IMMOBILIZED AND SOLUBLE ENZYME PREPARATIONS: THEIR USE IN UNSEGMENTED FLOW ANALYZERS

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CHAPTER I

INTRODUCTION

Both industries and clinical laboratories use continuous-flow analyzers. In a broad sense, continuous-flow analysis refers to any process in which concentrations of one or more analytes are measured, uninterruptedly, in a moving stream of fluid. This method eliminates the need for stepwise measurement, addition, and processing of samples and reagents.

An air-segmented continuous-flow system using a colorimetric method was first reported by Skeggs in 1957 (1). In this method samples of whole blood or serum were successively pumped into a flowing stream of diluent. The diluted sample was passed through a dialyzer where diffusable constituents passed the membrane and were picked up by a flowing stream of reagent. This stream was then either mixed with other reagents and heated, in order to produce a color related to the desired constituent, or processed in a continuous manner. The colored stream was finally passed through a flow-cell colorimeter equipped to record the results of the analysis. Urea nitrogen was successfully determined by this method (1).

The precision, accuracy, inherent errors, and kinetic parameters of continuous-flow analysis have been described (2, 3). Numerous papers have appeared since then, describing experiments directed towards method development. These developments include modification of the AutoAnalyzer

from single channel to multichannel and also from the use of photodetectors to the use of other different types of detectors. The AutoAnalyzer is a modular, automatic instrument that was developed and commercialized by the Technicon Instrument Company. It has proved to be an extremely versatile instrument that has answered the need for automation of repetitive chemical tests in laboratories, government, and academic laboratories and industrial testing facilities. Other laboratory instruments for continuous flow analysis are available, but by far the one most commonly used is the Technicon AutoAnalyzer.

In 1975 a technique known as flow-injection analysis was introduced (4). This is a modification of the previous continuous-flow methods in that no air segmentation is involved and the sample is injected directly into the flowing stream. Basically the AutoAnalyzer system uses a segmented flow while the flow-injection system uses unsegmented flow. Theoretical discussions of both are presented below.

Segmented-Flow Analysis (AutoAnalyzer)

Whatever sophistication has been developed in the AutoAnalyzer since its invention, the basic components that are needed to assemble it remained the same and are: a) sampling device, b) pump (peristaltic), c) mixing coil or mixing chamber, debubbler, d) detector, and e) readout.

The components come in modules and thus any required modification for further advancement can easily be performed. A schematic diagram of the basic AutoAnalyzer modules is shown in Figure 1.

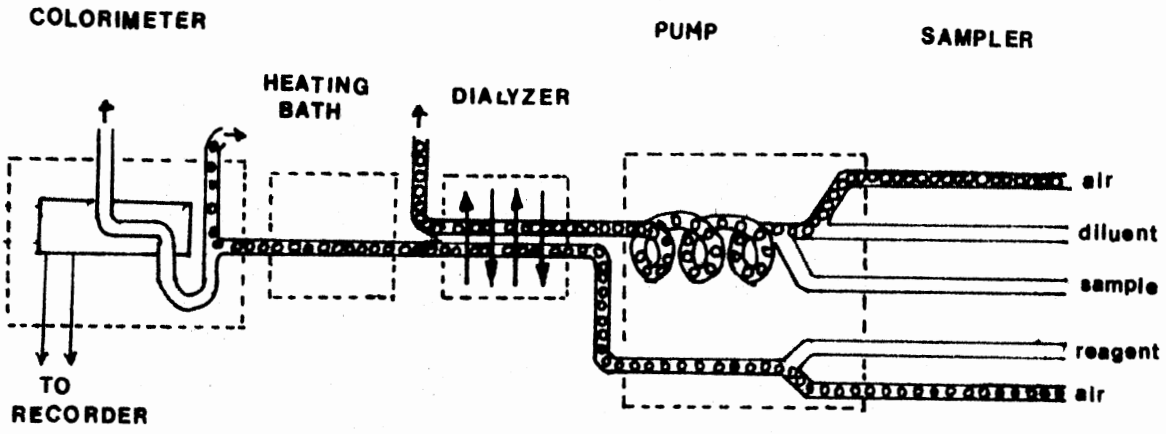


Figure 1. Schematic Representation of the Basic AutoAnalyzer Modules

The sampler consists of a circular plate that holds small plastic cups containing the samples to be analyzed. The plate is rotated in a timed sequence. With the help of the proportioning pump, the sample diluted to the required extent passes through the dialyzer. The dialyzer contains a semipermeable membrane which allows the required constituent to pass through it to the flowing stream while holding back high molecular-weight compounds (such as proteins), which are then discarded to waste. The flowing stream usually contains a reagent that develops color with the required constituent. A constant-temperature heating device is usually required for color development. The solution then flows to a detector for measurement of some physical parameter--usually color--that is a direct function of the concentration of the desired constituent in the original sample. The most wisely used detector is a simple filter photometer. However, electrochemical sensors, atomic absorption spectrophotometers, fluorometers, Υ-ray spectrometers,

scintillation counters, refractometers, and other spectrophotometers have been used as detectors. The change that occurs in the detector is then recorded usually by a strip-chart recorder; oscilloscopes and direct digital readouts have been also used.

Continuous-flow systems possess kinetic parameters of flow, unlike batch-analysis systems (manual methods). An ideal continuous-flow system would yield an output trace (peak) that corresponds exactly with input solute (peak) that corresponds exactly with input solute concentration changes, subject only to a time delay as required for chemical reactions involved. The peak is ideally expected to be a square-wave type.

> A sudden change of concentration at the sampler probe results in a gradual output transition, sigmoid in outline, from one steady state to another. The fluid stream linking sampler to detector imposes a deformation on the input solute concentration change, and this deformation is the limiting factor in sampling rate; it affects the extent to which peaks approach a plateau, the magnitude of interaction between one peak and the next, and the frequency of shoulder peaks in which a high sample obliterates the peak of a following low sample (5, p. 208).

Experimental work indicates that this deformation results mainly from occurrences in the segmented stream and in the unsegmented stream in the debubbler and flow cell (6).

The segmented stream deformation results because the solute molecules enter the segmented stream and move along the stream as a compact zone of solute occupying one (or at most two) fluid segments. When this zone arrives at the end of the segmented stream, it will have broadened and will have a symmetrical fall off in concentration ahead of and behind the zone of maximum concentration. Conclusions from experimental evidence indicate that solute molecules entering the seg-

mented stream travel with velocities normally distributed about the mean flow rate, so that the residence time of molecules in a segmented stream is distributed symmetrically about the mean residence time. As a result, a square wave at the input emerges from the segmented stream with a sigmoid front described by the integral of the normal probability curve (Figure 2a).

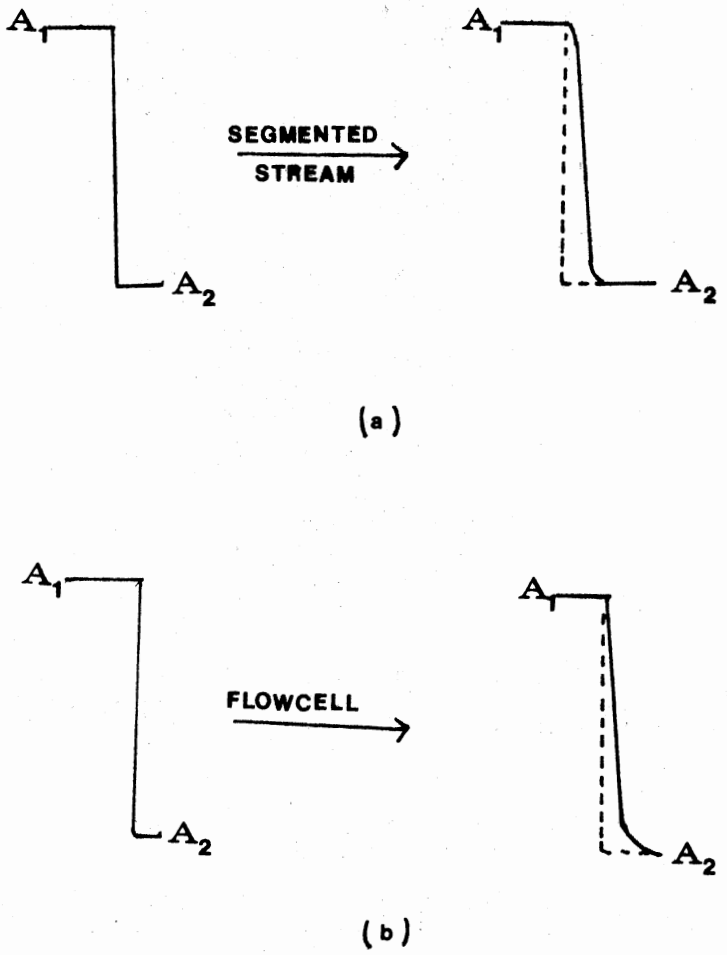


Figure 2. Square-wave Deformation of Signals (a) Due to Segmented Stream, (b) Exponential Deformation in Flow Cell

Under normal operating conditions, the deformation due to the flow-cell is found to conform closely to an exponential curve (Figure 2b). A further exponential deformation is introduced if the sample is diluted in the manifold and resampled after the diluted stream is debubbled.

Because of these factors a typical signal of the AutoAnalyzer looks like the one shown in Figure 3.

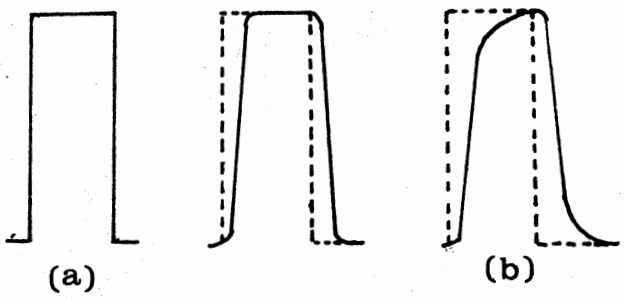


Figure 3. Signals of the AutoAnalyzer (a) Ideal (b) Typical

Unsegmented Flow Analysis (Flow-Injection Analysis)

Superficially the main features of unsegmented-flow analysis or "Flow-Injection Analysis" are the absence of air segmentation and the skill required in injecting the sample into the stream. This skill depends on a combination of three principles: (a) sample injection, (b) reproducible timing, and (c) controlled dispersion (8).

In flow-injection analysis a well-defined sample is injected into

a continuously moving stream in such a way that the movement of this stream is not disturbed. The total volume of sample and the length of sample zone at the point of injection must be reproducible. In the initial stages of the development of this technique a syringe and a flap valve (an injection block in which a silicone rubber disc squeezed between two blocks served as a septum) and sample loops operated by a set of magnetic valves (9) were used. This was then followed by the use of rotary valves. In the rotary valve, the carrier stream flows undisturbed while the core or loop is filled with sample. When the core of the valve is turned the stream now passes through the loop and the sample is injected into the system. This method of introducing the sample into a continuously flowing stream is more reproducible than the aspiration technique used in the air-segmented system; it allows the conventional concept of "steady-state signal" to be abandoned and thus permits analysis of larger numbers of samples in a given period of time.

In flow-injection analysis, highly reproducible timing is vital, since the signal is read on the ascending part of the curve rather on the flat part of the segmented-flow systems (Figure 4).

Since air can be compressed, any segmented stream pulsates and therefore neither the tube diameter nor the tube length can be decreased beneath a certain value if these pulsations are not adversely to affect the reproducibility of the timing required by the chemical reaction in question. Such a problem is not observed in an unsegmented system.

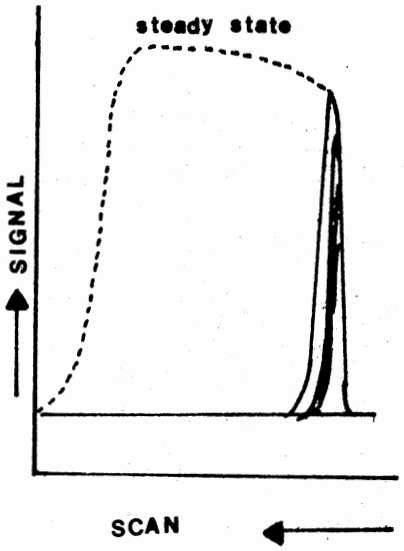


Figure 4. Typical Signals of AutoAnalyzer With Different Concentration Profiles

After injection the sample zone broadens as it moves towards the detector and its shape changes from asymmetrical to a more symmetrical and eventually to a Gaussian curve (Figure 5).

The flow parameters can be changed in unsegmented flow systems so that the dispersion (flow disturbance effect due to interaction between the injected sample and flowing stream) can be easily manipulated to satisfy the requirements of a particular analytical procedure and obtain optimum response at minimum time and reagent expense.

One limitation of continuous-flow analysis is the wasteful consumption of reagents. However, reagent usage can be decreased by the use of tubes of smaller diameter (which thus increases sampling rate), by using immobilizing enzymes, and by operating in a closed-flow system. Expensive enzymes or other reusable reagents can be physically or chemically

immobilized on the inside of tubing or other supports and used in continuous-flow analysis. Consumption of such reagents is thus decreased by about 100 to 10,000 times and the cost of analysis is decreased. Some examples of continuous-flow analysis in which an immobilized enzyme is utilized are the determination of urea using covalently attached urease (10), glucose with immobilized hexokinase and glucose phosphate dehydrogenase, cholesterol using cholesterol esterase and oxidase, triglycerides using pertinent immobilized enzymes (11), and uric acid using immobilized uricase (12, 13).

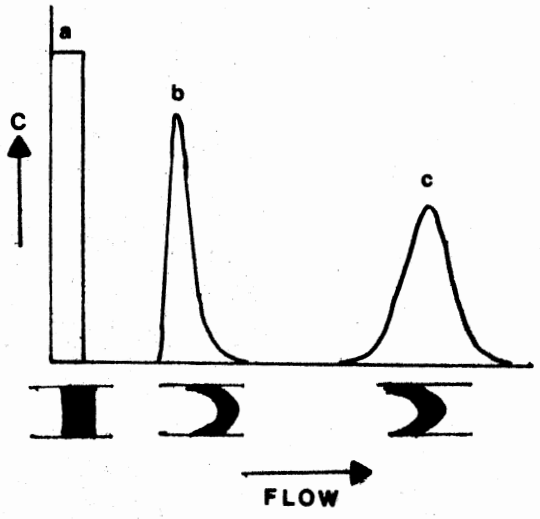


Figure 5. Typical Peak Profiles (a) at Point of Injection (b) Shortly After Injection (c) Long After Injection

The use of a closed continuous-flow system can also reduce the rea-

gent use and cost. A very good example is the one devised by Wolff and Mottola (14) in which a known concentration of glucose oxidase solution was continuously recirculated in analysis for glucose.

The studies described in this thesis were undertaken in an effort to apply the advantages of closed-flow-injection systems in the determination of enzymes (i.e., activity determination) and of substrates that utilize enzymes for reaction catalysis, with minimal quantities of reagents and subsequent reduction of cost of analysis.

The earlier success that Wolff and Mottola had with a closed system and amperometric detection (14) encouraged us to undertake the studies reported in this dissertation. Chemically they are based on:

$$\text{Substrate} + O_2 \xrightarrow{\text{Enzyme}} \text{Product(s)} + H_2O_2$$

and if needed:

$$H_2O_2 \xrightarrow{\text{Catalase}} 1/2\ O_2 + H_2O$$

These studies can be grouped as follows:

(1) Application of a closed-flow system for the determination of enzyme activity with potential usefulness in the quality control of enzyme preparations as they become used more and more as analytical reagents. Because of the system design of closed-flow, unsegmented analysis, this part of the work encompassed the design of an effective means of removing or rendering inactive the enzyme catalyst (this part of the work is reported in Chapter IV).

(2) Use of amino acid oxidases for the determination of D- and L-amino acids. This part of the work covered the use of both soluble and

insoluble (immobilized) enzyme preparations, and when pertinent included some kinetic considerations (work reported in Chapter V).

(3) Use of uricase for the determination of uric acid in biological fluids. Special attention was given here to the immobilization (chemical) of the enzyme since it represents a clear-cut case of an enzyme sufficiently unstable and expensive for routine use in soluble form (work reported in Chapter VI).

Analytical uses of immobilized enzymes in chemical analysis are becoming widespread. A detailed literature review on immobilized enzymes and their use in analytical application is presented in Chapter II.

CHAPTER II

IMMOBILIZATION OF ENZYMES AND THEIR APPLICATION IN ANALYTICAL CHEMISTRY

Immobilization of Enzymes

Introduction

Enzymes are biochemical protein catalysts for chemical reactions that occur "in vivo" in living systems. They are mostly linear chains formed by the head-to-tail polymerization of about 20 amino acids. Maximum activities shown by enzymes vary from one to another, but reaction rates corresponding to the turnover of 100 to 10,000 substrate molecules per molecule of enzyme per second may be regarded as typical. Synthetic catalysts made by man are nowhere close to giving these performances. Since increasing the sensitivity of a method is one of the goals of the analytical chemist, enzymes come to his rescue in this respect. Concentrations of substrates of 10^{-6} to 10^{-16} mole/L can often be determined by enzymic analysis.

