinc ions, in combination with the LKB 10700-1 microcalorimeter assembly to monitor enzymic activity.

Reagents

Alkaline phosphatase (Type 1-S), Activity 2 units per mg, immobilized (aragose-bound) alkaline phosphatase, and phenylphosphate (substrate) were purchased from Sigma (U.K.). All other reagents were obtained from B.D.H. or as described in Chapter 2.

All glassware was kept thoroughly clean; containers were washed with a detergent (DECON 90 or TEEPOL) followed

by 0.1M EDTA, and then washed thoroughly with distilled water before use to remove any adsorbed metal ions. All solutions were made up using distilled water (from a polythene container), or de-ionized water.

Preparation of immobilized alkaline phosphatase

Alkaline phosphatase (50 mg, 100 units) was bound to controlled porosity glass (0.2 g, 216.9 $m^2 g^{-1}$ surface area), and to glutaraldehyde-activated aminoethylcellulose, as described in Chaper 2 (Sections 1.2 and 1.1 respectively). However, in each case very little final activity was obtained, based on the heat-flux registered when a solution of 5×10^{-2}M phenyl phosphate was passed over the immobilized enzyme, in ethanolamine HCl buffer at pH 10.0, the pH associated with maximum enzyme activity, indicating little enzymatic activity in either immobilized enzyme preparation. Consequently, aragose-immobilized alkaline phosphatase (10 units) was purchased from Sigma U.K., and packed into the microcalorimeter. This gave satisfactory activity when ethanolamine HCl buffer (pH 10.0) containing 10^{-2}M substrate was passed through the enzyme column.

Preparation of the immobilized apo-enzyme

Experimentally, it was found that passage of a 20-25 min pulse of 0.1M EDTA, buffered to pH 10.0 with 0.3M ethanolamine HCl, at a flow rate of 0.072 $cm^3 min^{-1}$ (in the presence of 5×10^{-2}M phenyl phosphate) reduced the initial enzyme activity by over 90%. The remaining enzyme activity was found difficult to remove, even passage of EDTA for several hours did not completely remove all enzyme activity. It is speculated that this may have been due to competition between the immobilized enzyme and the EDTA to

bind the trace quantities of zinc ions still present in the buffer solution.

Re-activation of the immobilized apo-alkaline phosphatase.

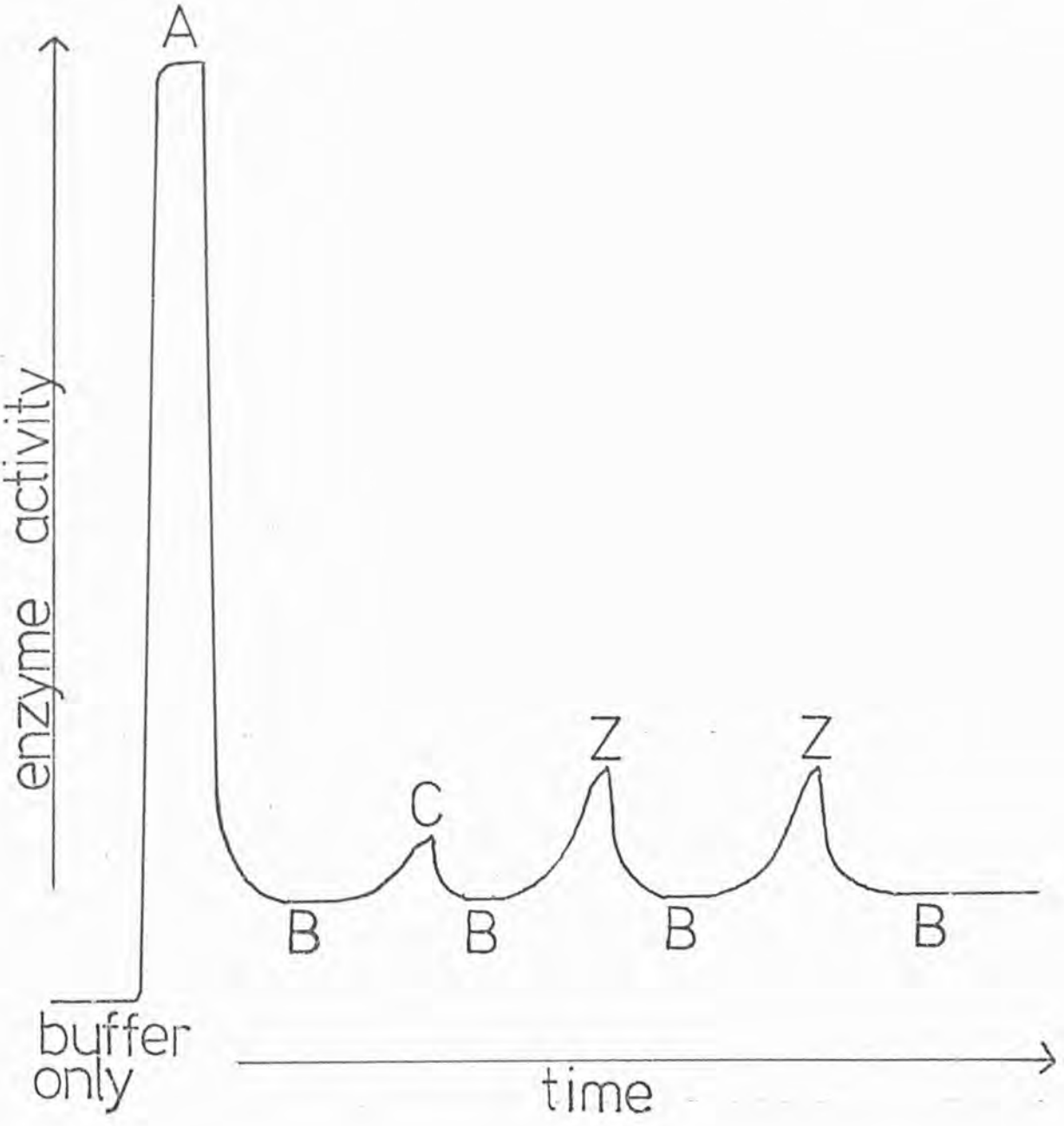
Immediately there was no EDTA solution flowing over the immobilized apo-enzyme, the apo-enzyme quickly began to regain activity, presumably due to it binding the trace amounts of zinc ions present in the buffer. This "blank" activation could be minimized by ensuring all containers were scrupulously clean, (see earlier), and using only de-ionized water but could never be completely eliminated. Addition of trace quantities of Zn^{2+} ($< 10^{-5}$M) were found to increase greatly this rate of re-activation. However, if much higher concentrations of zinc were used, the rate of activation was decreased, presumably due to the fact that excess zinc also inhibits alkaline phosphatase (231). Indeed, even the passage of an extremely dilute ($< 10^{-5}$M) solution of zinc ions for an extended period of time could be shown to inhibit the enzyme activity if the solution was passed long enough.

Fig. 6.1.2.1. shows the effect of EDTA, Zn^{2+} ions and the "blank" effect observed when the enzyme activity was continuously monitored by inclusion of 50×10^{-3}M phenyl phosphate in the buffer (ethanolamine HCl, pH 10.0).

Throughout all the series of experiments, a flow rate as low as was consistent with reasonable analysis times (0.072 $cm^3 min^{-1}$, giving an analysis rate of approximately 8 per 6 h), was used to maximize the sensitivity of the technique. Under these conditions, and using buffers and other solutions made up of de-ionized water, it was possible to detect 1 cm^3 of 0.00025 mg cm^{-3} (10^{-6}M) of $ZnSO_4.7H_2O$, corresponding roughly to 58 ng of Zn^{2+}.

FIG. 6.12.1. Effect of E.D.T.A. and Zn^{++} ions on immobilized alkaline phosphatase activity

A=buffer+50mM phenyl phosphate
B= " + " " " +0·1M EDTA
C= " + " " " +"blank"
Z= " + " " " +Zn^{++} ions



Interpretation of results.

Although it was thought at first that simply the measurement of the difference in enzyme activity between the residual activity, and the enzyme activity after a given time of passage of the zinc ions would be the most logical approach in practise, it was found that the relatively concentrated EDTA solution, diffusing back into the zinc solution, often gave anomalous results at the beginning of a run. This was shown by the initial concave curvature of the enzyme activity plot upon the slug of zinc ions reaching the immobilized apo-enzyme. Consequently, it was decided to take the maximum rate of reactivation (which usually occurred for several min), extrapolate this maximum rate (slope) back to the baseline, and measure the gradient of this line (as shown in Fig.6.1.2.2.). This would give an identical result to the increase in enzyme activity in a given time had there been no mixing whatsoever of the extremely dilute zinc solution with the preceding EDTA solution, and would thus eliminate effects due to mixing of EDTA and zinc solutions prior to their reaching the immobilized apoenzyme.