Enzyme-catalyzed reactions have been used for the determination of metabolites (substrates), enzyme activities, activators, and inhibitors. An important property of an enzyme which has been used to an advantage is its high selectivity. This helps in singling out a substrate from closely related homologs of that substrate, when performing an analysis (15).

Another attribute of enzymic analysis is that it avoids the lengthy separations and shortens the time required for the analysis. Owing to the mild reaction conditions used (neutral pH, moderate temperature), enzymes often allow the determination of labile substances that cannot be measured by some other analytical methods.

The development of enzyme chemistry is essential to the further evolution of new enzymatic methods, which finally find their way into clinical chemistry and other analytical fields. New methods contribute greatly to the development and advancement of applied enzymatic chemistry and biochemistry.

Structures of enzymes are continuously studied and thus can lead to new possibilities for the identification of enzymes. Analytical methods such as x-ray structural analysis, NMR, and ESR can produce detailed information on the fine structure and reactivity of the large molecules. At the present time almost all methods of functional analysis are physical methods. Kinetic constants and activities of enzymes are determined by means of a knowledge of physical parameters, measured by methods such as spectrophotometry, polarography, acidimetry, turbidimetry, and isotope effects.

In the past two decades the interest in immobilization of enzymes has been tremendous. Immobilization refers to the localization or confinement of enzymes during a process that allows them to be physically separated from the substrate and products for re-use. This tremendous surge of interest resulted from the many advantages that immobilized enzymes exhibit in comparison with the soluble ones. Some of these advantages that are of interest in analytical chemistry are:

1. Expensive enzymes when immobilized can be re-used. For example,

the same immobilized enzyme can be used for converting a large amount of substrate and still be active. This allows the analytical chemist to use a relatively large amount of enzymes to increase turnover of products and thus increase sensitivity.

2. The reaction process can be carried out continuously. This has been applied in continuous-flow analysis methods.

3. When an enzyme is immobilized, the products of the enzymatic reaction are free of the enzymes. This is applicable in the analysis of either pure products or those in which interference of the enzyme might be a problem in the analysis.

4. Some enzyme immobilizations involve a chemical change in the enzyme molecule and cause changes in its response to pH, temperature, denaturation, and specific substrates (16, 17), in a favorable way many times. Thus these enzymes can be used under adverse conditions (18).

5. Some enzymes can serve as models for the studies of enzymes bound to their natural cell membranes. This was one of the reasons that inspired work on enzyme immobilization in the first place.

Methods of Immobilization of Enzymes

Immobilization refers to the localization or confinement of enzymes during a process that allows the enzyme to be separated physically from the substrate and products for re-use. Many synonyms are used to describe immobilized enzymes, among which are insolubilized (classically enzymes have been immobilized by attaching them with water-insoluble materials), support-bound, carrier-bound, matrix-bound, and entrapped. Generally, immobilization can be achieved by chemically or physically attaching the enzyme to a support or confining the enzyme by means of a

semipermeable membrane.

The first reported enzyme immobilization was carried in 1916 by Nelson and Griffin (19). They observed that invertase (extracted from yeast) was adsorbed on charcoal and that the adsorbed enzyme showed the same activity as the native enzyme. Other adsorption experiments were done in subsequent years, such as adsorption of invertase on alum or charcoal, by Nelson and Hitchcocks (20), "monolayer" spreading of urease or pepsin on "conditioned" plates by Langmuir and Schaefer (21), and "monlyaer" of protein deposition on barium stearate step plate by Langmuir and Schaefer (22).

The first successful experiments of covalent binding of a variety of proteins including enzymes were carried out after World War II. In 1949, Michael and Evers (23) described the covalent binding of proteins to carboxymethyl cellulose oxide. These first steps were soon followed by other methods of coupling enzymes to polymers. However, enzyme immobilization did not become popular until the 1950s when Grubenhofer and Schleith (24, 25) reported coupling carboxypeptidase and amylase to diazotized poly(p-aminostyrene). These methods of immobilization, however, gave products with poor performances. Since then, tremendous interest has developed in immobilization of enzymes. With increasing interest the methodology, supports, and enzymes used become varied. As a result, supports of superior adsorptive properties were created. At the present time hundreds of papers can be cited that refer to immobilization of enzymes. The number of publications reached its apex in 1973. The funds available for such research have subsequently been switched to energy-related problems. On the other hand, analytical applications of immobilized enzymes are on the rise.

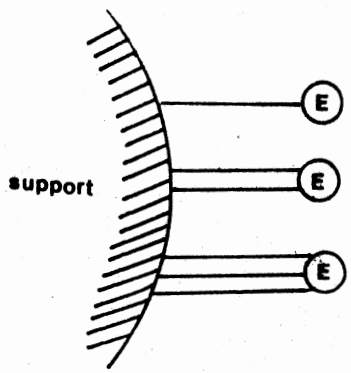
There are several ways of classifying immobilized enzymes, but in general the methods of immobilization can be divided into two broad classes: chemical and physical. [However, the first Enzyme Engineering Conference in 1971 proposed that they be classified as: 1) Entrapped (matrix entrapped and microencapsulated), 2) Bound (adsorbed and covalently bound) (26)].

In chemical immobilization, covalent-bond formation between some functional groups of the support and the enzyme or functional group of two or more enzyme molecules is involved. A usual feature of these chemical methods is the irreversibility of the reaction, i.e., the original enzyme molecules cannot be separated from the matrix and used as free enzyme again. There are, however, some cases in which the enzyme can be separated from the immobilizing matrix.

Among physical methods, different techniques are also included. There, the immobilization of enzymes is dependent on entrapment within microcompartments, or containment within special membranes, or on the operation of certain physical forces such as ionic bonds, electrostatic attractions, hydrogen bonds, and enzyme-enzyme interactions. Theoretically this method is completely reversible, but there are many exceptions.

Chemical Methods

Covalent Attachement. The covalent attachement of enzyme molecules (via amino acid residues not essential to function) to functionalized supports is the most prevalent method for immobilizing enzymes. In this respect one of three techniques can be employed: to activate the matrix for reaction with the groups of the enzyme, to use a coupling reagent to



bind the enzyme with the support, or to activate the enzyme molecule for coupling to the support material. In principle such covalent couplings offer the most stable, most versatile method for immobilizing enzymes.

Widely different supports have been used for the covalent fixation of enzymes. The ones that are commonly employed are shown in Table I.

TABLE I

SOME COMMONLY EMPLOYED SUPPORTS FOR COVALENT ATTACHMENT OF ENZYMES

Synthetic Supports	Natural Supports
Acrylamide-based polymers	Agarose (Sepharose)
Maleic anhydride-based polymers	Cellulose
Methacrylic acid-based polymers	Dextran (Sephadex)
Polypeptides	Glass
Styrene-based polymers	Starch

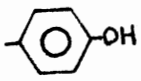
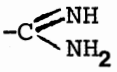
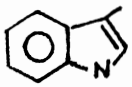
Source: (27).

In the case of enzymes the amino acid residues that are amenable to covalent binding can be limited mainly to the ones shown in Table II.

The relative concentrations of the amino acid residues in the protein molecule can be considered as one of the factors that determine which groups might be involved in covalent attachment to a solid support.

TABLE II

FUNCTIONAL GROUPS OF AMINO ACIDS

Functional Group	Derived From
$-NH_2$	Amino of lysine (Lys) and N-terminus amino groups
-SH	Mercapto of cysteine (Cys)
-COOH	Carboxyl of aspartate (Asp) and glutamate (Glu) and C-terminus carboxyl groups
	Phenolic of tyrosine (Tyr)
	Guanidino of arginine (Arg)
	Imidazole of histidine (His)
-S-S-	Disulfide of cystine
	Indole of tryptophan (Trp)
CH_3-S-	Thioether of methionine (Met)
$-CH_2OH$	Hydroxyl of serine (Ser) and threonine (Thr)

Source: (28).

Table III indicates the average composition of these amino acids with reactive residues for a number of proteins (29).

TABLE III

AVERAGE PERCENT COMPOSITION OF PROTEINS (REACTIVE AMINO ACID RESIDUES ONLY)

Residue	Percent
Ser	7.8
Lys	7
Thr	6.5
Asp	4.8
Glu	4.8
Arg	3.8
Tyr	3.4
Cys	3.4
His	2.3
Met	1.6
Trp	1.2

Another factor that has to be considered is the degree of the hydrophobic nature of the various amino acid residues. Increased hydrophobicity tends to increase the chances of a residue is being buried inside the protein.

Although a particular reaction and reaction condition dictate the reactivity of an amino acid residue, the number of reactions available for each residue can be compared. In a table listing side-chain reactivity Means, and Feeny (30) list the following number of reactions available for modification of these residues: Cys-31, Lys-27, Tyr-16, His-13, Met-7, Trp-7, Arg-6, (Glu, Asp)-4, and Ser, Thr-0.

The majority of reactions referred to above are carbonyl-type reactions (31) with nucleophiles, $-NH_2$, -SH and -OH. At high pH values (such as 9 or above) the NH_2 group would be the one of highest concentration compared to the other two ($-S^-$ and $-O^-$) and thus most likely to react. Although the S anion is more nucleophilic than the N and O (of comparable basicity), its esters are much less stable than the oxygen and nitrogen analogs. The nitrogen-substituted "esters" are the most stable among the three. Therefore, according to all these factors Lys would be predicted to be the most likely coupling residue followed by Cys, Tyr, His (Asp, Glu), Arg, Trp, (Ser, Thr), and Met.

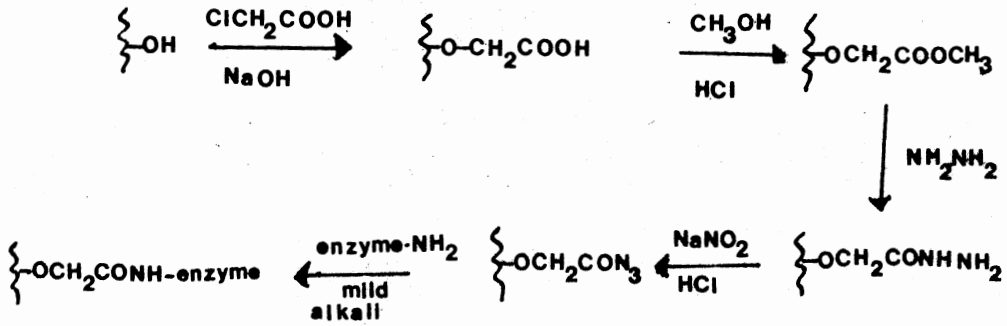
The amino acid residues at active sites have special properties and reactivities. In general, the structure of the enzyme is such that the residues of the active sites are located in a precise three-dimensional arrangement of charges and microenvironment for reaction. Frequently the reactive groups react with a coupling reagent to some degree. However, the coupling reagent interacts with many inactive residues of the protein and the active sites are not specifically favored for reaction. Also, the active sites of enzymes are usually in a recessed part of the enzyme.

Many times enzymes have been found to be inactivated during a coupling process. To prevent or at least minimize this, many precautions should be taken. One of these is to couple the enzyme with the support in the presence of a competitive inhibitor (32) or substrate. In this case the active sites will be occupied by the inhibitor or substrate and thus will not be accessible for bonding. Another one is to use a reversibly formed covalently linked enzyme-inhibitor complex (33, 34) for covalent attachement to the support. A third technique is chemically

to modify an enzyme and then use the newly modified residues for covalent linkage to the support (35). A zymogen (inactive enzyme) precursor has also been used for covalent attachment to a support (36).

To discuss all the different types of immobilization is out of the context of this dissertation. The methods can be classified according to the support types involved or the types of reactions involved. To avoid repetition of the various types of reactions involved with the many different types of supports (specific reaction types are involved in many cases) the latter has been chosen to be the criterion of classification. This classification itself includes a large number of reaction methods. Only some of the most common and often used for analytical purposes are described below.

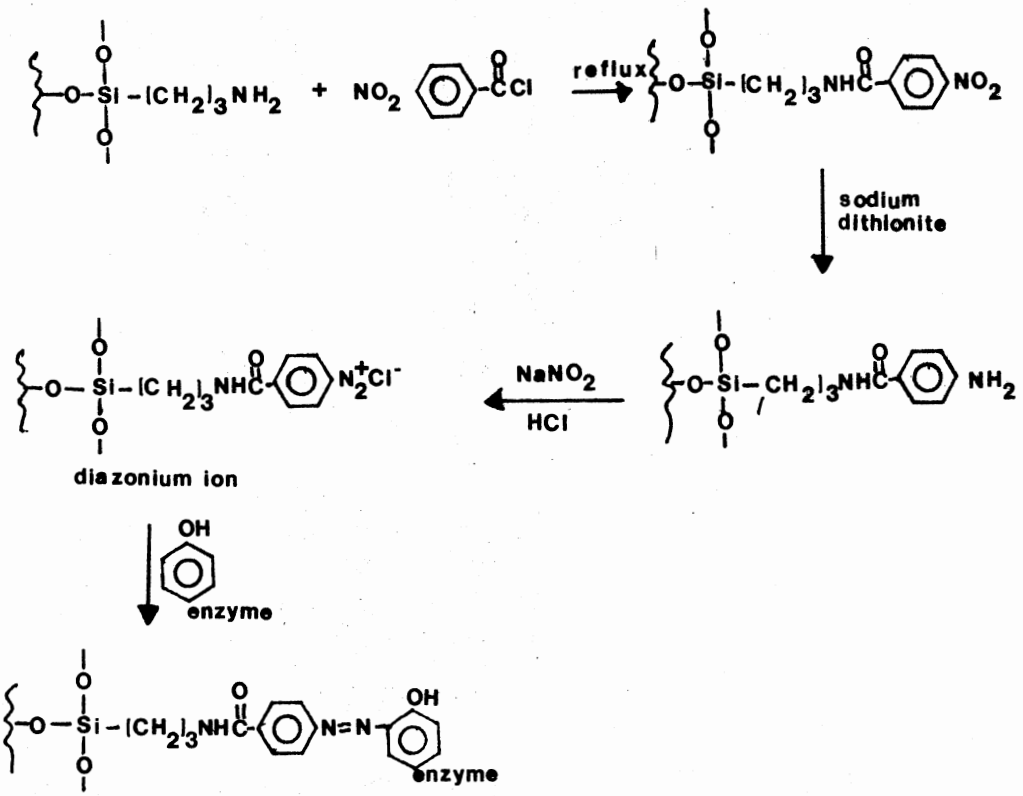
Azidocarbonylmethoxycellulose Reactions. This method was first applied by Mitz and Summaria (37) in 1961 (after modifying the work of Michael and Ewers (38)). This method is relatively simple and yields are relatively high. The reactions involved are:



The final amide formation involves primarily the ε-amino group of lysine, although other possible attachment sites implicated include those of tyrosine, cysteine, and serine. The experimental procedures

of this method have been described in detail by Crook, Brocklehurst, and Wharton (39).

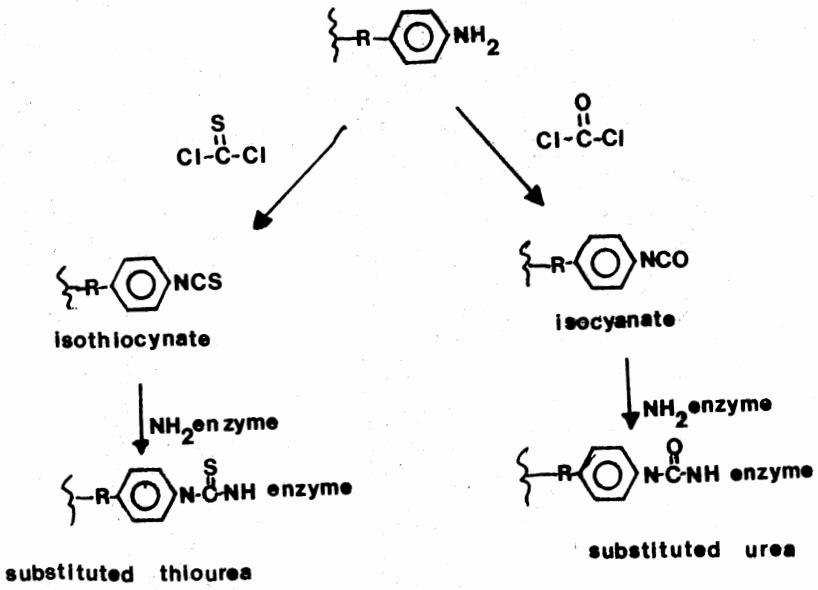
Reactions Forming the Azo Linkage. Substituted arylamines for this purpose are commonly prepared by reaction of p-nitrobenzoyl chloride with a support containing a covalently attached ω-aminoalkyl group, and reduction of the nitro group. The arylamines can then be diazotized and coupled to enzymes via an azo linkage.



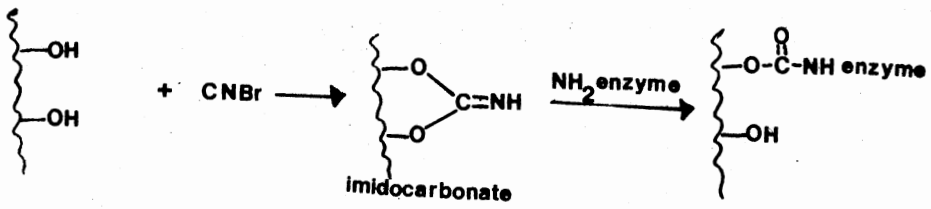
The amino acid residues (other than tyrosine) that may participate in this reaction are lysine, arginine, cysteine, and serine.

Reactions of Isocyanates and Isothiocyanates. Alkylamines and arylamines react with phosgene or thiophosgene to produce isocyanates or isothiocyanates, respectively. These will then react with amino groups of the enzyme to form the substituted urea or thiourea, respectively. It is mainly the ε-amino group of lysine which is involved in

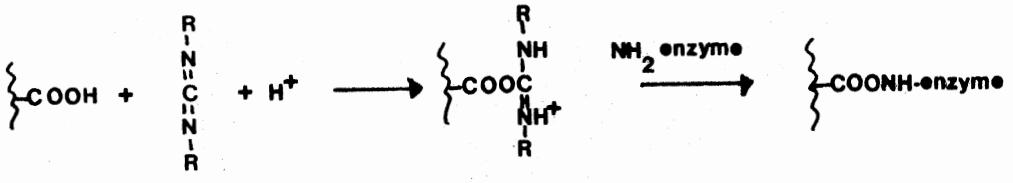
this coupling. The final reaction is carried out in slightly alkaline condition (pH 8.5 - 9).



Cyanogen Bromide Reactions. This method, devised by Axen, et al. (40), is one of the most important procedures for covalent coupling of enzymes to a carrier. This activation of crosslinked dextrans (Sepharose), agarose, or cellulose is a simple and attractive method. The -OH groups of the polysaccharide react with cyanogen bromide to form a reactive imidocarbonate group. This reactive polysaccharide then reacts with an amino group of the enzyme. Coupling of the enzyme is thought to be mainly at the amino groups of lysine and the α-amino group of the N-terminal amino acid. The coupling reaction works best at about pH 9.

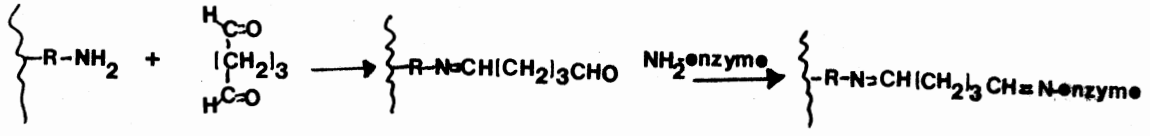


Carbodiimide Reactions. Soluble carbodiimides have been extensively used for the synthesis of nucleotides, coenzymes, and nucleic acids. These reactions commonly have broad applicability and take place at room temperature at neutral pH. The use of carbodiimides for immobilizing enzymes is useful if the enzyme must be coupled in an acidic solution, as in the case of pepsin (41). There is less chance of crosslinking to the carboxyl-activated carrier in this case.

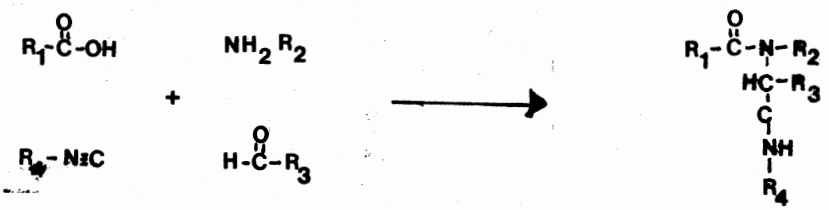


Amide groups instead of carboxyl groups have also been used to couple with carboxyl groups of an enzyme in the presence of carbodiimides, although there is a high possibility of cross-linking.

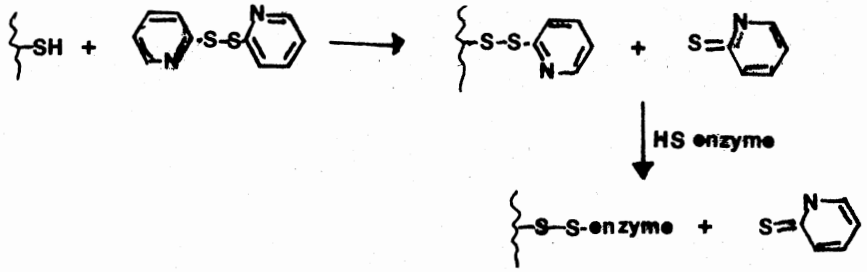
Glutaraldehyde Reaction. The nature of the reaction of glutaraldehyde with enzymes and carriers is not fully understood. However, this is one of the simplest, most gentle, and rapid of the coupling methods. The reaction can be carried out in aqueous solution within a rather wide range of pH values (5 to 9) with rate of reaction increasing with increasing pH. The modification, however, is usually carried out in neutral solution. In the supposed mechanism of the reaction, attachment is from the amine carrier to the ε-amino group of lysine in the enzyme. A simplified reaction mechanism is shown below:



Four-Component Condensation Method. This method is particularly interesting since at least 4 functional groups participate in the reactions which lead to the formation of an N-substituted amide (42) (Ugi reaction). The four functional groups are carboxyl, amino, carbonyl and isocyanide. In spite of the complex reactions taking place (no mechanism is shown below) the method is simple and extremely versatile (43). This method can be carried out in aqueous medium at neutral pH and allows for considerable maneuvering and high selectivity when applied to immobilization of enzymes. It has been optimized for proteins by Vretbland and Axen (44).



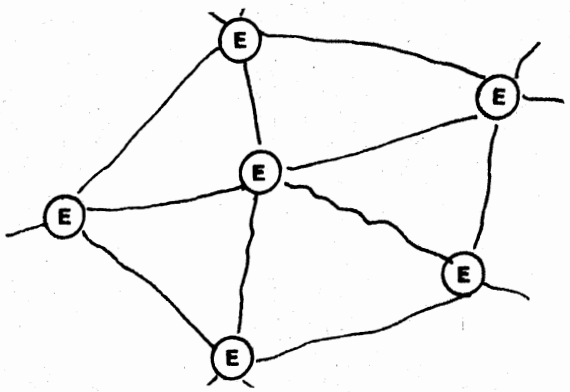
Thiol-Disulfide Interchange Reaction. Two recently published papers (45, 46) described the reversible coupling of enzymes to supports that are modified so as to contain mixed disulfide bonds via the thiol-disulfide interchange reactions. One example of this type has been reported by Carlsson (47) and is shown below:



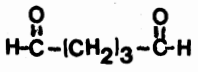
There are many other methods involving covalent coupling. The ones described above are, however, the ones most commonly used.

Cross-Linking

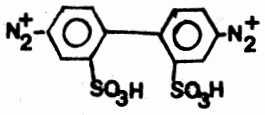
Enzymes can be covalently immobilized by cross-linking with low-molecular-weight bi-or multifunctional reagents by forming intermolecular bonds between the reagent and the enzyme. Enzymes that can be immobilized solely by their intermolecular linking into large aggregates may be grouped with this class too. Their application, however, is very



limited. Some of the frequently described reagents are diazobenzidine, diazobenzidine-3,3'-dianisidine, diazotized-benzidine-3, 3'-dicarboxylic acid, 4,4'-diisothiocyanatobiphenyl-2,2'-disulfonic acid, 1,5-difluoro-2, 4-dinitro benzene, glutaraldehyde, N,N'-hexamethylenebisiodoacetamide and hexamethylenediisocyanate. Among these crosslinking agents, only two have found widespread use in enzyme immobilization : glutaraldehyde and diazotized benzedine-2,2'-disulfonic acid.



glutaraldehyde



dizobenzidine-2,2'-disulfonic acid

The activity of crosslinked enzymes is dependent on many factors, including concentration of the enzyme, reagents, pH, ionic strength, and number of cross-links involved. Since in general high-molecular-weight substrates cannot come in contact with enzymes in the interstices of

such immobilized enzyme supports, the overall apparent activity of the derivative is dependent on the size of the substrate.

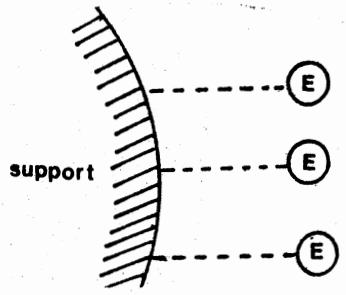
The reaction mechanisms involved in the immobilization have been described in part (a) above.

Physical Methods

This group includes all types of immobilization that do not involve covalent bonding. These methods comprise (i) adsorption, (ii) entrapment, (iii) microencapsulation, and (iv) containment within semipermeable membranes.

Adsorption

Adsorption is the oldest immobilization technique and is the easiest and frequently the most economical method of producing solid supported



enzyme conjugates. It consists of contacting an enzyme solution with a surface-active adsorbent and washing the resulting product to remove any nonadsorbed enzyme. The binding forces between the enzyme and the carrier are in most cases relatively weak. Some of the adsorption mechanisms are due to ion exchange, "physicochemical bonds" created by hydrophobic interactions, and Van der Waals forces (48, 49). The mode of adsorption depends greatly on the nature of the carrier. Recent work with titania carriers indicates that a covalent bond may be formed during the adsorption process (50). Some of the commonly used surface-

active materials are alumina, carbon, clay, ion-exchange resins (both cation and anion), calcium carbonate, cellulose, collagen, collodion, glass, diatomaceous earth, hydroxylapatite, and conditioned metal plates (like titania). Many cases occur in which nonspecific adsorption of enzymes results and thus leads to partial or total inactivation.

The adsorption of enzymes on a support is dependent on experimental variables such as pH, nature of solvent, ionic strength of the solution, concentration of the enzyme and active groups of the carrier, time, and temperature (51, 52, 53, 54).

The stability and activity of the adsorbed enzyme are dependent on assay and storage conditions, ionic strength of the solution, substrate concentration, and many of the same variables that affect the adsorption mechanism itself.

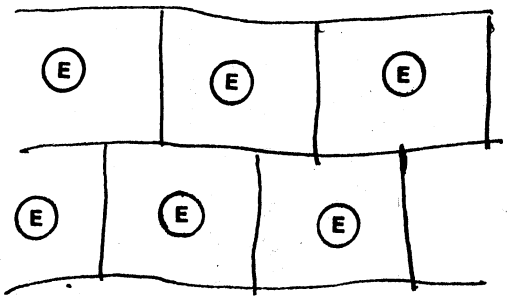
Many ions compete for the active sites on the carrier, and thus adsorption can be theoretically reversed by addition of salts (or high concentration of substrates sometimes), most enzymes being completely released at ionic strengths of 0.5 M and usually less. pH and or temperature change could serve the same purpose, too.

Early studies indicated some denaturation of the enzymes of hydrophilic carriers, but recent investigation on adsorption on hydrophilic matrices with hydrophobic side chains showed that the enzymes do not necessarily become denatured.

Promising new materials, such as controlled-pore-alumina, silica-alumina, silica and titania, developed by Messing (55), have been used to adsorb enzymes with extremely long half lives which can thus be successfully used repeatedly without activity losses (56).

Entrapment

Lattice entrapment of enzymes in the gel matrix of cross-linked polyacrylamide was first successfully employed by Bernfeld and Wan (57) in 1963. All entrapment methods, at least in principle, are based on the occlusion of an enzyme within a constraining structure tight enough to prevent the enzyme from migrating out into the surrounding medium while still allowing penetration of the substrate. To entrap an enzyme

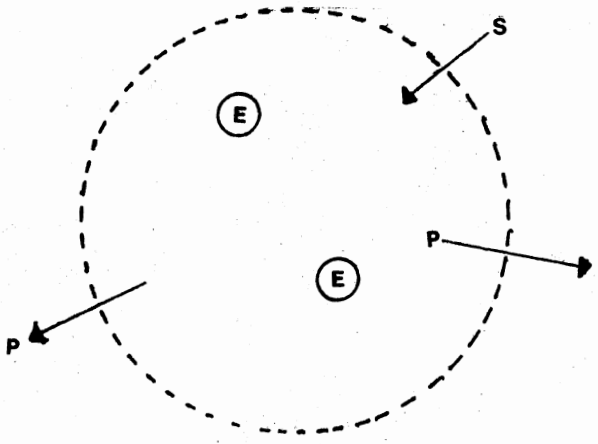


one must either form a cross-linked polymeric network around the enzyme molecule, or place an enzyme inside a polymeric material and then cross-link the polymer chains. The polymerization can be carried out by means of chemical initiators generating free radicals or by employing short-wave radiation (58). The most commonly employed cross-linking polymer for enzyme entrapment is the polyacrylamide gel system. However, silicone rubber (Silastic®) (59, 60), starch (61, 62), and silica gel (63, 64) have also been employed. When silica gel is used, part of the immobilization could be due to adsorption. With this method of immobilization no changes in the intrinsic properties of the enzymes are anticipated.

Microencapsulation

Enzymes can be immobilized by enveloping them with various forms of

semipermeable membranes. This technique, known as microencapsulation, was pioneered and perfected by Chang et al. (65, 66, 67), over a period of years, with the intention of applying it to medical purposes. The

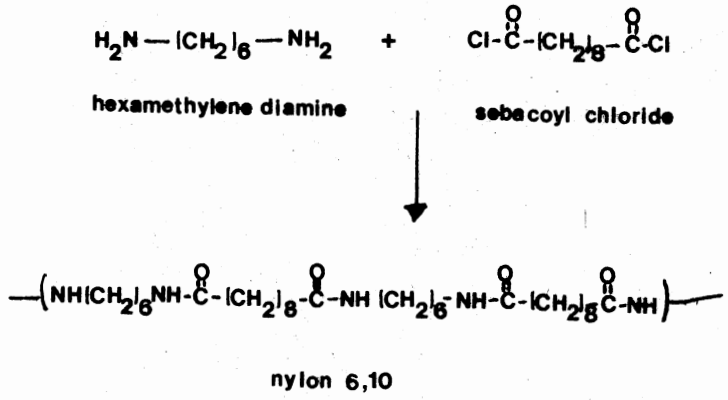


semipermeable membrane can be of the permanent or nonpermanent types. The permanent types are formed by interfacial polymerization or the phase separation of preformed polymers, while the nonpermanent ones are formed by the combination of suitable surfactants, "additives", and hydrocarbons. The formation of a microcapsule is dependent on the lower solubility of the polymer at the interface of a microdroplet. The interfacial polymerization method of forming a microcapsule is based on the synthesis of a water-insoluble copolymer at the interface of a microdroplet. The partition coefficient of the water-soluble reactant (the other component is soluble only in the organic phase) between the aqueous and the organic phases determines the properties of the membrane that is formed.

Invented by Dinelli (68), a form of microencapsulation and entrapment in fibers of cellulose triacetate utilizes the technology of wet spinning. The aqueous enzyme is dispersed in a methylene chloride solution of cellulose triacetate, and the emulsion is then extruded through fine orifices into toluene. Cellulose triacetate is insoluble in this medium and coagulates in the form of fibers, which when inter-

twined 100 to 200 times result in strong threads.

Permanent microcapsules, produced by phase separation, for the immobilization of enzymes have been made from cellulose nitrate (collodion) (69), polystyrene (70), ethylcellulose (71), benzalkonium heparin-collodion (72), and cellulose acetate butyrate (73). Permanent microcapsules produced by the interfacial polymerization method have been mainly made by the reaction of diamines with halides of dicarboxylic acids in order to form an amide copolymer such as a nylon.

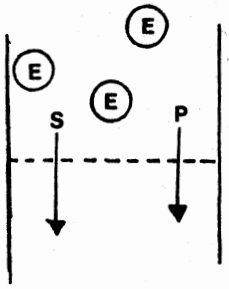


Immobilization of enzymes with nonpermanent microcapsules is based on the "liquid surfactant membrane" concept originated by Li (74). This involves encapsulating the enzyme solution within a semipermeable liquid surfactant membrane (75) (water-immiscible phase composed of surfactants, "additives" and a hydrocarbon solvent that contains emulsion-size aqueous droplets of various reagents or catalysts).

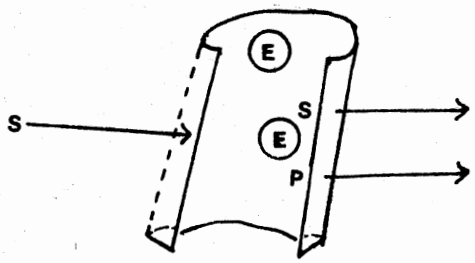
Theoretically, no changes in the inherent properties of the enzyme are expected upon immobilization by the method of microencapsulation. No chemical modification results and the enzyme molecules are free in solution.