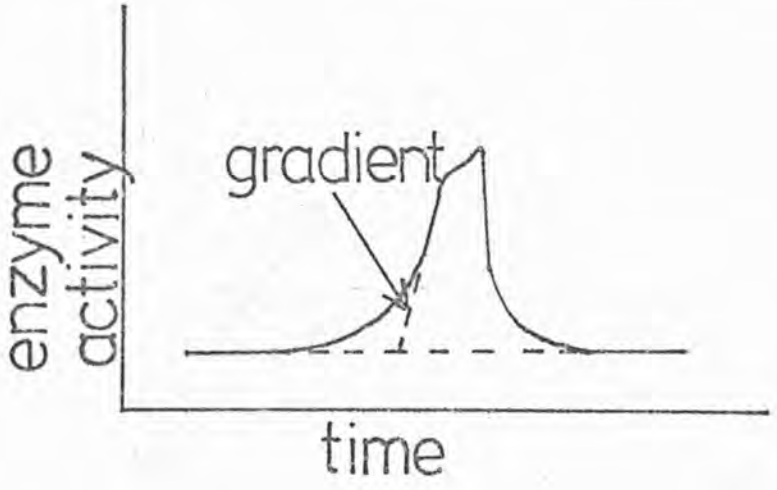
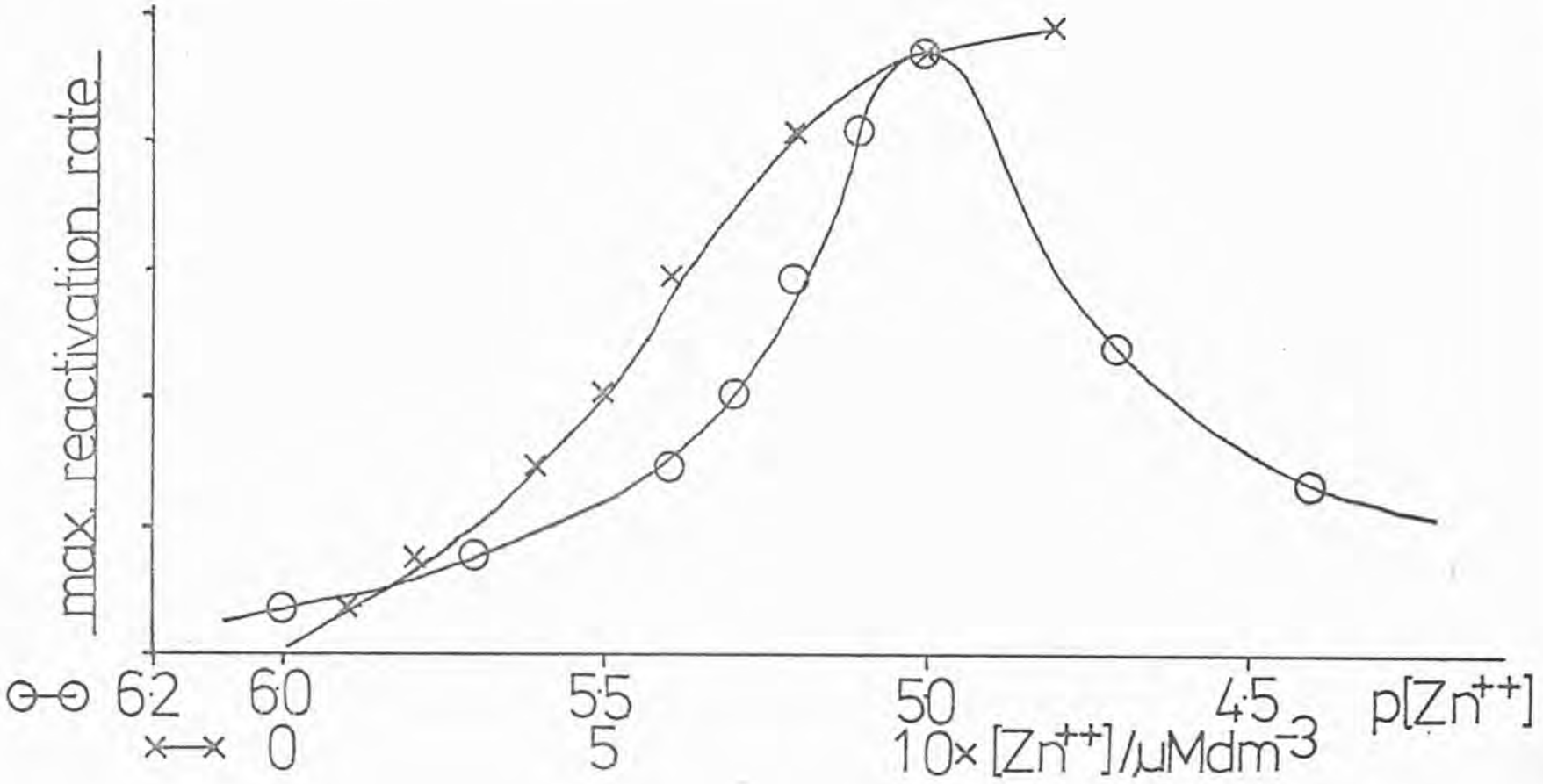


Fig. 6.1.2.2.

FIG. 6.12.3. Plots of maximum rate of reactivation of aragose-immobilized apoalkaline phosphatase vs. $-\log_{10}[Zn^{++}]$ (pZn^{++}) ⊖—⊖ and vs. $[Zn^{++}]$ ×—×



Plots of (maximum) rate of reactivation vs. -log (conc. of zinc ions) and against concentration of zinc ions , are shown in Fig. 6.1.2.3.

As in the copper/apopolyphenol oxidase system, reactivation and conc. of metal ionz were approximately linearly related at low concentrations of metal ions, and again reproducibility was poor, with variations between identical runs frequently being of the order of $\pm$ 10%.

Also, as in the case of apopolyphenol oxidase, other metal ions could be shown to interfere, particularly cobalt manganese and the alkaline earth metals.

More work is needed to establish quantitatively how much interference, if any, other metal ions will give, but further work was precluded by the relative instability of even the commercially prepared immobilized enzyme (showing a 90% loss of activity over a 100 h working period). Before this system will be of any analytical value immobilization techniques will have to be developed which render the apoenzyme much more stable.

6.1.3. A brief discussion of the merits of the apoenzyme reactivation technique as a means of analysis of trace quantities of metal ions, and possible other uses.

Of the two systems tried, each suffered from serious limitations. Common to both were the relative irreproducibility of reactivation using identical samples of the activating metal ion, and the interference of certain other metal ions. Regeneration of immobilized apopolyphenol oxidase was a long and tedious process using dangerous chemicals, whilst apoalkaline phosphatase, although easy to regenerate appears, in common with the free apoenzyme (232), rather unstable.

Although the sensitivity of each method was excellent, 50-100 ng of metal ion being detectable, the problems encountered would seem to make these particular immobilized enzymes unsuitable for routine analysis of trace metals, but that does not mean that some of the many other metalloenzymes available today might not be eminently suitable for such determinations, and (in the author's view), much more work is needed in this potentially interesting field (see Chapter 7).

One academically very interesting point concerning the binding mechanism in the reactivation of apopolyphenol oxidase has already been mentioned, and it appears that a technique such as the one described in this chapter is one of the few that will enable the activity of a constant amount of enzyme to be continuously monitored as many variables are altered, and such a technique should be of use in the study of enzyme mechanisms.

CHAPTER 7

Concluding remarks

Section 7.1

Achievements of the work described in this thesis.

This thesis is primarily concerned with the development of analytical techniques based on the combination of immobilized enzymes and heat measuring devices.

Until relatively recently, little calorimetric work had been performed on biological or biochemical systems, doubtless due to the lack of sensitivity and precision of calorimeters that were available. However, the more recent introduction of sophisticated commercial heat leak type microcalorimeters based on the original design by Wädso and others (133), and the advent of very small and highly sensitive temperature measuring devices has enabled very precise heat and temperature measurements to be carried out on small sample volumes.

Mosbach and co-workers at Lund, Sweden, and to a lesser extent, Carr's group in Georgia, U S A, have utilized immobilized enzymes to determine a number of enzyme substrates by measuring the temperature increase (caused by the enzyme-catalyzed transformation of the substrate to product) with a thermistor, but reports in the literature concerning the combination of heat-leak type flow microcalorimeters, and immobilized enzymes, number only three (154-156), and even in these, no serious attempt appears to have been made to optimize experimental conditions.

As a consequence, the majority of work described in this thesis

was carried out utilizing the well-recognized advantages of immobilized enzymes in combination with an LKB 10700-1 flow microcalorimeter, (described in Chapter 3, Section 1.1.) for measurement of heat. After optimizing the experimental conditions (Chapter 3, Section 1.2.), it was possible to determine a number of enzyme substrates (Chapter 3, Section 2) in concentrations of, typically, 2×10^{-5}M. This sensitivity appeared generally higher than those obtained by other groups, using immobilized enzymes in combination with either thermistors, or similar heat-leak type microcalorimeters, although the analysis rate was considerably slower than those using the former type of transducer, due to the relatively high time constant ($\simeq$130 sec) of the LKB microcalorimeter. The critical effect of the correct choice of buffer upon sensitivity in certain cases, (Chapter 3. Sections 2.1 and 2.2) has been demonstrated, and in other cases the limiting effect of factors such as dissolved oxygen on analytical ranges (Chapter 3. Section 3.5) has been emphasized.

Using a low flow rate, and large excess of immobilized enzyme, to ensure complete conversion of substrate to product, it has been possible to measure quantitatively the overall reaction enthalpy for the hydrolysis of urea, and also several semi-synthetic penicillins, in various buffers (Chapter 3. Section 2.1 and 2.2). In addition to semi-synthetic penicillins qualitative correlation of structure to overall enthalpy of hydrolysis has been possible. Groups using similar techniques, but incorporating thermistors as heat sensors, have not quantified such enthalpy changes due to difficulties in calibration.

The immobilized enzyme/heat-leak microcalorimeter combination appears to be the method of choice for determining overall enthalpies of such reactions, because of the greater number of determinations that can be carried out per unit time, and per unit enzyme by this technique, as opposed to the essentially batch techniques which must be used with soluble enzymes, necessitating long thermal equilibration periods between each run, and extravagant use of expensive enzymes.

Devices incorporating thermistors as heat sensors have also been constructed (Chapter 3. Section 3), but were found to show rather poor sensitivity than the LKB assembly. The combination of a nylon tube immobilized enzyme and thermal detector described in Chapter 3, Section 3.3. appears to be novel.

The two non-thermal detection systems utilizing immobilized enzymes described in Chapter 4 were intended primarily to serve as illustrations of some other reported techniques for the determining of enzyme substrate concentrations, and to enable first-hand insight into the relative advantages, disadvantages and limitations of the techniques to be gained. As no serious attempt was made to optimize experimental conditions, it was not felt fair to compare sensitivities or reproducibilities but, judging by reports appearing in the literature, the heat-leak type detector appears to compare well with most other detection systems with respect to both factors.

The extension of this technique to inhibitors is described in Chapter 5. Although an isolated paper by Mattiasson

et al (211) appeared at the same time as the urease inhibition work described in Chapter 5, Section 2, was being undertaken; no other reports concerning inhibitor determinations using immobilized enzymes have been found. Using this technique, Hg^{2+} and Ag^{+} can be determined in concentrations as low as 10^{-5}M. Although many reports (207) concerned with the determination of organophosphorous compounds, based on the inhibition of acetyl cholinesterase, have appeared in the literature, little work appears to have been carried out on the inhibition of immobilized cholinesterases by reversible, competitive inhibitors, despite the fact that the combination of immobilized enzymes and reversible inhibitors are able to give reasonably quick assays, and that some pharmacologically active alkaloids, e.g. eserine, owe their activity to an inhibitory mechanism (209).