Containment Within Semipermeable Membrane Devices

Enzymes have been immobilized by many devices that contain semipermeable membrane. These devices, which are commercially available, come in the form of flat disc-like membranes, cells, T-tubes, or hollow fibers, or other forms. The devices can be considered simply as containers for the confinement of the enzyme in much the same way as a beaker serves.



flat disc like
(ultrafiltration cells)



hollow fibers

The semipermeable membranes are of two types: homogeneous (or isotropic) and anisotropic. The homogeneous type have a uniform structure throughout the entire wall and the membrane has the same structure in the interior and the surface. Anisotropic membranes have one surface which is smooth ('skin') while the other side has a rough surface and is more porous in nature. The membrane is in general impermeable to the enzyme, but permeable to substrates and products.

Immobilization with ultrafiltration cells has been employed to carry out continuous reactions since the late 1960's (76, 77). Of course ultrafiltration cells have often been used for either concentration or purification of enzyme solutions.

Immobilization of enzymes in hollow fiber devices can function

either in the dialysis or the ultrafiltration mode. The beaker-type model can explain this. The enzyme can be contained either in the inside or on the outside of the semipermeable hollow fibers. Ultrafiltration equipment can be obtained from AMICON Corporation (offers the greatest choice), ABCOR, BIOMED, CHEMAPEL, GELMAN, MILLIPORE, SARTORIUS, and SCHLEICHER and SCHUELL, while the hollow fibers can be obtained from ABCOR, AMICON, BIORAD, DOW, DuPONT, GULF SOUTH RESEARCH INSTITUTE, MONSANTO, NORTH STAR RESEARCH INSTITUE, and ROMICON.

Characteristics of Immobilized Enzymes

When an enzyme is physically or chemically immobilized on a support several changes may occur in the apparent behavior of the enzymes. Some of the factors that have profound effects on properties of an immobilized enzymes are conformational and steric effects, local and net charges (electrostatic or hydrophobic interactions), environmental influences, and diffusional or mass-transfer effects. The net change in an enzyme property may not be due to only one of these changes of properties. It is very difficult to figure out exactly the cause and extent of an alteration in a property of an enzyme upon its immobilization. Some of the resultant changes are discussed below.

Activity

The activity of the immobilized enzyme can vary from zero (78, 79) to even higher values (80) than for the native enzyme. The activity obtained is dependent on the particular enzyme, the specific support, and the exact conditions employed by the experimenter. An observation that is gaining support is the fact that the specific activity of a

covalently attached enzyme increases as the solubility of the support in the medium increases.

pH Profiles

Every enzyme has an optimum pH at which maximum reaction rate takes place. Compared to the native enzyme the immobilized one may have broader (81), narrower (82), asymmetrical (83), or identical (84) pH profile. The optimum pH may shift depending on the nature of the carrier, chemical modification of the enzyme, and certain enzymatic reactions. According to Goldstein (85) and coworkers, an enzyme that is covalently attached to a negatively charged carrier will experience a locally higher hydrogen ion concentration, and thus its pH profile will indicate a shift towards alkaline pH values. The opposite may occur if the carrier is positively charged (86).

Many others observed the same behaviors with different positively or negatively charged supports using different enzymes (87, 88). This effect of the support on the apparent pH optimum of an enzyme suggests the possibility of tailoring a support specifically to permit an enzyme to function efficiently at an apparent pH not optimal for that enzyme when free. Immobilized enzymes may also be capable of operating at high concentrations of organic solvent. This is useful if the substrate is more soluble in an organic solvent than in water.

The pH profiles of an immobilized enzyme may also be shifted by an enzymatically generated pH gradient between the domain of the enzyme-polymer conjugate and the external solution. The degree of shifts is particularly dependent on the catalytic activity of the enzyme and the diffusion rates of the substrate and product from the reaction site.

Although most enzymatically shifted pH profiles have been observed with synthetic-membrane enzyme systems, they have also been observed with agarose gel beads (89).

In addition to these factors, chemical modification and charge effect can also contribute to the overall shifts in the pH profiles.

Kinetics of Immobilized Enzymes

The Michaelis-Menten constant, K_m is defined as the substrate concentration at $V = V_{max}/2$ in the familiar Michaelis-Menten relationship

$$V = V_{max}/(1 + K_m/[S])$$

where V is the initial reaction velocity, V_{max} is the maximum initial velocity, K_m is the Michael Menten constant and [S] is the substrate concentration.

K_m is a useful and important quantity to describe enzyme kinetics which can be determined for a substrate. From an analytical point of view a large K_m is advantageous since it implies that the linearity of the method extends to the substrate concentration which corresponds to K_m. Thus a large K_m will increase the dynamic range of analysis for that method, provided, of course, that the lower detection limit remains the same for both the soluble and the immobilized enzyme. K_m is independent of enzyme concentration and is expressed in units of M or mM of substrate.

When an enzyme is immobilized, its apparent K_m (K_m') is mostly found to be higher than that of the native enzyme. This is mainly accounted to diffusional limitations. If such diffusional limitations are overcome by reducing the size of the particles of the carrier that

contains the bonded enzyme, increasing the stirring speed of the suspended particles, or solubilizing the immobilized enzyme, then the apparent K_m is considerably reduced and can even approach the true K_m.

In addition to the diffusional effect, the increase in K_m is related to the charge of substrate or carrier and tertiary changes in the enzyme configuration. However, in some cases a decrease (90) or no change (91) in K_m is observed. Whenever the polarity of the charged carrier and the charged substrate differ, the K_m is found to be less for the immobilized than for the native enzyme (92). A few examples of changes of K_m are shown in Table IV.

Stability

Immobilized enzymes have been found to display both higher and lower conformational stabilities than the native ones (93). The conformational stability usually determines thermal, pH, organic solvent, and storage stability.

Both native and immobilized enzymes are found to be susceptible to thermal denaturation. In many cases, however, the rate of inactivation of the immobilized enzymes is found to be less than that of the negative enzyme. Several examples of both types can be found in the literature.

Enzymes that display excellent thermal stability do not necessarily show excellent operational stability, because other factors are introduced, such as support durability, inhibitor concentration, nature of organic solvent, or the presence of heavy metals.

Immobilized enzymes are often stored at or near $5^{o}C$ and such storage is found to be very beneficial in almost all cases. However,

TABLE IV

APPARENT K_m VALUES OF SOLUBLE AND IMMOBILIZED ENZYMES

Enzyme	Substrate	K_m (mM)	
		Soluble	Immobilized
Subtilopeptidase	N-Acetyltyrosine ethyl ester	9.4	17
Papain	Benzoylglycine ethyl ester	18	34
β-Fructofuranosidase	Sucrose	17	45
Thrombine	p-Tosyl-L-arginine methyl ester	3.7	3.7
Amyloglucosidase	Starch	1.22	0.3
Ficin	N-α-Benzoylarginine ethyl ester hydrochloride	20	2
Lactate dehydrogenase	Pyruvate	0.115	0.057

decreased storage stability of an immobilized enzyme has also been cited in the literature (94).

Some studies on the variation of stability with pH have also been reported on immobilized enzymes (95).

Immobilized enzymes may remain active for a much longer time than the soluble ones. Depending on the type of enzyme, this can vary from 10 days to a year. An example of this type of study is shown in Figure 6 (96).

The properties and the advantages of the different methods of immobilization are described in tabular form in Table V.

As a general rule, inorganic supporting materials have some advantages over their organic counterparts. These include (103): (a) structural stability, i.e., they are not susceptible to change by pH and solvent conditions as are the organic polymers, (b) lack of susceptibility to microbial attack, (c) ability to be shaped into a wide variety of configurations, (d) greater operational stability usually than those of the organic polymers.

Analytical Application of Immobilized Enzyme Preparations

The great potential of enzymes in analytical chemistry has been recognized for many years. However, high cost, unavailability, low purity, instability, and the length of time for analysis long prevented their widespread use. The problems are not so serious at the present time, since enzymes of high purity and high specific activity at reasonable prices are available. With the advancement of new analytical techniques the length of time required for analysis has been drastically

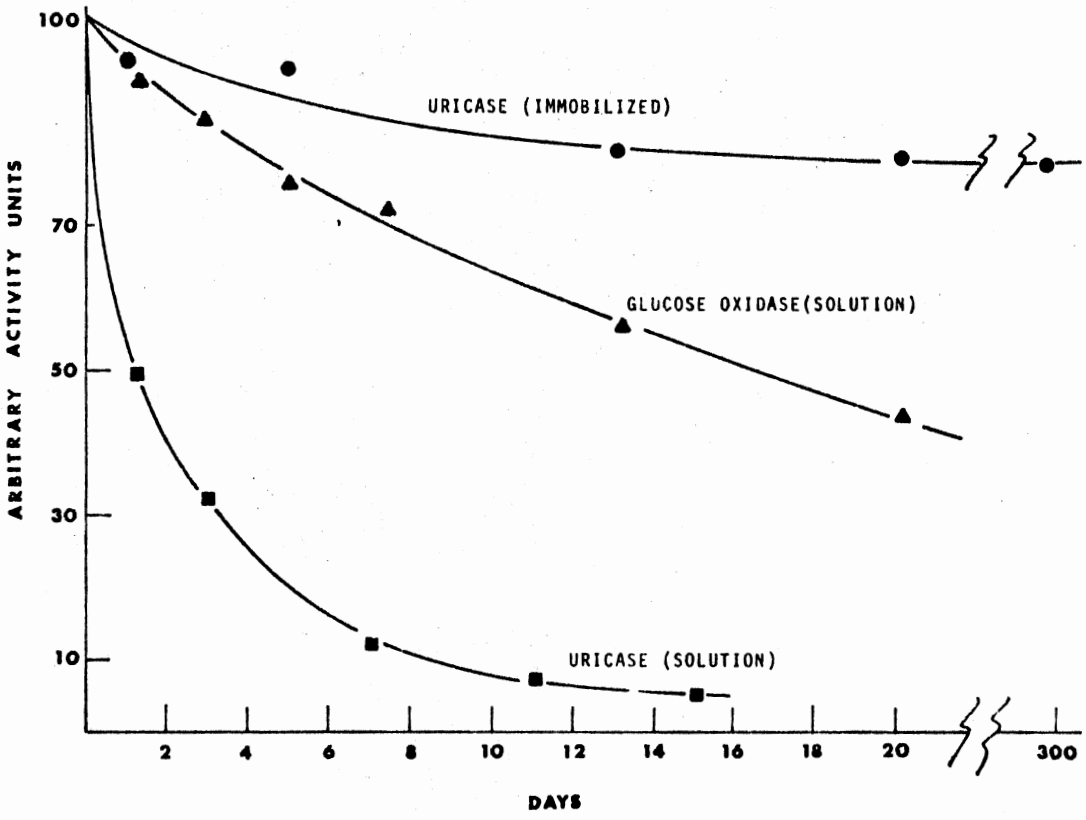


Figure 6. Long-Term Stability of Soluble Glucose Oxidase and Uricase and Covalently Bonded Immobilized Uricase

TABLE V

PROPERTIES, ADVANTAGES AND DISADVANTAGES OF THE DIFFERENT METHODS OF IMMOBILIZATION

1. Chemical

(a) Covalent

Properties	Advantages	Disadvantages
The activities of the enzymes vary; some of them have higher activity than the native. Depending on the charge of the support, the pH profile was found to shift either way. The apparent K_m was mostly increased but cases occurred in which it decreased or became identical to that of the native enzyme.	Binding is experimentally easy to carry out. It simply involves adding the enzyme to a suspension of the polymer, allowing the enzyme to be coupled and then washing the excess unbound enzyme. This solid product can easily be filtered out or centrifuged. This permits the control of the reaction since the solid catalyst can be easily removed from the reaction mixture at any time. The product is not contaminated with the enzyme. The immobilized enzyme can be used in continuous fashion by using different reaction systems (stirred tank, ultrafiltration, packed bed recycle, fluidized bed or tubular wall). The support shape can be as varied as needed, i.e., the	Knowledge of the structure of the enzyme and the amino acids it is made of is essential in many cases when immobilization by covalent attachment is required. Trial-and-error work can many times result in total inactivation of the enzyme. A second disadvantage could be the fact that the conditions (pH, ionic strength, etc.) required for preparing the enzyme-support conjugate may destroy enzyme activity. Thus the efficiency might be very low. Many times if the substrate molecule is large, steric repulsion can result in low catalytic efficiency.

TABLE V (Continued)

	form may be powder, fiber, large-size or small-size particle, etc. A first-rate feature of this method is its covalent bond formation which chemically alters the structure which may display excellent chemical or physical properties in comparison to the native one.	
(b) Cross-Linking		
The immobilized enzymes have been found to have varying degrees of catalytic activity depending on the amount of cross-linking reagent and some other factors. Occasionally, the pH profile of these cross-linked derivatives was also found to deviate from that of the native enzyme. The shapes depended on the nature of the products liberated and the kinetic parameters determining the types of reactions. Some cross-linked enzymes have been found to lose their specificity while others did not. Again the thermal stability of some cross-linked enzymes was higher than for the native, while for some others they were less. Not many studies on determination of K_m, pH stability and	In many instances this simple method gives a derivative that is almost pure enzyme. A single bi- or multifunctional reagent can be used to prepare many different types of immobilized enzymes, such as gels, membranes, adsorbed monolayer derivatives, and polymer-bound conjugates. The enzyme derivatives, particularly the gels, can be easily dispersed in aqueous solutions. The adsorbed monolayer enzyme derivatives have special advantages since large portions of the enzyme are accessible to the substrates for reaction.	The main disadvantage is the need of fairly precisely controlling the pH, the concentration of the enzyme, and the multifunctional reagent. Often a large amount of enzyme is required in order to create insolubilization and some inactivation always occurs by the chemical modification. Most of these gels or membranes which are gelatinous are not good enough for operation in columns.

TABLE V (Continued)

storage stability of cross-linked enzymes have been done.		
2. Physical		
(a) Adsorption		
Theoretically a change in pH, ionic strength, or temperature of the adsorbed enzyme should cause the adsorbed enzyme to be desorbed from the carrier and be released to the solution. However, this has not been found to be the case with some enzymes (97,98). The activity of the immobilized enzymes can vary anywhere from zero to high values. The pH profiles can also shift either towards lower or higher pHs. The explanation given for this is the same as that for the covalently attached enzymes, i.e., the change on the carrier can create micro-environments in the surroundings which can make the enzyme see an apparently different pH. Higher, lower, and the same K_m have been observed for adsorbed enzymes. Higher, lower and the same thermal and storage stabilities for adsorbed enzymes have been reported.	Historically the earliest method, it is also the easiest way of preparing solid-supported enzyme conjugates. The extensively varied choice of differently charged and shaped supports and the ability of this method to immobilize and purify simultaneously makes it to be of great value. Since the method is simple usually mild or no inactivation of the enzyme results.	Optimum conditions for achieving immobilization are often found by trial and error. The exact ionic strength, pH, temperature, etc., must be known to get optimum conditions. (These are the same parameters which cause desorption or denaturation of the enzymes.) Leakage or desorption of the enzyme can be a big problem in some cases, especially if strong binding between the enzyme and support does not exist.

TABLE V (Continued)

(b) Entrapment		
The inherent properties of the enzyme are not expected to change. The activity of the enzyme is dependent on the method of preparation. The relative activity of the entrapped enzymes is usually relatively low. The highest relative activity reported so far is 60% (99,100). These low activities are due to the diffusion and steric repulsion of the macromolecular species (substrates) in some examples. The pH profile can be shifted towards alkaline or towards acidic pHs or not at all compared with the native enzyme. The K_m' values were mostly found to be higher, but decreased and identical values compared with that of the native enzyme have also been observed. Both enhanced and diminished thermal stabilities have been observed.	The method in general is simple and needs only small quantities of the enzyme. Since no chemical change of the enzyme occurs, it still possesses all its inherent properties when entrapped. Many neutrally charged entrapping supports can be used in widely different physical forms.	Good mechanical properties and activity are critically dependent on experimental conditions, i.e., chemical and thermal denaturation can occur during entrapment and the physical nature of the gel is an indication of the activity of the enzyme. The entrapment technique suffers from another intrinsic drawback which is leakage of enzymes; this is particularly pronounced with enzymes of relatively low molecular weight. This is due to the fact that there is broad distribution of pore sizes in the entrapped gels. The larger the micropore the greater will be the leakage. This method is limited only to small-sized substrates.
(c) Microencapsulation		
The microcapsules are nearly always spherical. However, occasionally double or multiple or cup-shaped microcapsules can be produced. The	It allows an extremely large area of contact between the enzyme and the substrate with a small volume (the microcapsule). This technique	Quite large quantities of enzymes are required for microcapsule formation. Furthermore, inactivation may result sometimes and the enzyme might be lost by forming part of the wall

TABLE V (Continued)

mean diameter (usually between 5 to 300μ) of the flexible microcapsules is dependent among other things on the speed of reaction, concentration of surfactant and viscosity of the organic liquid and on the speed of the mechanical emulsification. The thickness of the membrane is largely influenced by the speed of capsule formation composition of the organic solvent (could be a mixture of organic solvents) and the make and concentration of the materials that make up the membrane. The enzyme molecules are free in the solution inside the microcapsules. The activities of the microcapsulated enzymes have been found to vary from about 10 to 100% activity. In the two enzymes in which the K_m was determined one was found to have higher(101) than, and the other one identical(102) to that of the native enzyme. The nature of the semipermeable membrane to a certain extent determines the specificity of the encapsulated enzymes. Enhanced stability of the microencapsulated enzymes have been reported.	by suitable preparations can be used for specifically reacting some substrates and not others. The technique permits the simultaneous immobilization of several enzymes in one step. Immobilization with nonpermanent microcapsules, i.e., liquid surfactant membrane immobilization has the distinct advantage of being reversible; thus it should be possible to recover the immobilized enzyme by breaking the emulsified microdroplets chemically or physically.	of the microcapsule. This technique is also limited to small-molecular-weight substrates.