The combination of a thermal detection system and immobilized cholinesterases was shown (Chapter 5. Section 1), to provide a quick assay method for some of the stronger inhibitors of cholinesterases without the need for a specific regenerating step to regain immobilized enzyme activity. The hypothesis has been advanced that such a technique could be used as an initial screening test for pharmacological activity of certain potential drugs active in the cholinergic nervous system, especially as enzymes could be immobilized on supports similar to those where the enzymes occur in vivo, thus providing a good in vitro model of an in vivo system that would probably show more realistic results than those obtained from any other enzyme inhibition system.

Chapter 6 describes attempts to adapt such techniques to apoenzyme activators. Much of this work was in many ways, rather disappointing, for reasons discussed in the chapter. However, there appears to be no doubt that re-activation of the apoenzyme is possible using extremely low concentrations of metal ions, the principle problems arising out of a lack of reproducibility, and rather poor specificity.

Section 7.2 Suggestions for future work

(a) Determination of enzyme substrates

It would appear that provided one can immobilize enough active enzyme, almost any enzyme substrate can be determined using thermal techniques similar to those described in Chapter 3 of this thesis, with high sensitivity, accuracy and reproducibility. Most commercially available enzymes have now been immobilized and used in conjunction with various heat-sensing detectors to determine substrates (see Table 1.3.3.1., and Chapter 3 of this thesis).

However, where an enzyme is not commercially available or is prohibitively expensive scope would appear to grow enzyme-containing cells in a fermenter, and subsequently to purify, at least partially, the enzyme by established methods described in the literature. One obvious example of a species of great interest to the analytical chemist is the nitrate ion (NO_3^-), which, although an essential nutrient for micro-organisms, can present a health-hazard to infants, livestock and during pregnancy (259). The enzyme nitrate reductase, even if immobilized, would appear prohibitively expensive (approx. £400 per g/10 units from Sigma in

January 1980) for routine assays. However, Senn et al (108) have described a method of preparation of nitrate reductase from anaerobically grown E.Coli (Crook's strain), and have indeed utilized immobilized nitrate reductase to determine the nitrate ion, in conjunction with a spectrophotometric detection system. Such a procedure should also be readily adaptable to thermal detection systems, with the added advantage of eliminating the need for subsequent auxiliary reactions to determine the nitrite-ion produced (e.g. diazo coupling).

In the opinion of the author, there would appear to be at least four other potentially interesting areas in which the combination of microcalorimetry and immobilized enzymes may have analytical potential. Two of these have been briefly described in Chapters 5 and 6 of this thesis, but the others have not yet been investigated for lack of time.

(b) Inhibitors of immobilized enzymes

Apart from analytical determinations of organophosphorous nerve gases and pesticides, little other work appears to have been performed that utilizes the monitoring of the activity of immobilized enzyme as a technique for the determination of inhibitors, although Mattiasson et al (211) have reported work of a similar nature to that described in Chapter 5, Section 2 of this thesis. Trace quantities of inhibitors have been determined by measurement of the reduction in activity, or decrease in reaction rate of many enzymes in solution. Some of these are shown in Table 7.2.1.

TABLE 7.2.1.

Inhibiting species determined	(soluble) enzyme	reference
I_2	invertase	(233)
Hg^{2+}, Ag^{+}	invertase	(234, 235)
Hg^{2+}, Ag^{+}	glucose oxidase	(236)
Hg^{2+}, Ag^{+}	xanthine oxidase	(237)
F^{-}	acid phosphatase	(238)
CN^{-}	hyaluroidase	(239)
S^{2-}	peroxidase	(240)
Be^{2+}, Zn^{2+}	alkaline phosphatase	(231)
Be^{2+}, Bi^{3+}	alkaline phosphatase	(241)

Additionally, many other species have been determined by their inhibition of the appropriate enzyme(s) in solution, and these have been reviewed by Guilbault (242).

All these systems have the potential to be adapted to immobilized enzymes (with thermal detection systems). Some may well prove more sensitive and reproducible than the systems already reported in this thesis.

(c) Activators of immobilized enzymes

As yet nothing appears in the literature concerning analytical determinations of immobilized apoenzymes, or immobilized inhibited enzyme reactivators, used in continuous flow systems.

The reactivation procedures described in Chapter 6 of this thesis appear, for a variety of reasons, to give rather disappointing reproducibility or selectivity, although the sensitivity of these techniques appears excellent.

Attention should be given to a report by Lehky and Stein (218), who have determined zinc ions using the reactivation of apoaminopeptidase, which appears ideal as the apoenzyme is very stable and highly specific. Additionally a specially made chelating agent (Chelex X-100) was used, which could easily be incorporated into techniques described in this thesis, thus avoiding problems associated with possible cyanide poisoning of the enzyme. Again, such a procedure should be readily adaptable to immobilized enzymes, and only a shortage of time prevented the author making a study of this system personally.

Baum and Czok (215) have reported that, in the absence of Mg^{2+} ions, the enzyme isocitric dehydrogenase (ICDH), shows no activity towards its substrate, isocitrate, and have used this effect to determine traces of magnesium ions. This procedure should be equally applicable to immobilized ICDH.

Another topic of interest, that does not appear in the literature, is the reactivation of heavy-metal inhibited immobilized enzymes. Mealor and Townshend (233) have reported such a technique using enzymes in solution, and have reported determinations of cyanide and sulphide, based on the reactivation of metal-inhibited invertase. Such a procedure should be easily adaptable to immobilized urease

(where it has already been demonstrated that reactivation of the metal-inhibited immobilized enzyme can be obtained by iodide), and, as in other cases, only a shortage of time has prevented the author from investigating this possibility.

(d) Immobilized whole cells (for review of immobilization methods for whole cells, see (243)).

The low stability of intracellular enzymes has sometimes caused difficulty in obtaining active immobilized enzyme preparations. In the case of determination of cephalosporins, Matsumoto et al (244) have found it advantageous to immobilize whole cells of citrobacter freundii (which produce cephalosporinase), rather than to immobilize the purified enzyme itself, because of its relative instability.

The fact that the living cell possesses enzyme activities for a broad spectrum of substrates could be advantageous when whole cells are used in the qualitative analysis of complex media (e.g. measurement of the total content of biodegradable materials in waste waters - known as biological oxygen demand, or when used as poison detectors). Glucose has been determined by Mattiasson et al (49), using the combination of immobilized saccharomyces cerviseae and a thermal detector.

However, apart from this isolated report, little else appears in the literature on the combination of immobilized whole cells and thermal detection systems.

Mattiasson (245) has recently reviewed the potential uses of immobilized whole cells, and there appear to be many

applications eminently suitable for use in conjunction with microcalorimetric detection systems, e.g. determination of nicotinic acid using immobilized lactobillus arabinosus, or glutamine using immobilized sarcina flava.

(e) Immunochemistry

The use of immunosorption for the assay of endogenous and exogenous compounds in biological fluids has received enormous attention during the last decade. Firstly, radioimmunoassay (RIA) was developed (246, 247). However, molecules labelled with gamma-emitting isotopes, such as iodine-125 have a relatively short shelf life; health hazards may be involved in their preparation; radioactive and organic waste must be safely disposed of, and the equipment required to measure radioactivity is expensive. A more recent, and possibly more useful technique that has been developed is known as an enzyme linked immunosorbent assay (ELISA) (248-250), and techniques based on both RIA and ELISA are now in use in clinical analysis. These, and other similar techniques, have recently been reviewed by O'Sullivan (251) and by Ishikawa (252).

The combination of the ELISA technique, with a thermal detection system (termed thermometric enzyme-linked immunosorbent assay, or TELISA), has recently been reported by Mattiasson et al (180), and allows a much wider choice of labelling enzymes than other techniques. As Mattiasson appears only to have investigated the determination of human serum albumin (HSA) in a model study, using the system anti HSA/HSA, with catalase as the marker enzyme, there would seem to be much scope to extend this technique

further, e.g. progesterone/anti progesterone, prostaglandin (A_1, B_1, E_1, E_2, $F_1\alpha$, or $F_2\alpha$)/corresponding antiprostaglandin, testosterone/anti testosterone. All the reagents are currently available from Sigma.

(f) Applications outside of analysis

Not to look beyond analysis for applications of immobilized enzymes would, in the opinion of the author, be very short-sighted. The versatility of these reagents has already brought about their use in the food industry (253-255). Table 7.2.2. summarizes industrial processes that are either in production, or at pilot plant stage, using immobilized enzymes.

TABLE 7.2.2.

product	starting material	immobilized enzyme(s)
glucose	corn starch	α-amylase + glucoamylase
glucose/fructose	glucose	glucose isomerase
L-amino acids	acetyl DL-amino acids	aminoacylase
6-aminopenicillanic acid	benzyl penicillin	penicillin amidase
L-aspartic acid	ammonium fumarate	aspartase
process		
reduction of lactose in milk		β-galactosidase
manufacture and modification of corticosteroids, for treatment of arthritis		various enzymes
Modication of edible fats and food products		various enzymes.

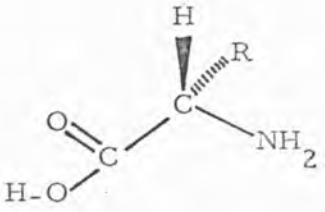
Berezin and Varfolomeev (256), have discussed some energy-related applications of immobilized enzymes and, in the opinion of the author, topics such as bioelectrocatalysis and electrode processes accelerated by immobilized enzymes should prove worthy of study.

Reports on other energy-related applications of immobilized enzymes, such as alcohol production by magnetically immobilised yeasts (257), and the use of adsorbed cellulase in the continuous conversion of cellulose to glucose (258), have also appeared. These are only a few of the ideas that readily spring to mind. Indeed, any process that is capable of being carried out by enzymes has the potential to be adapted to immobilized enzymes. Although such adaptation may, for any of a multitude of factors, not always be advantageous, in others it may meet with spectacular success. Despite the large volume of literature appearing on the subject over the last few years, applications of immobilized enzymes in many areas are still in their infancy, or even as yet unrealized.