TABLE V (Continued)

(d) Containment Within Semipermeable Membranes		
The intrinsic properties of the enzymes immobilized by this technique are not expected to change. Thus diffusion of substrate and product, activity, optimum pH, K_m, are expected to be the same as that of the native enzyme when contained under similar vessels. The stability of the enzyme, however, can be changed to some extent. Too violent stirring or adsorption of the enzyme on the membrane can cause inactivation of the enzyme.	The method of immobilization in ultrafiltration cells or hollow fibers is extremely simple; no irreversible modification takes place, and the method is especially suitable for high-molecular-weight substrates (soluble or insoluble). Separation of products could be done based purely on the size of molecules. Simultaneously many enzymes can be immobilized too. This method can be used to carry out continuous enzymatic conversion. In addition to the above named advantages the hollow fiber devices offer an exceptionally large surface-area-to-volume ratio, permit continuous operations at low pressure and can be used in more varied operational modes.	Vigorous agitation, adsorption on the membranes and high shear forces can be the causes for inactivation of the enzyme. Some limitations of the equipment which can cause control of pressure and filter selection can also be considered as disadvantages. Moreover the possibility of enzyme leakage and exact control of residence time for completion of reactions or controlled conversions of low-molecular-weight substrates can be considered as disadvantages of this technique. Not very many hollow fiber devices of different membranes are available.

reduced. The immobilization of enzymes has progressed tremendously in recent years and unprecedented possibilities have opened up for enzymatic analysis. Hicks and Updike (104) were the first to use immobilized enzymes for quantitative analytical purposes. They used polyacrylamide-bound glucose oxidase and lactate dehydrogenase in column fillings to determine glucose and lactic acid, respectively.

The versatility of immobilized enzymes in analytical application lies in the fact that they can be used either as part of the sensor or separated from the sensor. This criterion of classification is adapted here in discussing analytical applications of immobilized enzymes. Any other applications of immobilized enzymes which do not come under these categories are considered as miscellaneous. Both manual and automated techniques have been used in the analytical application of immobilized enzymes. Manual analysis in this context refers to procedures in which an immobilized enzyme in combination with a detection unit is used for analysis of discrete samples. In automated methods, however, large numbers of samples are analyzed for the same analyte by the uses of immobilized enzymes in combination with the detection instrument. Automated methods are used in routine analysis for some important metabolites or other compounds.

Application of Immobilized Enzymes Used as Part of the Sensor

Manual Analysis

These include the enzyme electrode systems which use either potentiometric or amperometric techniques and the enthalpimetric system.

Enzyme Electrode Systems. In this technique the enzyme derivative is placed on the tip of an electrochemical sensor. This method combines the selectivity of the enzyme and sensitivity of the electrochemical detection. Detection can be done either potentiometrically or amperometrically. Even though the amperometric electrode was the first enzyme probe reported in the literature (105), it did not receive much attention until rather recently. The potentiometric electrode's development and initial improvement are attributed to Clark and Lyons (106). The potentiometric mode of operation of artificial membrane enzyme probes is based on the diffusion-controlled movement of substrate through a thin layer of immobilized enzyme. The product of the enzyme-catalyzed reaction is detected at the surface of the sensor. Since many ion-selective electrodes are available to detect the electroactive product of the enzyme-catalyzed reaction, a large variety of enzyme electrodes has been reported. At the present time, ion-selective electrodes that are sensitive to particular cations and anions can be purchased at moderate cost. The dynamic range of these sensors generally ranges from 10^{-5} to 10^{-1}M. General schemes of the electrochemical sensors are shown in Figures 7 and 8.

Simplicity, reliability, and low cost are advantages of the potentiometric membrane probe. However, in order for this method to work, an ion-selective electrode for the product of the enzymatic reaction should be found. In addition, the interference of other materials should not be significant.

Some of the common enzyme electrodes and their characteristics are shown in Table VI.

Immobilized multienzyme electrodes have been reported (130). A

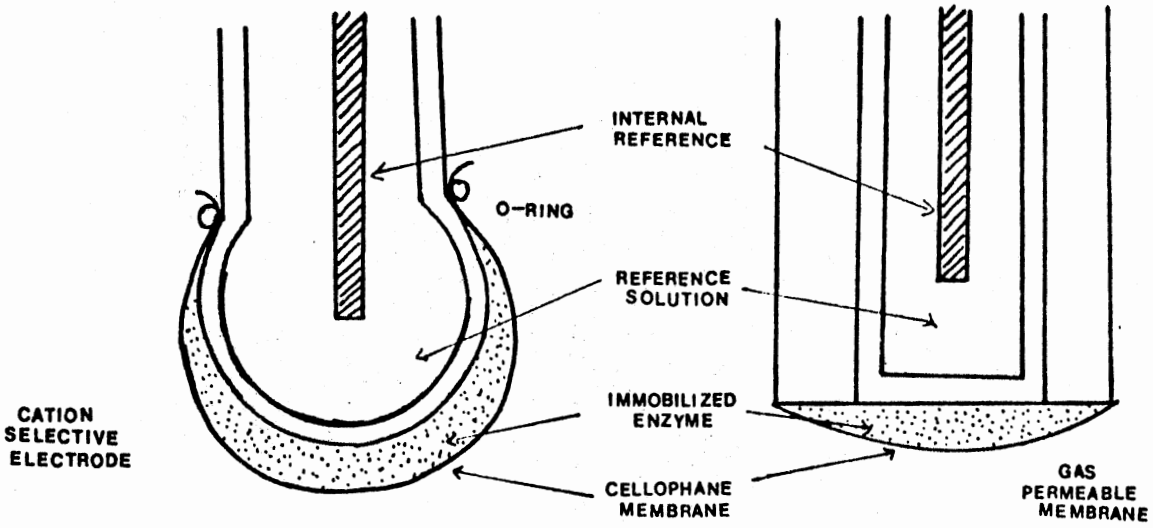


Figure 7. Schematic Diagram of Potentiometric Sensors

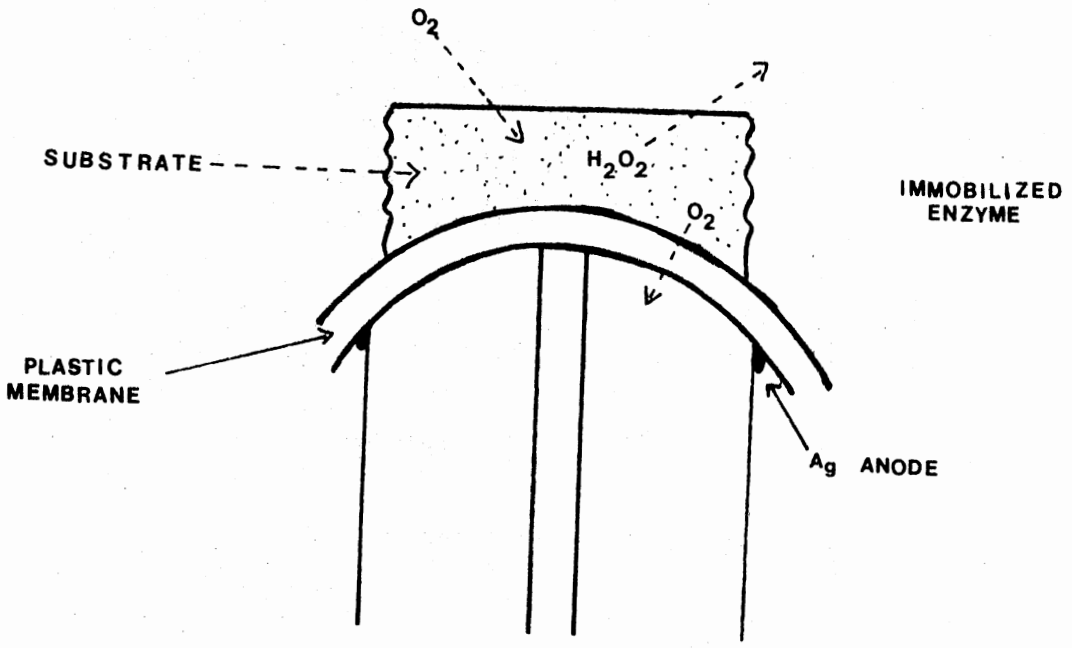


Figure 8. Schematic Diagram of an Enzyme Electrode
With Amperometic Sensor

TABLE VI

SOME COMMON ENZYME ELECTRODES AND THEIR CHARACTERISTICS

Electrode Type	Immobilized Enz.	Sensor	Range (M)	Reference
Glucose	Glucose Oxidase	O_2(Pt)	10^{-5} to 10^{-1}	107
		H_2O_2(Pt)	10^{-4} to $2x10^{-2}$	108
		O_2(Pt)	10^{-4} to 10^{-2}	109
		H^+(pH electrode)	10^{-3} to 10^{-1}	110
Urea	Urease	NH_4^+(glass electrode)	$5x10^{-4}$ to 10^{-2}	111
		H^+(pH electrode)	$5x10^{-5}$ to $5x10^{-3}$	112
		NH_3(air gap electrode)	$5x10^{-5}$ to $5x10^{-2}$	113
		CO_2(gas electrode)	10^{-4} to 10^{-1}	114
L-Amino Acids	L-Amino Acid Oxidase	NH_4^+(cation sel. ele.)	10^{-4} to 10^{-2}	115
		O_2(Pt)	10^{-5} to 10^{-3}	116
		NH_4^+(cation sel. ele.)	10^{-4} to 10^{-2}	117
L-Phenylalanine	L-Amino Acid Oxidase	NH_4^+(cation sel.ele.)	$5x10^{-5}$ to 10^{-2}	117
"	Peroxidase	I^-(I^- sel. ele.)	$5x10^{-5}$ to 10^{-3}	117
Penicillin	Penicillinase	H^+(pH glass electrode)	10^{-4} to $5x10^{-2}$	118
Ethanol	Alcohol Dehydrogenase	H_2O_2(Pt)	$5x10^{-2}$ to $5x10^{-1}$	119
Lactin Acid	Lactate Dehydrogenase	H_2O_2(Pt)	$7x10^{-3}$ to $7x10^{-2}$	119
Asparagine	Asparaginase	NH_4^+(cation sel. ele.)	10^{-2} to $5x10^{-2}$	120
Cholesterol	Cholesterol Oxidase	O_2(Pt)	$2x10^{-5}$ to 10^{-4}	121
Sucrose	Invertase (Mutarotase or Glucose Oxidase)	O_2(Pt)	10^{-3} to 10^{-2}	122

TABLE VI (Continued)

Electrode Type	Immobilized Enz.	Sensor	Range (M)	Reference
Pyruvate	Lactate Dehydrogenase	H^+(pH meter)	2.5×10^{-5} to 8×10^{-4}	123
Amygdalin	β-Glucosidase	CN^-(sol. state ele.)	10^{-5} to 10^{-2}	124
Uric Acid	Uricase	O_2(Pt)	10^{-4} to 10^{-2}	125
Nitrite	Nitrate Reductase	NH_4^+(cation sel. ele.)	10^{-4} to 10^{-2}	126
Phenol	Phenol Oxidase	O_2(Pt)	5×10^{-7} to 5×10^{-5}	127
Galactose	Galactose Oxidase	H_2O_2(Pt)	5.6×10^{-4} to 1.7×10^{-3}	128
Choline	Choline Oxidase	O_2(Pt)	to 10^{-4}	129

related device for the detection of phenol and related compounds has recently been described (131). This device is an electrochemical cell that consists of an anode (prepared by coating a conductive substrate with a gel containing a phenol oxidase such as phenolase or tyrosinase) and a cathode compartment connected by a salt bridge. The cell can be used to determine 3×10^{-7} to 10^{-4} M phenol.

Substrate electrodes for the assay of enzymes have also been made. The "immobilized" substrate in this case can determine the activity of the enzyme. One defect of this method is the fact that the substrate is continuously used up, unlike that in the immobilized enzyme electrode systems. The analysis involves kinetic rather than equilibrium measurements. Two examples that can be given of this technique are the urease (132) and cholinesterase (133) electrodes.

The advantages of these enzyme electrodes are their specificity, the simplicity of the setup, and the low cost of the instrumental components required.

A disadvantage of these electrodes is that they are not very sensitive and the length of time for analysis is relatively long. The enzyme loses activity with time, and thus a downward shift of calibration plots results. Interference of some active species on the electrode sensor can be a problem in some cases.

Enthalpimetric Methods. The enthalpy change of a chemical reaction using an immobilized enzyme sensor has been used to determine the concentration of substrates such as H_2O_2, glucose, and urea (134). This technique could theoretically be applied to all enzymatic reactions if their enthalpy change is large enough to be detected by thermistors.

Two thermistors, both of which are cross-linked proteins on their sensitive parts, are used for this purpose. One of them, the measuring thermistor, is covered with a mixture of albumin and enzyme immobilized together by glutaraldehyde. The other one, which is the reference, is coated with albumin alone. The differential device eliminates the effect of temperature fluctuations induced by the thermostat. Figure 9 shows the diagram of an enzyme-bound thermistor.

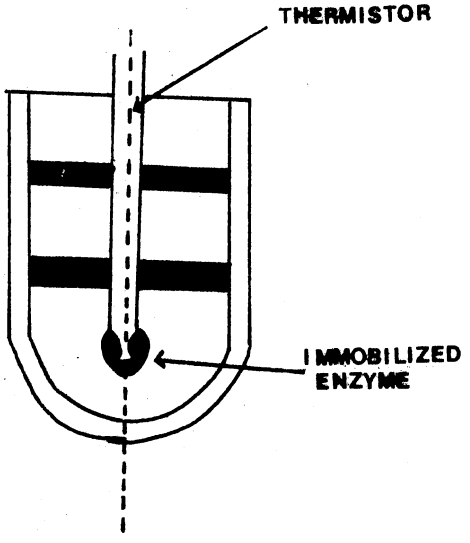


Figure 9. Enzyme-Bound Thermistor

A similar device has also been recently described (135). A differential thermometer was prepared that measures the steady state temperature differential (ΔT), which is directly proportional to the substrate concentration. Immobilized hexokinase was used as a test with satisfactory results.

A special enzyme thermistor device with a probe and a reference thermistor has been used to determine cholesterol, glucose, lactose, and uric acid in standard solutions as well as biological samples. Linear relationships between temperature changes and substrate concentrations were observed (136).

A calorimetric method of analysis utilizing immobilized enzymes has been reported (137). A heat-sensing detector is used in which the thermopile consists of a series of n-p semiconductor junctions. The heat of an enzyme-catalyzed reaction causes generation of a potential and this signal is amplified and recorded. Experimental trials with immobilized catalase indicated rapid and sensitive analysis.

There are several advantages of enthalpimetric methods. They can in principle be used for any enzymatic reactions, i.e., they are not restricted to the detection of a single chemical species. Enthalpy change occurs in all enzymatic reactions. There is no need for auxiliary enzymes and their cofactors. Overall information from complex events may be obtained. The enzyme need not be transparent or highly pure for this purpose. Thermistors are relatively simple and cheap compared with those required for microcalorimetry.

A disadvantage of this method is the fact that the sample size, pH, and composition of the solution must be critically controlled. The accuracy of the thermistors is low when compared with those used in microcalorimetry.

Automated Analysis

Automated systems in which enzyme electrodes have been used include the glucose analyzer (marketed by Yellow Springs Instrument

Company) and the organophosphate and carbamate analyzer (marketed by Midwest Research Institute).

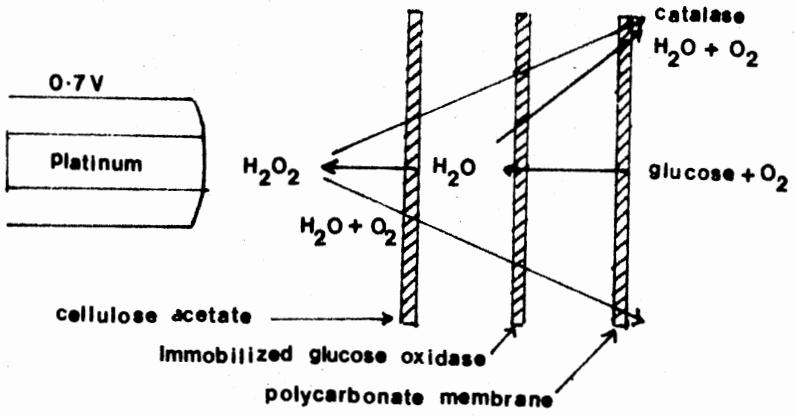


Figure 10. Probe Membrane Combination Used in the Yellow Spring Instrument Company's Glucose Analyzer

The electrode system used in the glucose analyzer (138), as shown in Figure 10, consists of a platinum anode (0.7 V), at which the H_2O_2 decomposes, and a silver cathode. The platinum electrode is covered by a cellulose acetate membrane followed by a layer of glucose oxidase and then finally by a polycarbonate membrane. The polycarbonate membrane prevents proteins, other macromolecules, and catalase from coming in contact with the platinum, and also prevents the passage of glucose oxidase into the solution. The innermost layer, which is made of the cellulose acetate membrane, has such small pore size that only molecules

the size of hydrogen peroxide, oxygen, and water can contact the probe. The reaction product of the reaction between glucose and oxygen in the presence of glucose oxidase, H_2O_2, is detected by the platinum electrode. The instrument operates in a kinetic mode and thus the temperature must be maintained at a constant $37^{o}C$ with a heater block.

The Midwest Research Institute analyzers (139, 140) are used to determine organophosphates and carbamates. These instruments measure inhibitors (organophosphates and carbamates) rather than the enzyme (cholinesterase). The electrochemical cell of these instruments consists of cholinesterase immobilized on an open-pore disc of polyurethane from which is placed between two electrodes having an applied current of 2 μa. The rate of formation of the easily oxidizable thiocholine iodide from butyrylcholine iodide, in the absence of the inhibitors, is measured by the cell. The resulting voltage can then be related to the concentration of the inhibitors. The immobilization of cholinesterase was carried out by entrapping it in starch gel and then adsorbing it on aluminum hydroxide gel. This was then finally applied to polyurethane foam.

Application of Immobilized Enzymes Used Separated From the Sensor

Procedures in this category have the advantage of using the immobilized enzymes under optimum conditions such as temperature, ionic strength, and pH, while the sensor is in a different environment adjusted to maximum sensor sensitivity and durability.

Manual Analysis

The solid-surface fluorescence method and the chemiluminescent (or bioluminescent) methods can be categorized in this section.

Solid Surface Fluorescence Method. Among the methods used for the determination of enzyme activity, spectrophotometry has generally been preferred. This is because of its simplicity, rapidity, and ability to detect reasonalby low enzyme and substrate concentrations. Fluorescence measurements, however, are many orders of magnitude more sensitive than colorimetric measurements. They have greater selectivity and the accuracy is independent of the region of measurement. In this technique a fluorescent compound is produced by the reaction between the enzyme and substrate. The rate of production of the fluorescent compound, which depends on the concentration of the enzyme and substrate, can be measured with a fluorometer. The problems associated with the wet chemistry study of fluorescence methods--the high cost of enzymes, coenzymes, substrates and cofactors--have been overcome by the development of solid-surface fluorometric methods.

> Guilbault and coworkers have developed solid-surface fluorometric methods, using what is called a 'reagentless' system, for the assay of enzymes, substrates, activators and inhibitors. An attachment to an AMINCO filter fluorometer has been adapted to accept, instead of a glass cuvette, a metal slide (a cell), painted black to reduce the background. A silicone rubber pad is placed on the side. All the reagents for a quantitative assay are placed in a form of solid reactant film on the surface of the pad. The change in fluorescence with time is measured and related to the concentration of the substance determined (141, p. 619).

These reagent pads are simple to prepare and can be stable for a month or more under the right conditions. Some clinical analyses per-

formed using this method include determinations of cholinesterase in blood (142), alkaline phosphatase in blood (143), lactate dehydrogenase in serum (144), creatine phosphokinase in serum (145), urea nitrogen in serum (146), creatine in urine (147), and uric acid in serum (148).

There are some advantages of this method, in addition to the ones mentioned above. Once the pad is prepared the time of analysis is short (~ 4 min.). The samples required for analysis can be as small as 3 µl and the amounts of reagents required are small. The reagents, once prepared in a solid form in the silicone rubber pad, can be kept for quite a long time. The temperature need not be critically controlled as in other enzymatic assays.

The handicaps of this technique are (1) the necessity for dissolution of the reagent on the silicone rubber pad, (2) the relatively high level of background fluorescence, and (3) the involved pad preparation procedure.

Chemiluminescence or Bioluminescence Methods. Oxidizable substrates or cofactors like NADH (nicotinamide adenine dinucleotide, reduced form) and NADPH (nicotineamide adenine dinucleotide phosphate, reduced form) have also been determined by bioluminescent or chemiluminescent methods (149). For this purpose fine activated glass beads were cemented with epoxy resin to glass rods and coated with a mixture of luciferase and FMN (flavin mononucleotide) reductase. The intensity of the light emitted by the immobilized enzyme system was linearly dependent on the amount of NADH and NADPH present when the rods were immersed in buffered solutions containing FMNH and decanal. Linearity

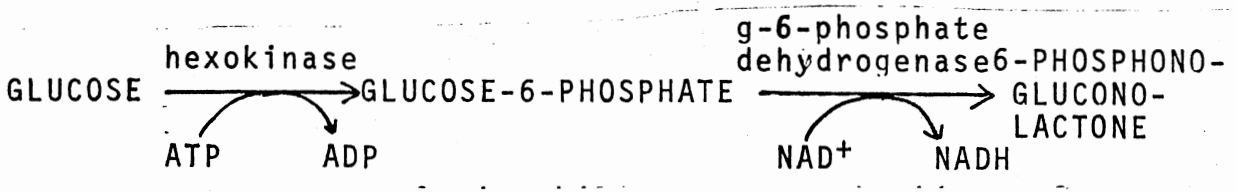
for NADH and NADPH was observed in the range of 1×10^{-12} to 5×10^{-8} and 1×10^{-11} to 2×10^{-7} mols, respectively.

The very high sensitivity made possible by the chemiluminescent methods can be taken as an advantage. The long time required for analysis (15-20 mins. at best) is a disadvantage of this method.

Automated Analysis

Automated analyzers can operate in either a kinetic or an equilibrium mode. The use of a flowing stream allows the stream to be split so that more than one test can be run on a single sample and lends itself easily to a continuous monitoring mode. Instruments of this type are available in large numbers. The types of sensors are found to be varied, and thus this section is subdivided accordingly. Since some of these are commercially available, the names of the companies marketing them will be referred to, whenever relevant.

Spectrophotometry. Setups similar to those shown in Figure 11 are used in many of them. In the commercially available Technicon module, hexokinase and glucose-6-phosphate dehydrogenase are immobilized on the surface of plastic tubing, which is placed in the flowing stream. The reaction product NADH is spectrophotometrically measured.



Enzymes covalently immobilized on the inside surface of nylon tubing have also been incorporated into the flow system of a modified

continuous-flow analyzer (Technicon AutoAnalyzer) to determine pyruvate, lactate, creatinine, and creatine (150, 151). A microcolumn of immobilized enzymes which is covalently bound to aminoalkyl derivatives of porous glass has similarly been used to determine uric acid in blood serum and urine. In this AutoAnalyzer I continuous flow system, H_2O_2 produced by the oxidases was colorimetrically determined using horseradish peroxidase and hydrogen acceptor in solution (152). Small-bore (AUTOZYME) tubes with immobilized enzymes on the inner walls were developed for the Technicon SMAC high-speed continuous-flow analyzer (153). Glucose, triglycerides, and ATP were measured by colorimetric determination of the H_2O_2 produced in the reactions of the respective enzymes.

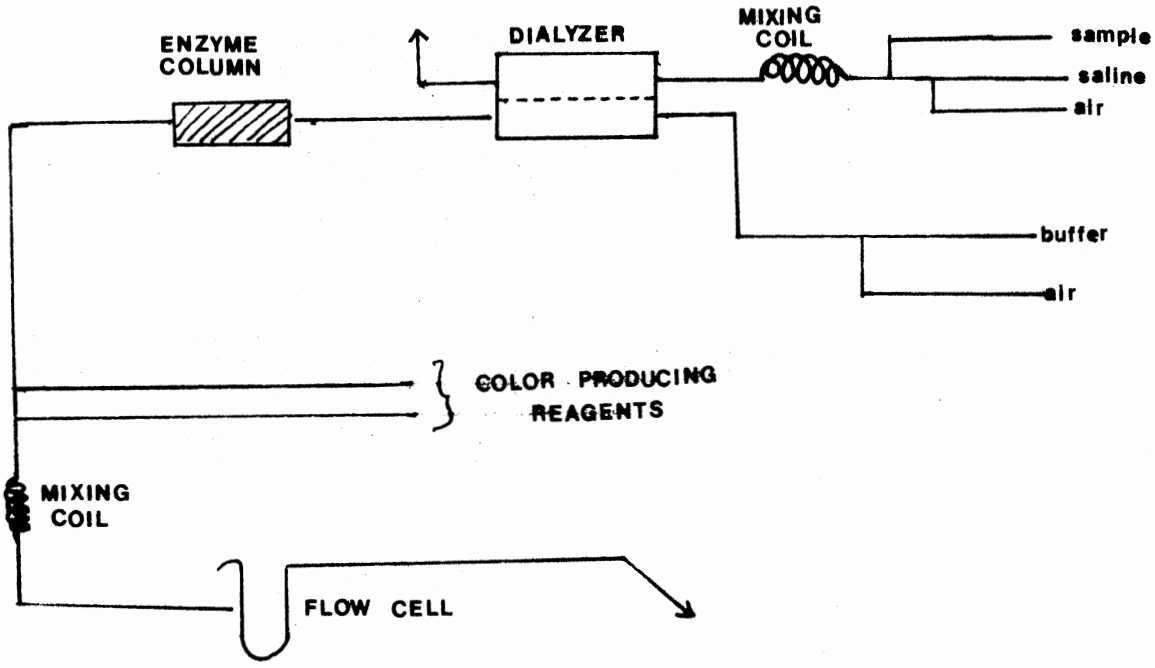


Figure 11. A Simplified Flow Diagram for Spectrophotometric Analysis Using Immobilized Enzyme Column Reactor Integrated Into the AutoAnalyzer I

A closed system containing an immobilized redox enzyme and spectrophotometer has also been described (154). In the example cited, a urine sample was injected into a stream of buffer solution containing NAD. This stream passed through a spectrophotometer and then through a column containing glucose oxidase immobilized on agarose. The stream again passed through the spectrophotometer where the NADH was measured and used to determine the glucose content of the urine. The stream finally passed through another column in which NAD was regenerated by reaction of glutathione reductase in the column. Miles Laboratories, Inc., has produced enzymes immobilized on the surface of nylon tubes by covalent attachment. The coated nylon tubes can be incorporated in the typical stream with a spectrophotometric detector.

Amperometry. The commerically available glucose, glucose-sucrose, and glucose-lactose analyzers (Leeds and Northrup Company) (155, 156) are types that fall into this category. In these analyzers the stream is split, after the sample is dialyzed and diluted with buffer, and glucose is detected in one stream by using immobilized glucose oxidase and a three-electrode amperometric detector sensitive to H_2O_2. The other stream is passed over immobilized mutarotase, invertase and glucose oxidase, and the hydrogen peroxide formed is detected the same way. The reactions involved are:

$$\text{Sucrose} \xrightarrow{\text{Invertase}} \text{Glucose} + \text{Frucrose} \xrightarrow{\text{Invertase}} \text{Glucose}$$

$$\text{Glucose} + O_2 \xrightarrow[\text{Oxidase}]{\text{Glucose}} \text{Gluconic Acid} + H_2O_2$$

A partitioned enzyme-sensor system which incorporates an immobilized substrate (starch) and three or more discrete immobilized enzymes

(glucose oxidase, catalase, glucoamylase or maltase) has been described (157). Here α-amylase activity is assayed by first passing the sample through a column of starch and then exposing the resulting oligosaccharides to columns of immobilized glucose oxidase, catalase, glucoamylase or maltase, and glucose oxidase successively. The resulting H_2O_2 is then amperometrically determined.

A recently published paper indicated that glucose oxidase could be determined by sample injection in closed-flow systems by measuring the depletion of oxygen amperometrically (158). In this technique the injected glucose oxidase was successfully removed after detection by immobilizing it (by adsorption), on phenoxyacetylcellulose traps.

Potentiometry

An interesting clinical development that makes use of a potentiometic sensor is the blood urea nitrogen (BUN) analyzer (Kimble) (159, 160). In this case urease is immobilized on porous particulate alumina packed on a cartridge. The detector is an ammonia sensor which consists of a pH electrode and reference surrounded by a filling solution, which is kept separate from the solution to be measured by a membrane.

Coulometry. Coulometric analysis has been done to determine NADH (161) and urea (162) using the respective immobilized-enzyme reactors. In the analysis for NADH the substrate was oxidized coulometrically at a rotating Pt gauze electrode at 0.7 V vs. SCE at pH 9. The produced NAD was reduced enzymatically in a reactor containing immobilized alcohol dehydrogenase. The coulometric results were found to be in agreement with those obtained by spectrophotometric measurements.

A flow coulometric immobilized-enzyme analyzer based on the use of a fast, totally electrochemical pH-stat has been developed. In this pH-stat (a device which measures the total quantity of acid or base produced by a chemical reaction while holding the pH constant), pH changes were measured with a glass electrode and coulometrically restored to a preset value (163). By this technique urea was determined with good precision (3%) in both simulated serums and in quality control reference serums. A scheme of the flow coulometric immobilized-enzyme analyzers is shown in Figure 12.

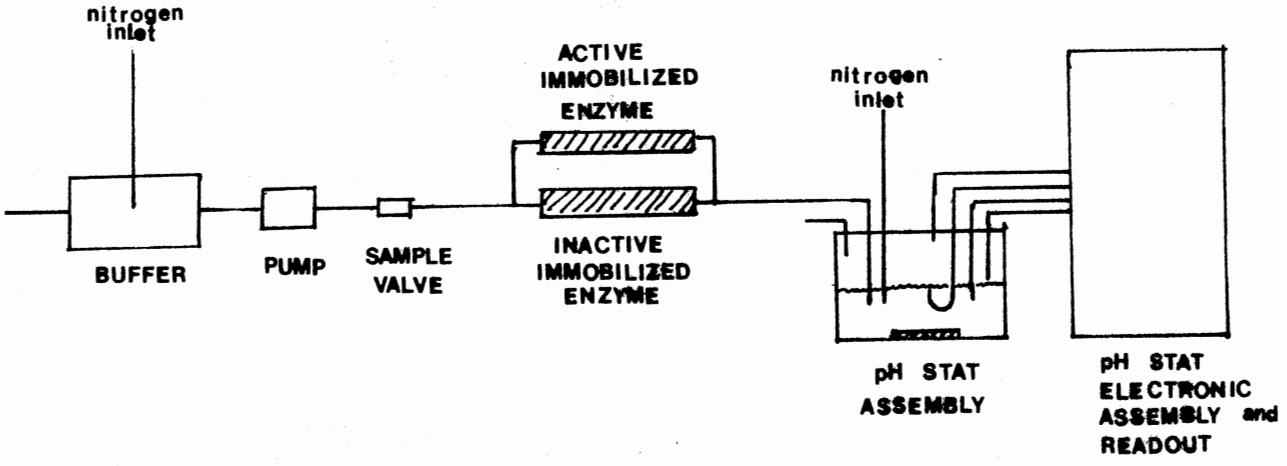


Figure 12. Block Diagram of Flow Coulometric Immobilized Enzyme Analyzers

Enthalpimetric Methods. A microcalorimetric method for substrate determination in flow systems with immobilized enzymes has been

described (164). An LKB flow calorimeter was used to determine the concentration of urea (0.5 to 5 μmole) and glucose (0.03 to 0.5 μmole) by taking the enthalpy of the process catalyzed by the relevant immobilized enzyme in the reaction cell. Accuracy for this was 2 to 5% and the time needed for one determination was 20 min. A more refined device was developed which used a combination of sensitive thermistors and immobilized enzymes. Glucose concentrations of 0.05 to 1 μmol could be determined with an accuracy of 0.03 μmol within 4 min. for analysis of urine and serum samples.