Appendix 1

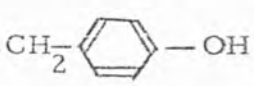
Structures of L-amino acids of importance in protein chemistry

All L-amino acids are based on the following structure, where the R-group distinguishes uniquely one particular amino acid from any other.

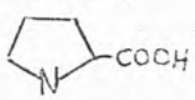
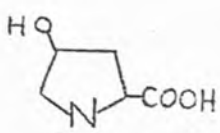


Name	R-group
Alanine	$-CH_3$
Arginine	$-CH_2-CH_2-CH_2-NH-C(=NH)-NH_2$
Aspartic acid	$-CH_2-COOH$
Aspargine	$-CH_2-CONH_2$
Cysteine	$-CH_2-SH$
Glutamic acid	$-CH_2-CH_2-COOH$
Glutamine	$-CH_2-CH_2-CONH_2$
Glycine	$-H$
Histidine	$-CH_2-C_3H_3N_2$ (imidazole)
Isoleucine	$-CH(CH_3)-CH_2-CH_3$
Leucine	$-CH_2-CH-(CH_3)_2$
Lysine	$-CH_2-CH_2-CH_2-CH_2-NH_2$

Appendix 1 - continued -

Name	R-group
Methionine	$-CH_2-CH_2-S-CH_3$
Phenylalanine	$-CH_2-C_6H_5$
Serine	$-CH_2-OH$
Threonine	$-\underset{\underset{OH}{|}}{CH}-CH_3$
Trytophan	
Tyrosine	
Valine	$-CH(CH_3)_2$

Two alpha-imino acids are also of importance in protein chemistry, namely:-

Name	Structure
Proline	
Hydroxyproline	

Appendix 2

Estimation of the concentration of functional groups on the surface of controlled porosity glass.

Reagents

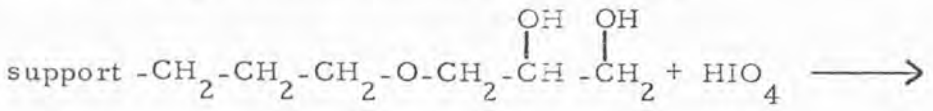
3-glycidoxypropyl trimethoxy silane (GPTS) was obtained from Phase Separations Ltd, Deeside Industrial Est., Queensferry, Clwyd CH5 2LR, U.K.
Sodium metaperiodate was obtained from B.D.H. Chemicals Ltd., Poole, U.K.
Potassium iodide (99.5+ % pure) was obtained from May and Baker Ltd., Dagenham, U.K.
All other reagents were obtained as stated in Chapter 2.

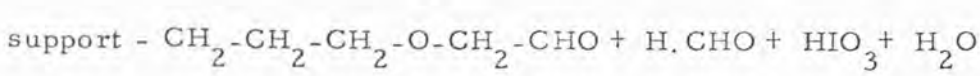
Experimental procedures

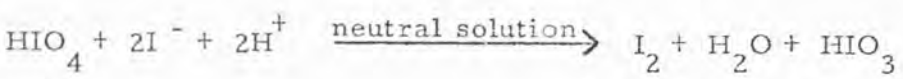
The 3-glycidoxypropyl derivative of glass was prepared in an identical manner to the 3-aminopropyl derivative described in Chapter 2, Section 1.2., by using 3-glycidoxypropyl trimethoxy silane (GPTS), instead of 3-aminopropyltriethoxy silane. It was assumed that equal concentrations of functional groups would be present in both derivatives.

Having effectively replaced an $-NH_2$ group with a $\begin{matrix} CH\text{-}CH_2 \\ | \quad\quad | \\ OH \quad OH \end{matrix}$ group, it was then possible to determine the concentration of diol groups using a modification of Malaprade's method for determination of vicinal diols, based on oxidation and cleavage with excess metaperiodic acid, followed by reaction of the excess metaperiodic acid with acidified potassium iodide, and titration of the liberated iodine with standard thiosulphate

solution, using starch as indicator (260-262), as shown in the following reaction scheme (Fig. A.2.1.)







unreacted excess

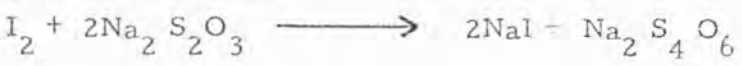


Fig. A.2.1

Sodium metaperiodate solution (approx. 50×10^{-3}M) was prepared by dissolving 10.7 g of sodium metaperiodate in distilled water containing 25.0 cm^3 of 0.1M H_2SO_4, and making up to one litre of solution.

200 cm^3 of approximately 0.25M potassium iodide solution was prepared by dissolving 8.3 g in 200 cm^3 of 0.1M phosphate buffer, and adjusting the pH to 7.5.

Two "blank" titrations were run, by pipetting 20.00 cm^3 of each solution into a 100 cm^3 conical flask, allowing to react for 10 min, and titrating the liberated iodine with 0.10044M standardized sodium thiosulphate solution, using starch as indicator over the last 1-2 cm^3 of the titration.

20.00 cm^3 aliquots of sodium metaperiodate solution were then allowed to react with 1.00 g of functionalized controlled

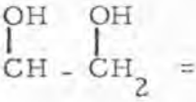
porosity glass for 2 h at room temperature (with magnetic stirring), and followed by addition of the buffered potassium iodide (20 cm^3), the liberated iodine being titrated as previously described (see Table A. 2. 2.).

From the difference in titration volumes between the blank and the functionalized glass, the concentration of functional groups (diol groups) on the glass could be calculated.

Table A. 2. 2.

Titration of liberated iodine with 0. 10044M sodium thiosulphate soln.

	Vol. of 0. 10044M $Na_2S_2O_3$ required for equivalence/ cm^3		mean/cm^3	mean conc. of functional (diol) groups (μ-equiv g^{-1} (see calculation below)
blank	19. 70,	19. 60	19. 65	-
controlled porosity glass (13. 3m^2 g^{-1} surface area)	18. 50,	18. 80	18. 65	50. 2
controlled porosity glass (216. 9 m^2g^{-1} surface area)	13. 85,	13. 80	13. 82	292. 8



as $-CH(OH)-CH_2(OH) = 2Na_2S_2O_3$, the concentration of functional groups on the surface of the controlled porosity glass is given by

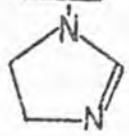
$$\text{conc.}(\mu\text{-equivs } g^{-1}) = \frac{(\text{blank titration} - \text{glass titration}) \times 0.10044 \times 10^6}{2 \times 1000}$$

APPENDIX 3

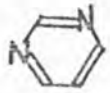
Key to structural characteristics of cholinesterase inhibitors

(See Chapter 5, Section I.)

A = 

B = 

C = 

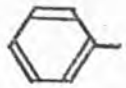
D= 

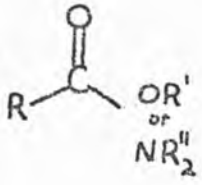
E= 

F = NR_4^+ (R ≠ H)

G = NR_3 (R ≠ H)

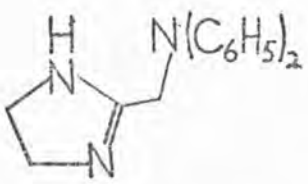
H = NR_3 (R = H, alkyl or aryl)

I = 

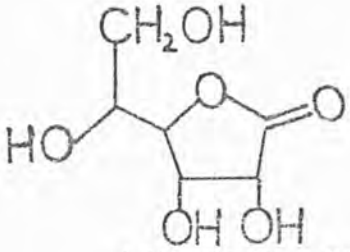
J = 

APPENDIX 4

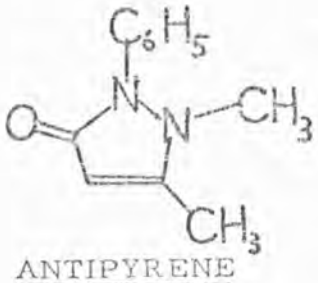
Structures of cholinesterase inhibitors



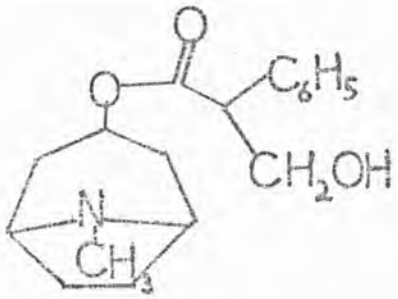
ANTAZOLINE



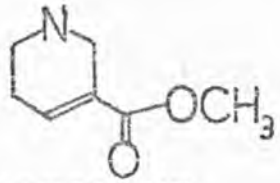
ASCORBIC ACID



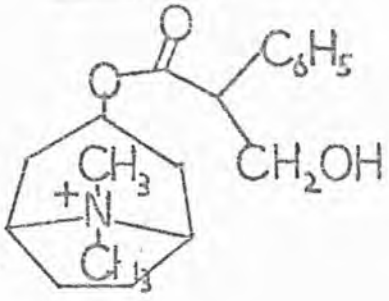
ANTIPYRENE



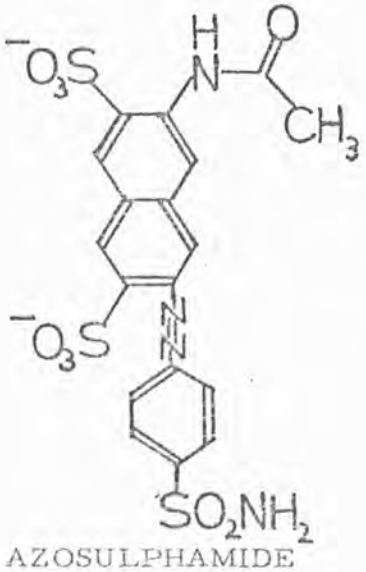
ATROPINE



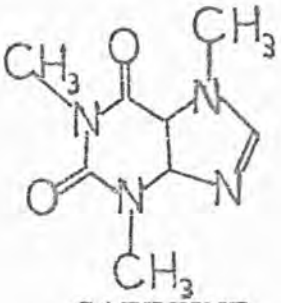
ARECOLINE



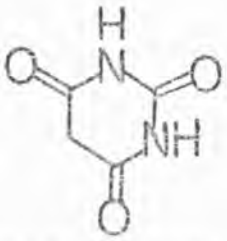
ATROPINE METHYL (NO_3)