The enzyme thermistor used for the manual operation mode was modified for automation. Two changes are described in which the thermistor is positioned closer to the site of reaction and in the path of the heat flow, with consequent improvement of the efficiency (165). Single- or double-probe thermistors could be used. The introduction of a reference electrode in the double-probe thermistors allows elimination of the variation in the baseline (due to fluctuations in the ambient temperature). The assembly is connected to a Wheatstone bridge, the voltage output of which corresponds to a certain change in temperature which in turn is proportional to the concentration of substrate. Regular use of this technique in which the immobilization is done on controlled-pore glass beads, allowed the determination of penicillin, urea, and glucose. Immobilized urease has also been used for the determination of heavy metal ions (166). The assay is based on the susceptibility of urease to inhibition by silver, mercury, and copper. The degree of inhibition of urease was determined after first passing a 30-sec. pulse of 0.5 M urea through the enzyme thermistor, then a pulse containing the inhibitor, and then another pulse of urea. The degree

of inhibition was expressed as the ratio of the temperature peaks obtained before and after introduction of the inhibitor. The enzyme column could then be regenerated by passing a solution containing NaI and EDTA through the system for three minutes. The metal ion concentration required for 50% inhibition of a urease column with high enzymatic activity were 50, 300, and 40 x 10^{-6} M for Hg, Cu, and Ag, respectively.

Chemiluminescent and Bioluminescent Methods. Chemiluminescent determinations of trace amounts of cholesterol with immobilized enzymes have been reported (167). In this technique the samples are injected into an analyzer (see Figure 13) containing two enzyme reactors (cholesterol ester hydrolase and cholesterol oxidase). The sample is carried through in a buffer stream. Effluent from the cholesterol oxidase column is mixed with a buffered dioxane-based reagent containing bis(3,4,6-trichlorophenyl)oxlate, 9,10-diphenylanthracene, and triethylamine; the chemiluminescence produced by this reagent and H_2O_2 from the oxidase reaction is measured photometrically in a quartz flow-through cuvet. The enzymes are immobilized on ZrO^- coated porous quartz beads. The lower limit of detection was 1 x 10^{-9} M cholesterol in a 100 µl plasma sample. Linearity in response appeared from 10^{-8} to 5 x 10^{-4} M for both free and esterified cholesterol with < 5% error of determination.

The utility of the bacterial luciferase complex, which has an absorption requirement for NADH and may therefore be used to assay many oxidoreductase enzymes and their substrates, was investigated in a continuous system incorporating a novel flow cell and detection system

(168). This method was used to determine ethanol via alcohol dehydrogenase immobilized on nylon coils. A curvilinear relation between peak light output and ethanol concentration was found. The detection limit was about 2 µmol/L and carryover was 2 to 8%.

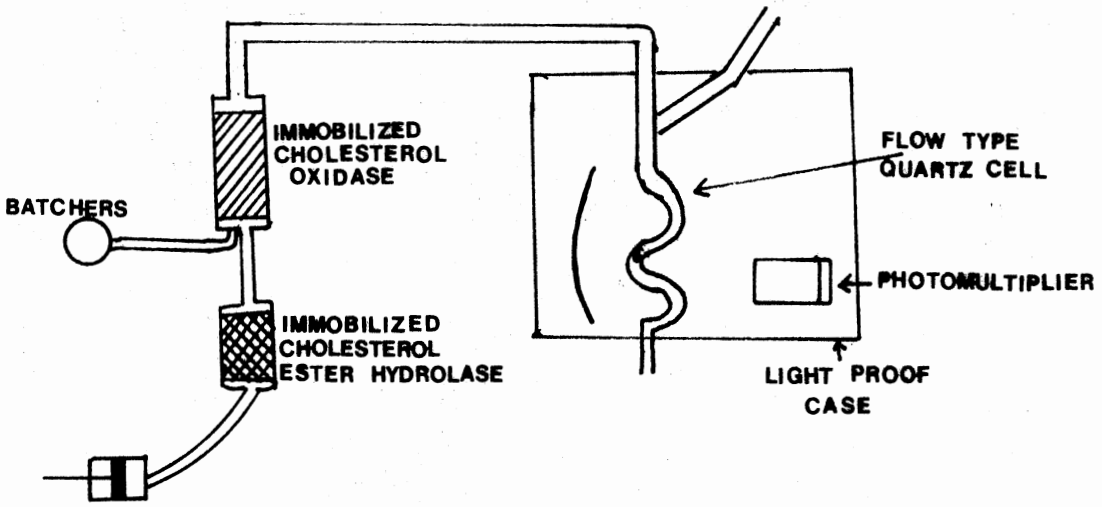


Figure 13. Schematic Diagram of Setup for Treating Sample and Measuring the Chemiluminescence

Fluorescence. Methods for the simultaneous, continuous in vivo monitoring of glucose, lactate, alanine, and 3-hydroxybutyrate have been described (169). They use dialysis and immobilized enzyme and measure the generated reduced pyridine nucleotides fluorometrically. The methods are accurate, sensitive, and suitable for use in emergency situations or in clinical investigations.

Another new approach is one that involves a membrane sandwich

reactor (170). Such reactors have been used for automated determination (171) of uric acid in which the resulting product (H_2O_2) in the presence of peroxidase oxidizes p-hydroxyphenylacetic acid to a fluorescent derivative. The uricase involved was entrapped in an immobilized-enzyme sandwich reactor.

Mass Spectrometry. A method for determining the concentration of reactants present and an apparatus which monitors a product of a reaction catalyzed with an immobilized enzyme have been described (172). The apparatus has a membrane permeable to a gaseous product and an immobilized enzyme adjacent to the membrane. A mass spectrometer also adjacent to the membrane surface is used to analyze the gaseous product. In the samples analyzed for urea, the CO_2 produced was determined by using a quadrupole mass spectrometer. Urease was immobilized on a singly backed dimethylsilicone membrane. From the calibration curves, 10^{-3} to 2.5 x 10^{-2} M urea in solution could be determined. This approach is, however, open to criticism since the same or even better results can be obtained by other means that are much easier to manipulate and which require simpler and less expensive instrumentation.

Miscellaneous Studies

Structural studies of macromolecules (proteins, nucleic acids, etc.), studies of mechanisms of enzyme reactions, and determinations of microenvironment about the support have been done with immobilized enzymes. On complete degradation of biopolymers by various hydrolases, much information about the primary structures and amino acid or base composition can be gathered. Conventional methods are complicated by the fact that

the amino acids, peptides, or bases are all mixed together and that extensive separation techniques have to be followed, many times without much success. These problems, however, are absent when immobilized enzymes are used instead. The limited or altered hyrolysis of proteins can give more information about the structure of the protein (173, 174, 175, 176, 177). Much more literature could be cited to this effect, but the areas covered are somewhat outside the scope of this dissertation.

Chemical studies using relevant immobilized enzymes have been used to examine the mechanism of coagulation of blood, which involves complex enzymatic reactions. The mechanisms of activating zymogens using immobilized enzymes have been studied; an example is the conversion of chymotrypsinogen to chymotrypsin using the azidocarbonylmethoxycellulose method of immobilizing trypsin (178).

Immobilized enzymes have been proposed as model systems for investigating the characteristics and behaviors of natural membrane-bound enzymes (e.g., respiratory enzymes). From these studies the possible influence of the support on the enzyme could be recorded. Comparative studies of the behavior of membrane-bound and immobilized sarcoplasmic reticulum ATPase (179) have been done. Their optimum pH, inhibition by some compounds, and metal ion stimulation have also been investigated. Similarly the mechanism of the hydrolysis reaction catalyzed by papain has been elucidated for the enzyme adsorbed on a collodion membrane (180).

The function of enzyme subunits has also been studied by immobilization of enzymes. The immobilization of aldolase (which contains four subunits) on CNBr-activated Sepharose has been thoroughly studied (181). When 8 M urea containing 2-mercaptoethanol was added, 3 subunits were

found to be released and these could then be studied. The remaining denatured immobilized subunit was then subjected to dialysis to remove the urea which renatured on the addition of excess aldolase. This method offers the opportunity to study the nature of individual-subunits or individual-subunit interactions of an enzyme.

One very interesting use of the immobilization process is for controlling the activity of an enzyme using an electric field (182). Lipase was entrapped in a collagen membrane containing a liquid crystal material 4-methoxybenzylidene-4'-n-butylaniline. Linearity was observed for the activity of the liquid-crystal in the presence of an electric field, and reversal to a basal level on removal of the field occurred cyclically. This type of activity control of immobilized enzymes is desirable for switching devices of a bioreactor.

Another immobilization that has been recently reported is the preparation of mechanosensitive and sound-sensitive immobilized enzymes that could be used as chemical amplifiers of weak signals (183).

Conclusion

The analytical application of immobilized enzymes lies in the fact that they can be used in the analysis of specific substrates when coupled with the relevant sensors. The advantages of immobilization of enzymes are that they can be re-used many times, reaction processes can be carried out continuously, and reaction products are free from enzyme contamination.

A wide variety of methods of immobilization exists and a few of these are quite useful for analytical purposes. Among these are the four component condensation method which can be used on supports that have

different functional groups ($-NH_2$, $-\overset{O}{\overset{\|}{C}}-R$, -COOH, or $-C\equiv N$) and the glutaraldehyde method. The versatility of the latter depends on the fact that it can be used for crosslinking enzyme molecules with each other or for binding enzyme to controlled-pore glass or other supports. Furthermore it is one of the simplest methods of immobilization of enzymes. Immobilization of enzymes on controlled-pore glass of large particle size has the advantage of lowering the back-pressure that occurs in packed columns used in continuous-flow techniques. Immobilization of enzymes on the inside surface of nylon tubes and glass tubings can also find wide analytical application in continuous-flow analysis if sufficient activity can be preserved in the immobilized preparation.

The availability of a variety of analytical sensors has helped spread the analytical applications of immobilized enzymes.

Both manual and automated techniques have been used in analyses carried out with immobilized enzymes. Only a few simple modifications are required in the manual technique in order to use it in continuous analysis (automated techniques). In each case the immobilized enzyme was used either as part of the sensor or separated from the sensor. Although the use of immobilized enzymes as part of the sensor has the advantage of simplicity and often of portability, it cannot be used with many of the sensors present in analytical instruments; it is commonly limited to electrochemical sensors. When an immobilized enzyme separated from the sensor is used, both the enzyme and the sensor can be kept at their respective optimum operational conditions. Thus many instruments that use this principle are on the market.

The cost and time of analysis are significantly reduced when automated techniques are applied. Closed systems can further reduce the

cost of analysis. The automated techniques enumerated above will find wide applications in clinical laboratories too (for routine determinations of metabolites, in blood serum, urine, etc.).

The nature of substrate (or other reactants) or product determines the type of sensor to be used for quantitative work. Furthermore the range of detection required determines the sensor type to be used. For example, although the chemiluminescent method of analysis is the most sensitive one, it is limited to the analysis for substances that are easily oxidizable (such as NADH or NADPH). Enthalpimetric methods are most nearly applicable since heat is nearly always released or absorbed during reaction. The poor limit of detection found at the present time can be improved by the use of thermistors with higher α (percent resistance change per ^{o}C). Miniaturization of enzyme bound thermistors may permit their use in vivo in the future, since very small thermistors are easily constructed. The simplicity of formation of enzyme electrodes together with the availability of the various electrochemical sensors makes this method promising.

The other analytical application of immobilized enzyme is in the elucidation of structures of some macromolecules.

CHAPTER II

EXPERIMENTAL METHODS AND PROCEDURES

Apparatus

The setup used for closed-flow analysis is shown in Figure 14. When an immobilized enzyme was present in the coil-packed bed reactor, the pump was positioned just before the injection point, in order to force the reagents through the packed reactor.

The circuit diagram in Figure 15 gives details of the potentiostat arrangement. All modular units were from MP-SYSTEM 1000 (Pacific). Where highly active enzymes were used (e.g., glucose oxidase), backing up the potential was not required.

The detector of the system was an amperometric three-electrode system. It was designed in such a way that it had fast response and the working electrode surface was continuously washed by the flow.

The design of the electrode cell is shown in Figure 16. It was all glass, since plastic allows oxygen diffusion. The working electrode was a bare platinum wire. A platinum wire was also used as counter electrode. The counter electrode was physically separated from the flowing solution as shwon in the figure by Agar-agar-filled sintered glass. A 7g/L solution of NaCl (saline concentration in serum is 7 g per L) was used as an electrolyte inside the housing. The passage of current through the cell was conducted by Cl^- ions which were oxidized to Cl_2. The ref-

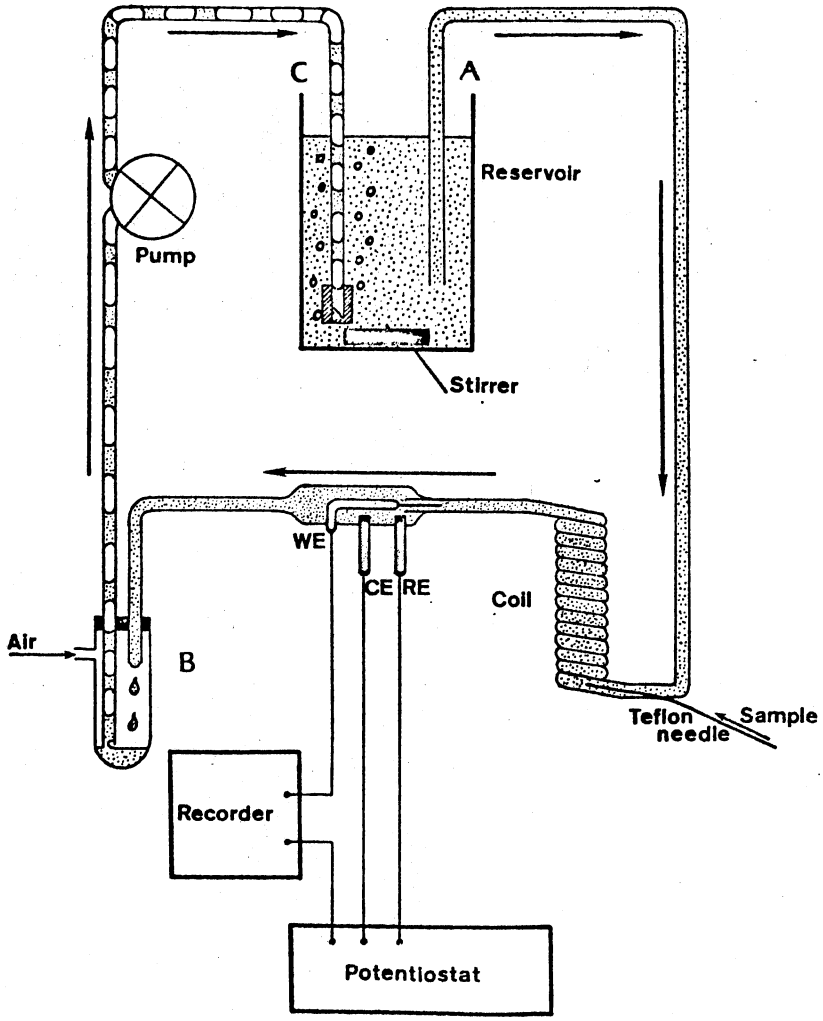


Figure 14. Experimental Setup Used for the Amperometric Determination of Enzymes or Substrates

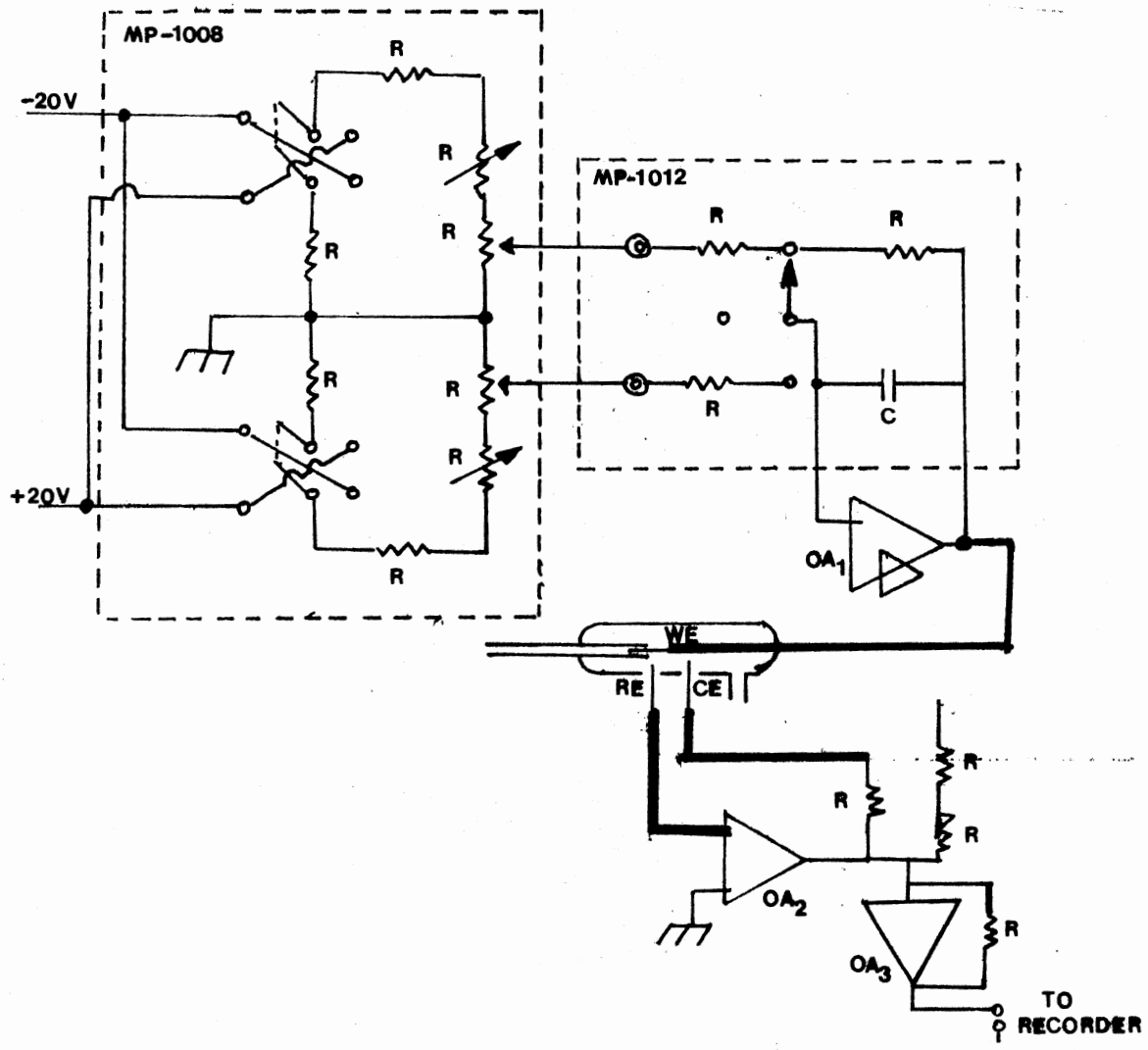


Figure 15. Potentiostat Arrangement for Amperometric Determinations

erence electrode was a calomel electrode from a Coleman 3-511 electrode (or Sargent Welch pH combination electrode) removed from its sleeve and inserted into a T unit with a platinum contact wire at its bottom. The electrolyte inside the reference electrode was a saturated KCl solution. The working electrode, a bare platinum electrode wire (0.5 mm 0.d.), was connected to a copper wire at position c as shown in Figure 16. This connection was made by coiling copper wire on the platinum wire and melting the tip of the copper wire on the platinum (with a Bunsen burner) and then dipping it in sodium borate; it was finally heated until the borate covered the junction as a glassy material. This was done in order to protect the copper at this junction from being oxidized to CuO and thus rendered nonconductive. Epoxy cement was used at position d in order to seal the platinum to the glass.