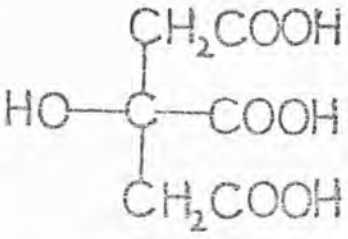
AZOSULPHAMIDE



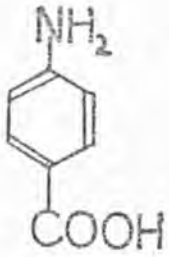
CAFFEINE



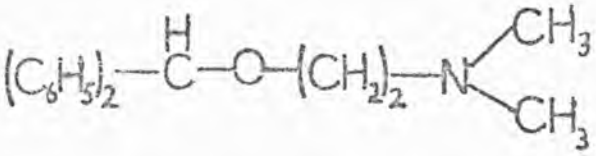
BARBITURIC ACID



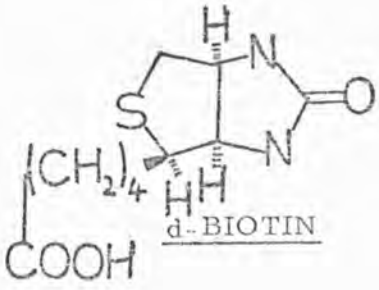
CITRIC ACID



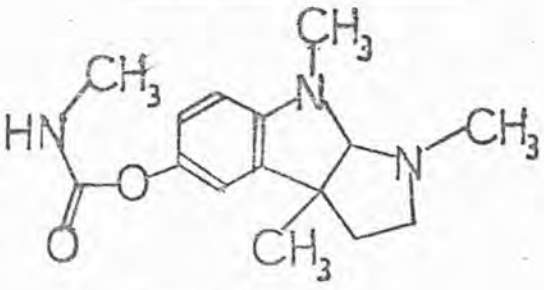
p-AMINOBENZOIC ACID



DIPHENYLDRAMINE

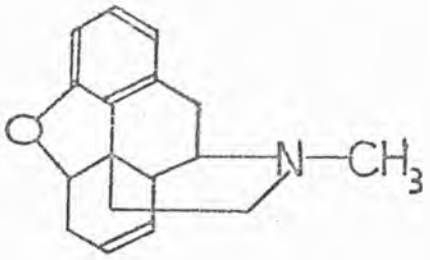


d-BIOTIN

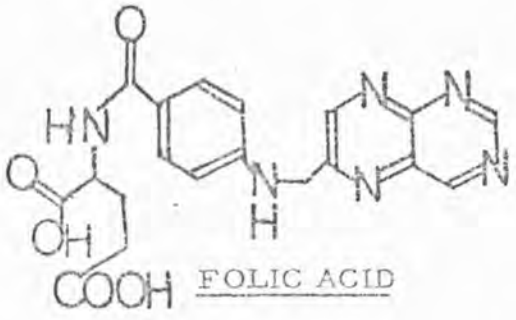


ESERINE

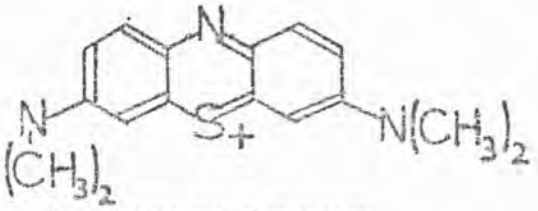
FLUORIDE



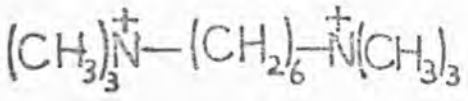
MORPHINE



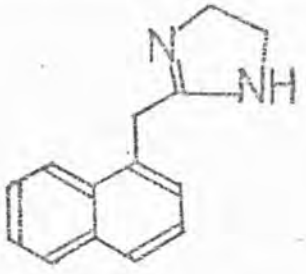
FOLIC ACID



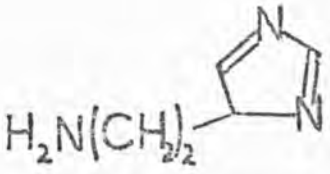
METHYLENE BLUE



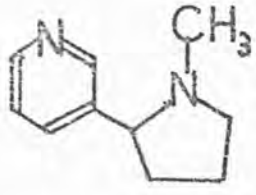
HEXAMETHONIUM (Br)



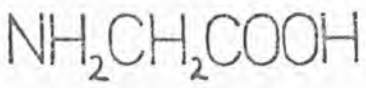
NAPHAZOLINE



HISTAMINE

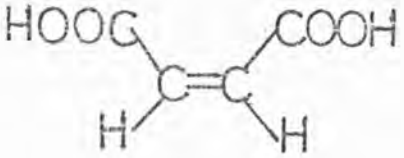


NICOTINE

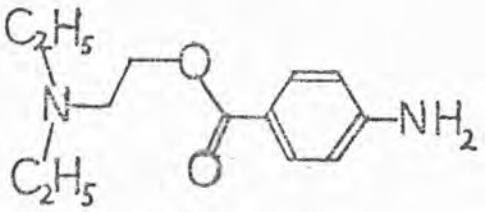


GLYCINE

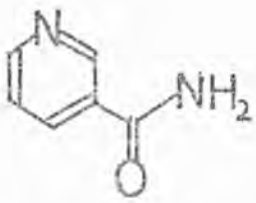
IMIDAZOLE



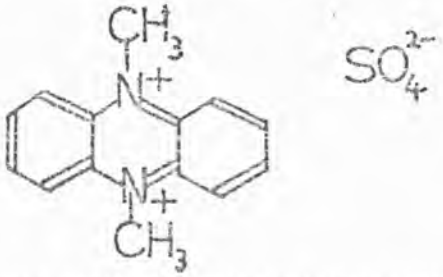
MALEIC ACID



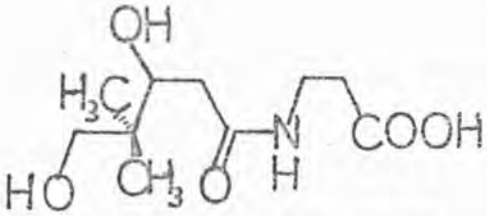
PROCAINE



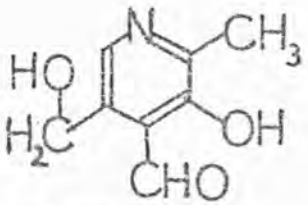
NIACINAMIDE



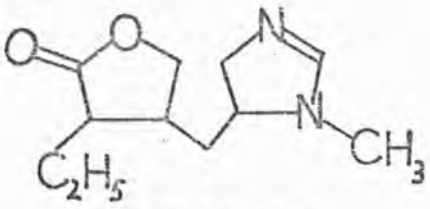
PHENAZINE METHOSULPHATE



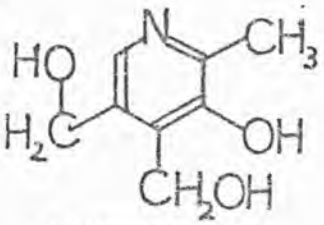
PANTOTHENIC ACID



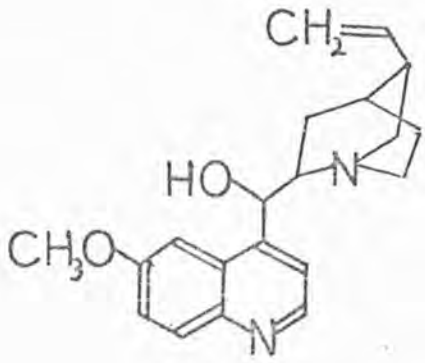
PYRIDOXAL



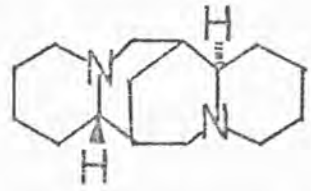
PILOCARPINE



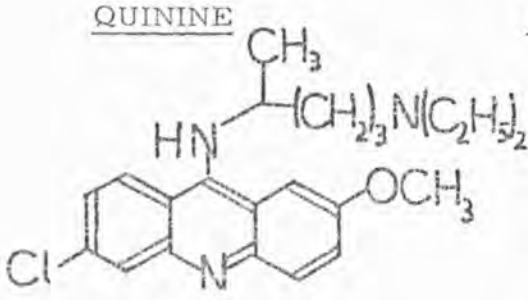
PYRIDOXINE



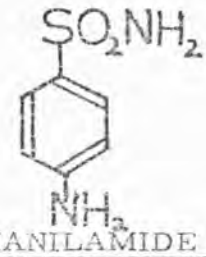
QUININE



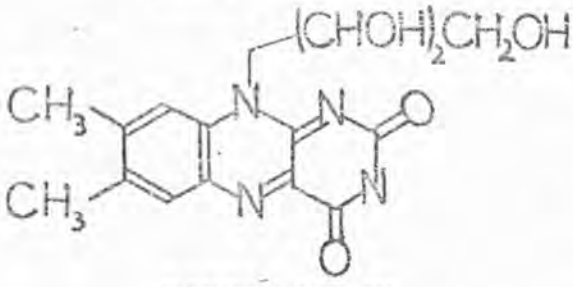
L(-)SPARTEINE



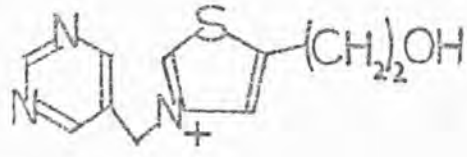
QUINACRINE



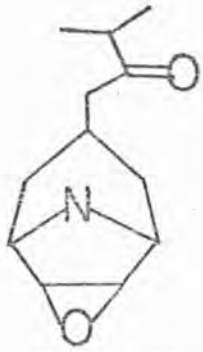
SULPHANILAMIDE



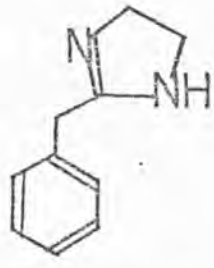
RIBOFLAVINE



THIAMINE



SCOPOLAMINE



TOLAZOLINE

$HPO_4^{2-} + H_2PO_4^-$

PHOSPHATE (pH 7.0)

$(CH_3)_4N^+$

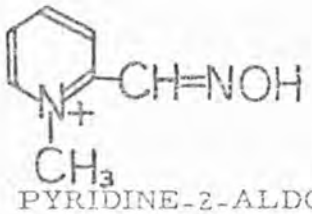
TETRAMETHYL AMMONIUM

$NH_2-(CH_2)_4-NH_2$

PUTRESCENE

$(C_2H_5)_4N^+$

TETRAETHYL AMMONIUM



PYRIDINE-2-ALDOXIME METHIODIDE

$(n\text{-}C_3H_7)_4N^+$

TETRA (n-PROPYL) AMMONIUM

$C_6H_5N^+(CH_3)_3$

PHENYLTRIMETHYL AMMONIUM

$(n\text{-}C_4H_9)_4N^+$

TETRA(n-BUTYL) AMMONIUM

REFERENCES

1. Payen, A., Persoz, J. Ann.Chim.(Phys). 53, 73 (1833).
2. Kühne, W. Unters. a. d. physiol Institut der Univ. Heidelberg 1, 291 (1878).
3. Michaelis, L.,Menten, M. L. Biochem Z, 49, 333 (1913).
4. Sumner, J. B. J. Biol. Chem., 69, 435 (1926).
5. Lartigue, D. J. in "Immobilized enzymes for industrial reactors" (ed. Messing, R. A.), Academic Press, New York 1975, p. 11.
6. Wetlaufer, D. B.,Ristow, S. Ann. Rev. Biochem., 42, 135-58 (1973).
7. Osann, G. Poggendorfs Ann., 67, 372 (1845).
8. "Enzymatic Method of Analysis"(by G. G. Guilbault) Pergamon Press, Oxford, London, New York (1966).
9. "Principles of Enzymatic Analysis"(ed. Bergmeyer, H. U.), Pub. Verlag Chemie, Weinheim, New York (1978).
10. Nelson, J. M., Griffin, E. G. J. Amer. Chem. Soc., 38, 1109-15 (1916).
11. Sumner, J. B. Science, 108, 410 (1948).
12. Tosa, T.,Mori, T.,Chibata, I. Agr. Biol. Chem. 33, 1047-52 (1969).
13. Venkatosubramanian, K., Saini, R.,Vieth,,W. R. J. Ferment. Technol, 52, 268-74 (1974).
14. Mitz, M. A. Science, 123, 1076-7 (1956).
15. Tosa, T., Mori, T.,Fuse, N.,Chibata, I. Enzymologica, 31, 214-24 (1966).
16. Tosa, T.,Mori. T., Fuse, N.,Chibata,I. Biotechnol. Bioeng. 9, 603 (1967)
17. Guilbault, G. G., Lubrano, G. J. Anal. Chim. Acta. 64, 439-55 (1973).
18. Guilbault, G. G., Lubrano, G. J. Anal. Chim. Acta. 69, 183-88 (1974).

19. Mitz, M.A., Summaria, L.J., Nature (London) 189, 576-7 (1961).

20. Weetall, H.H., Weliky, N. Nature (London), 204, 896 (1969).

21. Weetall, H.H , Science, 166, 615-6 (1969).

22. Davis, R.V., Blanken, R.M., Beagle, R.J, Biochemistry, 8, 2706-11 (1969).

23. Weetall, H.H., Nature (London), 223, 959-60 (1969)

24. Weetall, H.H., Hersh, L.S. Biochim.et Biophys.Acta 185, 464-5 (1969).

25. Bowers, L.D., Canning, L.M , Sayers, C.N., Carr, P.W., Clin-Chem 22 (8), 1314-1318 (1976).

26. Regan, D.L., Dunnill, P., Lilly, M.D., Biotechnol. Bioeng. 16, 333-43 (1974).

27. Stanley, W.L., Walters, G.G., Chan, B G , Mercer, J.M , Biotechnol.Bioeng., 17, 315-26 (1975).

28. Broun, G., Thomas, D., Gellf, G., et al Biotechnol Bioeng. 15, 359-75 (1973).

29. Weston, P.D., Aurameas, S., Biochim Biophys.Res. Comm. 45, 1574-80 (1971).

30. Ogle, J.D., Glassmeyer, C.K., Biochemistry, 10, 786-91 (1971).

31. Robinson, P.J., Dunnill, P., Lilly, M.D. Biochim. Biophys. Acta, 242, 659-661 (1971).

32. Morris, D.L., Campbell, J., Hornby, W.E. Biochem.J., 147, 593-603 (1975).

33. Campbell, J., Hornby, W.E., Morris, D.L. Biochim. Biophys.Acta, 384, 307-316 (1975).

34. Guilbault, G.G., Nanjo, M. Anal.Chem. 46, 1769-72 (1974).

35. Guilbault, G.G., Nanjo, M Anal.Chim.Acta 73, 367-71 (1974).

36. Porath, J., Axen, R., Ernbach, S. Nature (London) 215, 1491-2 (1967).

37. Mosbach, K., Mattiasson, B., Acta.Chem.Scand. 24, 2093-2100 (1970).

38. Axen, R., Porath, J., Ernbach, S. Nature (London) 214, 1302-4 (1967).

39. Ngo, T.T., Yam, C.F., Int.J.Biochem, 9 (9), 685-9 (1978)

40 Horvath, C., Biochim. Biophys. Acta, 358, 164 (1974)

41. Werner, M., Mohrbacker, R.J. et al. Clin-Chem. (Winston-Salem) 25, (1) 20-23 (1979).

42. Sundaram, P.V., Hindustan Antibiol.Bull. 20,(1-2), 1-4 (1977)

43. Mosbach, K Methods in Enzymology Vol. 44, Academic Press, New York (1976).

44. Avrameas, S. Immunochemistry 6, 43-52 (1969).

45. Avrameas, S. Ternynck, T. Immunochemistry 6, 53-66 (1969).

46. Ferrier, L.K., Richardson, T., Olson, N.F. Enzymologia, 42, 273-83 (1972).

47. Ozawa, H. J.Biochem., 62, 419-23 (1967).

48. Bernfield, P., Bieber, R.E., MacDonnell, P.C. Arch.Biochem.Biophys., 127, 779-86 (1968).

49. Mattiasson, B. Larsson, P.O., Mosbach, K. Nature(London), 268, 519-20 (1977).

50. Tosa, T. Sato, T., Mori, T., Chibata I. et al., Appl. Microbiol, 27, 886-9 (1974).

51. Chang, T.M.S., Johnson, L.J., Ransome, O.J., Can.J.Physiol, 45, 705-15 (1967).

52. Chang, T.M.S., Biochem.Biophys.Res.Comm. 44, 1531-6 (1971).

53. Hoffman La Roche and Co., British Patent 1329520 (1973).

54. Sharp, A.K., Kay, G., Lilly, M.D. Biotechnol. Bioeng., 11, 363-80 (1969).

55. Axen, R., Vretblad, P., Poratn, J. Acta Chem.Scand. 25, 1129-32 (1971).

56. Haynes, R., Walsh, K.A., Biochim.Biophys.Res. Comm., 36, 235-42 (1969).

57. Kleine, R., Spangenberg, P., Flemming, C. Hoppe-Seyler's Z.Physiol.Chem. 357, 629-39 (1976).

58. Goldstein, L. Katchalski, E., Z.Anal.Chem., 243, 375-96 (1968).

59. Goldstein, L., Levin, Y., Katchalski, E. Biochemistry 3, 1913-19 (1964).

60. Axen, R., Myrin, P.A., Janson, J.C. Biopolymers, 9, 401-13 (1970).

61. Melrose, G.J.H. Rev.Pure and Appl.Chem. 21, 83-119 (1971).

62. Maeda, H., Suzuki, H., Agr Biol.Chem. 36, 1581-93 (1972).

63. Mori, T., Tosa, T., Chibata, I. Cancer Res. 34, 3066-8 (1974).

64. Mori, T., Tosa, T., Chibata, I. Biochim.Biophys. Acta. 321, 653-61 (1973).

65. Hornby, W.E., Lilly, M.D., Crook, E.M. Biochem.J. 98, 420-5 (1966).

66. Hornby, W.E., Lilly, M.D., Crook, E.M. Biochem J. 107, 669-74 (1968).

67. Goldstein, L. Biochemistry, 11, 4072-84 (1972).

68. Guilbault, G.G., Lubrano, G.J. Anal. Chim. Acta, 60, 254-6 (1972).

69. Nagy, G., von Storp, L.H., Guilbault, G.G. Anal. Chim. Acta, 66, 443-55 (1973)

70. Williams, D.L., Doig, A.R., Korosi, A. Anal. Chem. 42, 118-121 (1970).

71. Nilsson, H., Akerlund, A., Mosbach, K. Biochim. Biophys. Acta, 320, 529-34 (1973).

72. Updike, S.J., Schults, M.C., Busby, M. J. Lab. Clin. Med., 93 (4), 518-27 (1979).

73. Kunz, H.J., Stastny, M. Clin. Chem. 20, (8) 1018-22 (1974).

74. Weibel, M.K., Dritschilo, W. et al. Anal. Biochem, 52, 402-14 (1973).

75. Inman, D.J., Hornby, W.E Biochem. J. 129, 255-62 (1972).

76. Filippusson, H., Hornby, W.E. McDonald, A. FEBS Lett. 20, 291-3 (1972).

77. Marconi, W. Bartoli, S. et. al. Process Biochem. 9, (4), 22-4 (1974).

78. Leon, L.P., Narayanan, S. et al. Clin. Chem. 22 (7), 1017-23 (1976).

79. Chirillo, R., Caenaro, G. et al. Clin. Chem. 25, (10), 1744-48 (1979).

80. Puget, K., Michaelson, A.M. Biochimie, 58 (6) 757-8

81. Williams, D.C. et al. Clin Chem. W.S) 22, 372-4 (1976).

82. Moeller, K.G., Wandrey, C. Eur. J. Appl. Microbiol. 3, 81-9 (1976).

83. Miller, J.N., Rocks, B.F., Burns, D.T. Anal.Chim. Acta., 93, 353-6 (1977).

84. Wease, D.F., Anderson, Y.J., Ducharme, D.M. Clin. Chem. 25, (7), 1346-7 (1979).

85. Guilbault, G.G., Montalvo, J.G., J. Amer. Chem. Soc. 91, 2164-5 (1969).

86. Guilbault, G.G., Montalvo, J.G. J. Amer. Chem. Soc. 92, 2533-8 (1970).

87. Guilbault, G.G., Hrabankova, E. Anal. Chim. Acta. 52, 287-94 (1970).

88. Guilbault, G.G., Nagy, G. Anal Chem, 45, 417-9 (1973).

89. Guilbault, G.G., Nagy, G. Anal Chim Acta, 67, 195-201 (1973).

90. Anfalt, T., Granelli, A., Jagner, D. Anal. Letts. 6, 969-75 (1973).

91. Guilbault, G.G., Tarp, M. Anal Chim. Acta. 73, 355-65 (1974).

92. Guilbault, G.G., Shu, F. Anal. Chem. 44, 2161-66 (1972).

93. Tran-Minh, C., Broun, G. Anal.Chem. 47, 1359-64 (1975).

94. Mascini, M., Guilbault, G.G. Anal Chem. 49, 795-8 (1977).

95. James, D.R., Pring, B. Clin. Chem. Acta. 62, 435-8 (1975).

96. Kuan, J-C.W., Lau, H.K.Y., Guilbault, G.G. Clin. Chem. 21 (1), 67-70 (1975).

97. Durand, P., David, A., Thomas, D. Biochim et Biophys. Acta. 527, 277-81 (1978).

98. Ngo, T. T., Yam, C.F. Int. J. Biochem. 9, 685-8 (1978).

99. Guilbault, G.G., Nagy, G. Anal. Lett. 6, 301-12 (1973).

100. Guilbault, G.G., Hrabankova, E. Anal. Chim. Acta. 56, 285-90 (1971).

101. Cullen, L.F., Rusling, J.F. et al. Anal. Chem. 46, 1955-61 (1974).

102. Nilsson, H., Mosbach, K., Enfors, S-O., Molin, N., Biotechnol. Bioeng. 20, 527-39 (1978).

103. Enfors, S-O., Nilsson, H. Enzyme Microb. Technol. 1, (4), 260-4 (1979).

104. Paparello, G.J., Mukherji, A.K., Shearer, C.M. Anal. Chem. 45, 790-92 (1973).

105. Guilbault, G.G., Lubrano, G.J. Anal. Chim. Acta 69, 189-94 (1974).

106. Guilbault, G.G., Nan jo, M. Anal. Chim. Acta 75, 169-80 (1975).

107. Toftgard, R., Anfalt, T., Graneli, A. Anal. Chim. Acta 99, 383-5 (1978).

108. Senn, D.R., Carr, P.W., Klatt, L.K. Anal. Chem. 48, (7), 954-8 (1976).

109. Lau, H.K., Guilbault, G.G., Clin. Chem. Acta 53, 209-16 (1974).

110. Huang, N.F., Kuan, J.W., Guilbault, G.G. Clin. Chem. 21, 1605-8 (1975).

111. Pesce, M.A., Bodourian, S.H. Clin. Chem. (W-S) 23, 757 (1977).

112. Patsch, W. et al. J. Lipid Res., 17, 182-5 (1976).

113. Yamanaka, K. Agric. Biol. Chem., 39, 2227-34, (1975).

114. Taylor, P.J. et.al. Anal. Chem., 49, 789-94 (1977).

115. Holtzman, J.C., Anal. Chem., 48, 229-30 (1976).

116. Haard, N.F. et. al. Anal. Biochem., 69, 627-31 (1975).

117. Kondo, H. et al. Agric. Biol. Chem., 41, 631-4 (1977).

118. Duval, C. "Inorganic Thermogravimetric Analysis" 2nd ed. Elesevier, 1963.

119. Ewing, G. W. "Instrumental methods of chemical analysis", McGraw-Hill, 1969.

120. Hume, D.N., Jordan, J. Anal. Chem., 30, 2064-5 (1958).

121. Wasilewski, J.C., Pei, P.T.S., Jordan, J. Anal. Chem., 36, 2131-3 (1964).

122. Carr, P.W. C.R.C. Crit. Rev. Anal. Chem., 491-557 (Jan. 1972).

123. Jordan, J., Dumbaugh, W.H. Anal. Chem., 31, 210-13 (1959).

124. Vaughan, G.A., Swithenbank, J.J., Analyst, 92, 364-70 (1967).

125. Miller, F.J., Thomason, P.F. Anal. Chim. Acta, 21, 112-16 (1959).

126. Jordan, J., Billingham, E.J. Anal. Chem., 33, 120-3 (1961).

127. Jordan, J., Alleman, T.G. Anal. Chem., 29, 9-13 (1957).

128. Jordan, J., Henry, R.A., Wasilewski, J.C. Mickrochem. J., 19, (10), 260-72 (1966).

129. Schafer, H., Wilde, E. Fresnius' Z. Anal. Chem., 130, 396-401 (1950).

130. Everson, W.L., Ramirez, E.M. Anal. Chem., 37, 806-11 (1965).

131. Lavoisier, A.L., deLaplace, P.S. in "Sur la Chaleur" Paris, 1780.

132. Kitzinger, C., Benzinger, T.H. Methods Biochem. Anal., 8, 309-60 (1960).

133. Wädso, I., Acta Chem. Scand., 22, 927-37 (1968).

134. Linde, H.W., Rogers, L.B., Hume, D.N. Anal. Chem. 25, 404-7 (1953).

135. McGlothlin, C.D., Jordan, J. Clin. Chem. 21, (6), 741-5 (1975).

136. McGlothlin, C.D., Jordan, J. Anal. Chem. 47 (8), 1479-81 (1975).

137. McGlothlin, C.D., Jordan, J. Anal. Chem. 47 (6), 786-790 (1975).

138. Goldberg, R.N., Prosen, E.J. et al. Anal. Biochem., 64, 68-73 (1975).

139. Goldberg, R.N., Biophys. Chem., 3, 192-205 (1975).

140. Goldberg, R.N., Biophys. Chem., 4, 215-221 (1976).

141. Goldberg, R.N., Clin. Chem. 22 (10), 1685-91 (1976).

142. Prosen, E.J., Goldberg, R.N. et al. in "Thermal Analysis: Comparative Studies on Materials" (ed. Kambe, H., Garn, P.) pub. Kodansha and John Wiley, Tokyo-New York, pp. 253-89 (1974).

143. Monk, P., Wädso, I. Acta Chem. Scand. 23 (1), 29-36 (1969).

144. Pennington, S.N. Enzyme Technol. Digest 3 (2), 105-114 (1974).

145. Jespersen, N.D. J. Amer. Chem. Soc., 97, 1662-7 (1975).

146. Beezer, A.E., Steenson, T.I., Tyrell, H.J.V. Talanta 21, 467-74 (1974).

147. Rehak, N.N., Janes, G., Young, D.S. Clin.Chem. 23, 195-99 (1977).

148. Grime, J.K., Tan.B. Anal.Chim.Acta, 107, 319-26 (1979).

149. Donnovan, L., Barclay, K. et al. Thermochimica Acta, 11, 151-6 (1975).

150. Carr, P.W., Bostick, W.O., Canning, L.M., Callicott, R.H. American Laboratory, 8(1), 45-53 (1976).

151. Wädso, I. Biochem.Soc.Trans., 4, 561-5 (1976) (563rd Meeting, London).

152. Beezer, A.E., Tyrrell, H.J.V. Science Tools, 19(1), 13-16 (1972).

153. Beezer, A.E., Proc.Anal.Div., Chem.Soc. 14 (5), 104-6 (1977).

154. Johansson, A. Protides Biol.Fluids, Proc.Colloquia, 1972, 20, 567-70 (1973).

155. Johansson, A. Lundberg, J., Mattiassen, B., Mosbach, K. Biochim.Biophys.Acta 304, 217-21 (1973).

156. Schmidt, H-L, Krisam, G., Grenner, G. Biochim. Biophys.Acta, 429, 283-90 (1976).

157. Mosbach, K., Danielsson, B., Borgerud, A., Scott, M. Biochim.Biophys.Acta, 403, 256-65 (1975).

158. Mattiassen, B., Danielsson, B., Mosbach, K. Anal.Letts. 9(3), 217-34 (1976).

159. Mattiassen, B., Danielsson, B., Mosbach, K. Anal.Letts. 9(10), 867-89 (1976).

160. Danielsson, B., Gadd, K., Mattiassen, B., Mosbach, K. Clin.Chim.Acta, 81, 163-75 (1977).

161. Danielsson, B., Mattiassen, B., Karlsson, R., Wingvist, F. Biotechnol. Bioeng., 21(10), 1749-66 (1979).

162. Tran-Minh, C., Vallin, D. Anal. Chem., 50(13), 1874-8 (1978).

163. Weaver, J.C., Cooney, C.L., Fulton, S.P. et al. Biochim. Biophys. Acta, 452, 285-91 (1976).

164. Bowers, L.D., Carr, P.W. Clin. Chem. 22(9), 1427-33 (1976).

165. Pennington, S.N., Anal. Biochem., 72, 230-7 (1976).

166. Canning, L.M., Carr, P.W., Anal. Letts. 8, (5), 359-67 (1975).

167. Bowers, L.D., Canning, L.M., Sayers, C.N., Carr, P.W. Clin. Chem. 22(8), 1314-18 (1976).

168. Danielsson, B., Gadd, K., Mattiassen, B., Mosbach, K. Anal. Letts. 9(11), 987-1001 (1976).

169. Schifreen, R.S., Hanna, A.D., Bowers, L.O., Carr, P.W., Anal. Chem. 49 (13), 1929-39, (1977).

170. Mosbach, K., Danielsson, B. Biochem. Biophys. Acta 364, 140-5 (1974).

171. Mosbach, K., U.S. patent 4,021,307 (May 3rd 1977).

172. Forrester, L.J., Yourtee, D.M., Brown, H.D., Anal. Letts. 7 (8 and 9), 599-608 (1974).

173. Pennington, S.N. Natl. Sci. Found. Res. Appl. Natl. Needs (Rep) NSF/RA (U.S.) 1975 (MSF/RA-760032 Enzyme Technol) Grantees-Users Conf PB-265 548 pp. 124-8.

174. Mattiasson, B., Danielsson, B., Abstract 3847, 12th FEBS Meeting, Dresden (1978).

175. Mattiasson, B., Danielsson, B., Mosbach, K., in Enzyme Engineering (Pye, E.K., Weethall, H.H. eds.) Vol. 3, pp. 453-60, (Plenum, 1978).

176. Danielsson, B., Johansson, B.G., Mattiasson, B., Acta Soc. Med. Suec., 86(4), 282 (1977).

177. Danielsson, B., Mattiasson, B., Mosbach, K. Pure Appl. Chem., 51, 1443-57 (1979).

178. Mattiasson, B. FEBS Letts. 77, 107-110 (1977).

179. Mattiasson, B., Mosbach, K., Svenson, A. Biotechnol. Bioeng. 19 (11), 1643-51 (1977).

180. Mattiasson, B., Borrebaeck, C., Sanfridson, B., Mosbach, K. Biochim. Biophys. Acta, 483, 221-7 (1977).

181. Monzan, P., Puzo, G., Mazarguil, H. Biochimie, 57, 1281-92 (1975).

182. Meerwein, H. Organic Syntheses (Vol. 46) 113-5 (1966) pub. John Wiley and Sons, London, New York, Sydney.

183. Alicino, J.F. Ind. Chem. Eng. Anal. Ed. 18, 619-20 (1946).

184. Hamilton-Miller, J.M.T., Smith, J.T., Knox, R. J. Pharm. Pharmacol, 15, 81-91 (1963).

185. Ilver, K., Johansen, O.I., Reimers, F., Acta Pharm Int. 1, 225-41 (1950).

186. Hornby, W.E. in Enzyme Engineering (Ed. Pye and Wingard), Plenum, New York, 2, 401 (1974).

187. Bark, L.S., Grime, J.K. Anal. Chim. Acta, 64, 276-9 (1973).

188. Oljelund, G., Wädso, I. Acta Chem. Scand., 22, 2691-9 (1968).

189. Watt, G.D., Sturtevant, J.M. Biochemistry, 8, 4567-71 (1969).

190. Fearon, W.R. Biochem. J., 33, 902-7 (1939).

191. Natelson, S., Scott, M.L., Beffa, C. Am. J. Clin. Pathol., 21, 275-81 (1951).

192. Evans, R.T. J.Clin.Pathol., 21, 527 (1965).

193. Chaney, A.L., Marbach, E.P. Clin.Chem., 8, 130-2 (1962).

194. Kaltwasser, H., Schlegal, H.G. Anal.Biochem. 16, 132-8 (1966)

195. Roch-Ramel, F. Anal.Biochem., 21, 372-81 (1967).

196. Jespersen, N.D. J.Amer.Chem.Soc. 97 (7) 1662-7 (1975).

197. Borrebaeck, C., Börjeson, J. Scand.J.Clin.Lab.Invest., 40, 169-172 (1980).

198. Pileggi, V.J., Szustkiewicz, C.P. in Clinical Chemistry: Principles and Techniques, 2nd edition, ed. Henry, Cannon and Winkelman, pub. Harper, New York, 1974 page 1274.

199. Martinek, R.G. J.Am.Med.Technol, 31, 530-70 (1969).

200. Monosaccharide Chemistry (ed. Ferrier and Collins), Penguin, London, 1972 p.34.

201. Slein, M.W. in Methods of enzymatic analysis (ed. Bergmeyer), Academic Press, New York, page 117 (1965).

202. Widdowson, G.M., Penton, J.R. Clin.Chem.(Winston-Salem) 18, 299-300 (1972).

203. Young, D.S., Pestaner, L.C., Gibberman, V. Clin.Chem. 21, (5) 1D (1975).

204. Enzymes (ed. Dixon, M and Webb, E.C.) pub. Longmans, London (1958) p.286.

205. Cramer, F.B. Woodward, G.E. J.Franklin Inst., 253, 354-60 (1952).

206. Beezer, A.E., Stubbs, C.D. Talanta, 20, 27-31 (1973).

207. Konickova, J., Wädso, I. Acta Chem.Scand., 25, 2360-2 (1971).

208. Grime, J.K., Tan, B. Anal.Chim.Acta 106, (1), 39-49 (1979).

209. Mongar, J.L. in Enzymes in drug action (ed. de Rench, A.V.S.) pub. Little and Brown, Boston, 1962.

210. Todrick, A. Brit. J. Pharmacol. 9, 76-83 (1954).

211. Mattiasson, B., Danielsson, B., Mosbach, K. FEBS Letts. 85 (2), 203-6 (1978).

212. Enzymes (ed. Dixon M, and Webb E.C.) pub. Longmans, London (1958) p. 381.

213. Baldridge, J.N., Jesperson, N.D. Anal. Letts., 8(9), 683-96 (1975).

214. Toren, E.C., Burger, F.J. Mikrochim. Acta, 1968 (5) 1049-58.

215. Baum, P., Czok, R. Biochem. Z. 332, 121-30 (1959)

216. Townshend, A., Vaughan, A. Anal. Chim. Acta, 49, 366-7 (1970).

217. Townshend, A., Vaughan, A. Talanta, 17(4), 289-98 (1970).

218. Lehky, P., Stein, E.A. Anal. Chim. Acta, 70, 85-93 (1974).

219. Kobayashi, K., Fujiwara, K. et al. Bull. Chem. Soc. Jap. 52(7), 1932-36 (1979).

220. Stone, J.V., Townshend, A. J.C.S. Dalton, 1973, 495-501.

221. Kobowitz, F. Biochem. Z. 296, 443 (1938.

222. Kobowitz, F. Biochem. Z. 299, 32-57 (1938).

223. Allen, T.H., Bodine, J.H. Science, 94, 443-4 (1941).

224. Lerner, A.B., Fitzpatrick, T.B. et al. J. Biol. Chem. 187, 793-802 (1950).

225. Lerner, A.B. Arch. Biochem. Biophys. 36, 473-81 (1952).

226. Kertesz, D. Nature 168, 697 (1951).

227. Kertesz, D., Rotilio, G., et al. Biochem. Biophys. Res. Comm. 49, (5), 1208-15 (1972).

228. Engström, L. Biochim.et Biophys.Acta , 52, 36-48 (1961)

229. Plocke, D.J., Vallee, B.L. Biochemistry, I, 1039-43 (1962)

230. Morton, R.K., Biochem.J. 65, 674-82 (1957).

231. Townshend, A., Vaughan, A. Talanta 16, 929-37 (1969).

232. Tait, G.H., Vallee, B.L. Proc.Nat.Aca.Sci. U.S., 56, 1247 (1966).

233. Mealor, D., Townshend, A. Talanta, 15, 1477-9 (1968).

234. Mealor, D., Townshend, A. Talanta, 15, 747-58 (1968).

235. Mealor, D., Townshend, A. Talanta, 15, 1371-6 (1968.

236. Toren, E.C., Burger, F.J. Mikrochim.Acta, 1968, 538-45.

237. Guilbault, G.G., Kramer, D.M., Cannon, P.L. Anal. Chem., 36, 606-10 (1964)

238. Setter, H. Chem.Ber., 81, 532-9 (1948).

239. Guilbault, G.G., Kramer, D.N., Hackley, E. Anal.Biochem. 18, 241-7 (1967).

240. Guilbault, G.G., Brignac, P., Zimmer, M. Anal.Chem. 40, 190-6 (1968).

241. Guilbault, G.G., Sadar, M.H., Zimmer, M.Anal.Chim. Acta, 44, 361-7 (1969).

242. Guilbault, G.G., in Enzymatic Methods of Analysis (pub. Pergamon), Oxford, 1970. pp.197-234.

243. Kennedy, J.F. in Immobilized Microbial Cells (ed. Venkatsubramanian) pub. Amer.Chem.Soc, 1979 pp.119-131.

244. Matsumoto, K., Seijo, H., Watanabe, T. Anal.Chim.Acta 105 , 429-32 (1979).

245. Mattiasson, B. A.C.S.Symposium Series 106, 203-20 (1979).

246. Yalow, R.S., Berson, S., Nature, 184, 1648-9 (1959).

247. Ekins, R.P. Clin. Chem. Acta, 5, 453-9 (1960).

248. Avrameas, S., Guibert, B. C.R. Acad. Sci. Ser. D. 273, 2705-7 (1971).

249. Engvall, E., Perlmann, P. Immunochemistry, 8, 871-4 (1971).

250. Van Weeman, B., Schuurs, A. FEBS Letts. 15, 232-6 (1971).

251. O'Sullivan, M.J., Bridges, J.W., Marks, V. Annals of Clin. Biochem., 16, 221-39 (1979).

252. Ishikawa, E. Techniques in Metabolic Research, B217, 1-18 (1979).

253. Weetall, H.H. Food Prod. Develop. 7(3), 46-52 (1973).

254. Weetall, H.H. Food Prod. Develop. 7(3),94-100 (1973).

255. Kilara, A., Shahani, K.M. C.R.C. Crit. Rev. Food Sci. and Nutrition, 12(2), 161-98 (1979).

256. Berezin, I.V., Varfolomeev, S.D. Appl. Biochem. Bioeng. 2, 259-90 (1979).

257. Larsson, P.O., Mosbach, K. Biotechnol. Letts., 1(12), 501-6 (1979).

258. Mandels, M., Kostick, J., Parizek, R. J. Polymer Sci., Part C 36, 445-59 (1971).

259. Camp, T.R. in "Water and its impurities" pub. Reinhold, New York) Chpt. 5 (1963).

260. Malaprade, L. Bull. Soc. Chim. France (5e) 1, 833 (1934).

261. Malaprade, L. Bull. Soc. Chim. France (4e) 43, 628 (1929).

262. Fundamentals of Analytical Chemistry. (ed. Skoog and West), pub. Holt International, London (1970).