The counter electrode housing was made large so that it could contain as much as possible of the sodium chloride solution (7 g/L), in order that more Cl^- ions could be oxidized and thus more current allowed to pass through. For the counter electrode, tantalum wire could have been used since its coefficient of expansion is the same as that of glass. However, to be on the safe side expoxy cement was used to close any tiny opening that might remain. The connection between the platinum or tantalum wire and copper wire was made in the same way as that for the working electrode.

The contact wire for the reference electrode could also be made of tantalum wire instead of platinum wire. The wire connection was made very smooth on the outside in order that it would not create any flow disturbance.

Gas-tight syringes (Hamilton) and a Hamilton PB600-1 repeating dis-

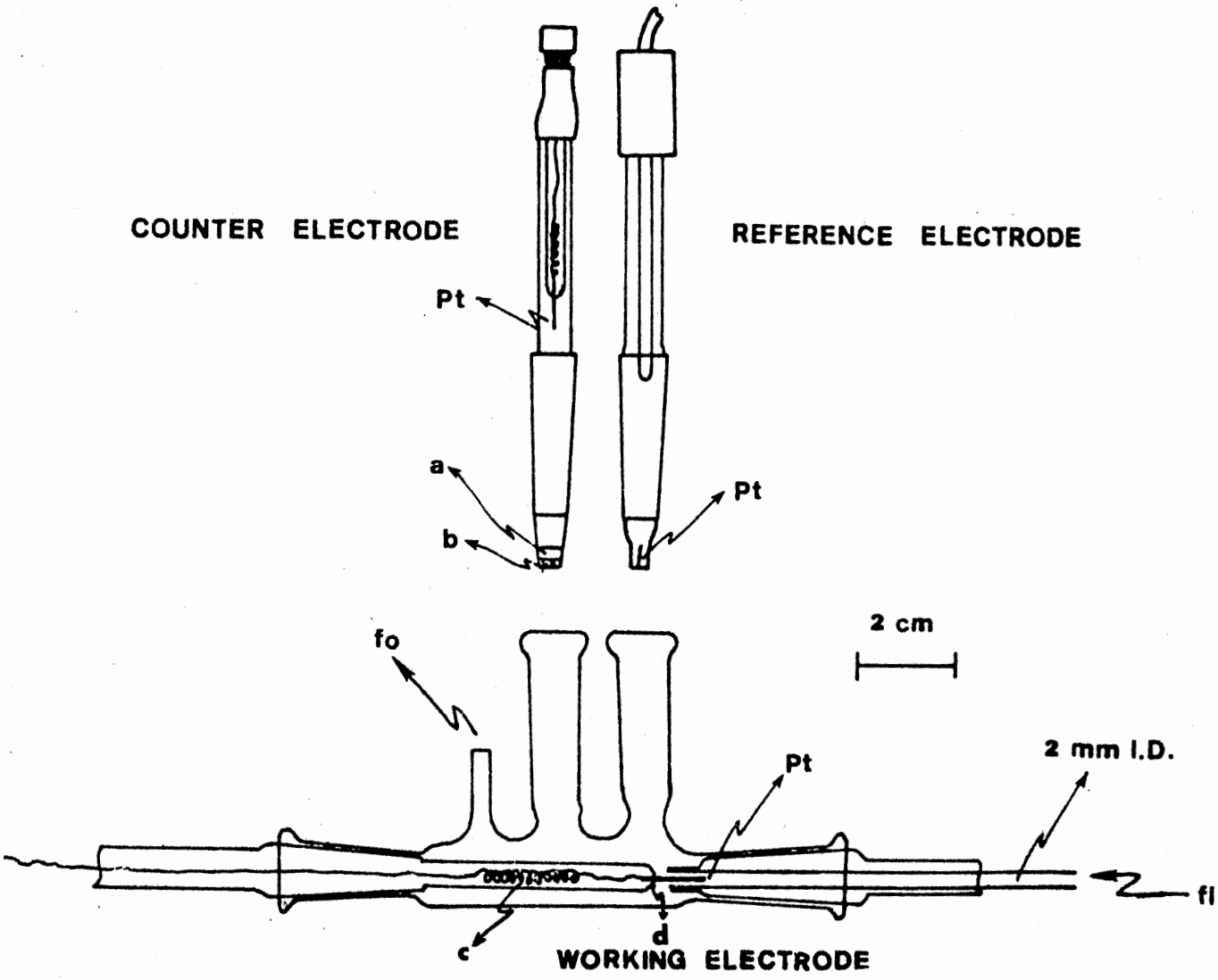


Figure 16. Design of the Amperometric Three Electrode System

penser were used for injecting samples into the flow system. A rotary valve similar to the one described by Ruzicka et al. (184) with the required sample loop was also used in some experiments.

The pump shown in Figure 14 was a peristaltic type (Masterflex with SRC model 7020c speed controller and 7014 pump head).

The recorder used for the system was a Sargent strip chart recorder model SRG.

A Beckman Zeromatic pH meter was used for all pH measurements.

Some of the spectrophotometric measurements were made with a Bausch and Lomb Spectronic 505 Spectrophotometer. A pair of matched quartz cells for the UV and glass cells for the visible region were used to measure absorbance readings at the pertinent wavelengths. In the latter part of the experimental studies a Beckman Model 25 instrument was used for spectrophotometric measurements.

When temperature control was required, a LAUDA K-2/R model thermostated circulator (Brinkman Inst.) was used.

In experiments that required mixing a reagent with a solid material for prolonged periods of time a shaker was used. The modified shaker was based on an air-driven mechanism provided by a vacuum motor for a car windshield wiper. It was normally operated at 25 strokes/min.

Reagents and Solutions

Water

Aqueous solutions of reagents or buffers were mostly made up with deionized-distilled water. In some cases when the need for distilled water did not seem critical, plain deionized water was used.

Acetate Buffer

Glacial acetic acid, ACS (Octagon Process, Inc.) (2.84 mL), and 1.46 g of NaCl, AR (Mallinckrodt, Inc.), were dissolved in about 900 mL of distilled water. Adjustment to pH 5.1 was done with 30% NaOH. The solution was then diluted to 1 liter.

Tris Buffer (0.05 M)

Tris buffer was prepared by dissolving 0.605 g of tris, $H_2N—C(CH_2OH)_3$, (Eastman Organic Chemicals) in 90 mL of distilled water and then diluting to 100 mL after adjusting to pH 7.00.

Triton X-100 (0.1%)

Triton X-100 (alkyl phenoxy polyethoxyethanol) (0.10 mL) (from Rohn and Haas) was dissolved in 100 mL of tris buffer, 0.05 M, pH 7.0.

Phosphate Buffer (0.02 M)

A solution containing 7.16 g/L of $Na_2HPO_4 \cdot 12H_2O$ (Baker Analyzed Reagent) (85 mL) and 15 mL of a solution prepared by dissolving 2.75 g/L of $NaH_2PO_4 \cdot H_2O$ were mixed and then diluted to 200 mL with distilled water.

Phosphate Buffer (0.1 M)

KH_2PO_4 (Baker Analyzed Reagent) (2.70 g) and 1.4 g NaCl were dissolved in about 170 mL of distilled water. After the pH was adjusted to 7.0 with 30% NaOH, the solution was diluted to 200 mL.

Agar-agar

Agar-agar suspensions were prepared by dissolving 0.3 g of agarose

(Marine Colloids Inc.) in 100 mL of distilled water. After boiling for ≈ 10 minutes, the solution was then allowed to cool to room temperature and reach the desired consistency.

o-Dianisidine 1%

o-dianisidine (Sigma Chemical Co.) (1% solution) in anhydrous ethanol.

EDTA Solution

Disodium ethylenediaminetetracetate (Fisher Scientific Co.) (0.372 g) was dissolved in about 900 mL of distilled water. The pH was adjusted to 7.00 and the solution diluted to 1 liter with distilled water.

Borate Buffer (0.1 M)

A borate buffer was prepared by dissolving 38.14 g of sodium borate, $Na_2B_4O_7 \cdot 10H_2O$ (Baker Analyzed Reagent) and 13.2 g of ammonium sulfate $(NH_4)_2\ SO_4$ (Baker Analyzed Reagent) in about 950 mL of distilled deionized water. The resulting solution had a pH between 9.2 and 9.3. Either dilute HCl or 30% NaOH was then added to adjust it to the required pH.

3-Aminopropyltriethoxysilane 2% Solution

A 2% solution was prepared by dissolving 2.0 mL of 3-aminopropyltriethoxysilane (Aldrich) in 117 mL of anhydrous acetone (Mallinckrodt).

Glutaraldehyde Solution 1%

Glutaraldehyde (Eastman) (2 mL) was dissolved in 100 mL of distilled

water.

Pyrophosphate Buffer

Sodium pyrophosphate, $Na_4P_2O_7 \cdot 10H_2O$, (Fisher Scientific Co.) (8.92 g) was dissolved in about 900 mL of distilled water, adjusted to pH 8.3, and then diluted to 1 liter with distilled water.

Triethanolamine Buffer (0.2 M)

Triethanolamine (Baker Analyzed Reagent) (26.6 mL) was dissolved in 950 mL of distilled water. The pH was adjusted to 7.6 and the solution diluted to 1 liter with distilled water.

Peroxidase 0.001%

Peroxidase (Sigma Chemical Co.) (10 mg) was dissolved in 100 mL of distilled water.

Ammonium Hydrogen Fluoride 5% W/V

Ammonium hydrogen fluoride (Fisher Scientific Co.) (5 g) was added to 95 mL of methanol (Mallinckrodt) and the mixture thoroughly stirred in order to dissolve as much of the solid as possible.

Glycine Buffer (0.1 M)

Glycine powder (Matheson Coleman & Bell) (0.75 g) was dissolved in distilled water; the pH of the solution was adjusted to 9.4 with 30% NaOH.

Other chemicals that were used:

-Glucose (Mallinckrodt)

-Ethanol 95%

-Pyridine (Fisher), dried over molecular sieve type 4A

-Dimethylformamide (Eastman Organic Chemicals)

-Phenoxyacetyl chloride (Aldrich)

-Apiezon-M

-Enzorb-A (Regis Chemical Co.), phenoxyacetylcellulose powder

-Isopropyl alcohol (Isopropanol) (Wood Scientific Inc.)

-Glutaraldehyde

-Acetone (Mallinckrodt)

-DL-Isoleucine (Nutritional Biochemical Corporation)

Enzymes Used for Study

(a) Glucose Oxidase (Sigma) from Asperigillus niger; EC No. 1.1.3.4.

Two types were used, one was reported to have an activity of 20,300 units/g solid and the other, 52,600 units/g solid. Both types contained catalase as an impurity.

Unit definition for glucose oxidase: 1 unit is that amount of enzyme which causes the oxidation of 1.0 μM of glucose to gluconic acid per minute at pH 5.1 and at $35^{o}C$.

(b) Catalase (Sigma) from bovine liver.

The activity of the catalase used varied from 10×10^{3} Sigma units/mg to 16×10^{3} Sigma units/mg.

One sigma unit is the amount of enzyme that decomposes 1 μmole of H_2O_2 per min. at pH 7.0 and $25^{o}C$, while the H_2O_2 concentration falls from 10.3 to 9.2 μmoles per mL of reaction mixture.

(c) D-Amino Acid Oxidase (Sigma) from hog kidney; EC No. 1.4.3.3. Its activity was 0.2 units/mg solid. One unit will oxidatively deaminate 1.0 µmole of D-alanine to pyruvate per minute at pH 8.3 and 25°C in the presence of catalase.

(d) L-Amino Acid Oxidase (Sigma) from Crotalus atrox venom. The reported activity was 0.33 units/mg solid. One unit of this enzyme will oxidatively deaminate 1.0 µmole of L-Phenylalanine per minute at pH 6.5 and 37°C. (L-Leucine is deaminated at the same rate at pH 7.5 at 37°C).

(e) The Uricase used (Sigma), (EC No. 1.7.3.3) came from one of two sources: 1) Candida Utilis, 2) hog liver. Those from the latter were impure types; their activity varied from only 20 units/gm to 36 units/g. The one from Candida Utilis was purer and contained 2.9 units/mg protein. One unit will convert 1.0 µmole of uric acid to allantoin per minute at pH 8.5 and 25°C.

An impure uricase (Worthington) of 9 units/g was also used at one point in our study.

Materials Used as Supports for Immobilizations of Enzymes

(a) Controlled-Pore Glass (Electro-Nucleonics, Inc.)

Two varieties of controlled-pore glass were used: CPG 00500C and CPG 3000. Their characteristics follow.

	CPG 005000	CPG 3000
Mesh Size	200/400	80/120
Mean pore diameter (A)	544	2869
Pore distribution (+%)	5.3	8.3
Pore volume (cc/g)	1.24	1.06
Surface area (M/g)	57	8.9

(b) Enzorb-A (Regis Chemical Co.)

A phenoxyacetylcellulose powder.

(c) Apiezon-M

A high pressure vacuum grease.

(d) Filter Paper (Duren)

Hydrophobic filter paper, type MN 617WA, 9-cm diameter.

(e) Sponge

A cellulose sponge was chemically treated to convert it to a hydrophobic phenoxyacetylcellulose.

(f) Silica Gel

Large particles of silica gel (without indicator), 6-12 mesh.

(g) CPG/CDI-Glycophase (Pierce)

A modified ready-to-use controlled-pore glass Glycophase. It had to be activated by the carbonyldiimidazole (CDI) method (185).

(h) Whiskers

Whiskers were grown on the inside surface of Pyrex glass by etching with ammonium hydrogen fluoride (186).

Procedure

The instrumental setup required for the closed-flow analysis has already been shown in Figure 14. The amperometric response of the platinum electrode to oxygen is very sensitive to changes in flow rate. In the case when the immobilized enzyme was contained in the chamber or when no immobilized enzyme was used, the solution flowed from the reservoir to the electrode systems by gravity. After passing the detector, the solution was taken back to the reservoir by means of a peristaltic pump. The reservoir solution was buffered as required for the particular enzyme or

When controlled-pore glass attached (fused) to the inside surface of a glass coil was used as a support, the solutions were caused to pass through the coil with the aid of a peristaltic pump.

Once the whiskers were developed, the method of immobilizing uricase on them was the same as above since they contain silica moiety just like the controlled-pore glass.

A similar procedure was also followed for immobilizing uricase on silica gel.

CHAPTER IV

DETERMINATION OF GLUCOSE OXIDASE ACTIVITY

An Example of Catalyst Determination in Closed-loop Flow Systems

Enzyme assays are of importance in clinical medicine, the food industry, and agriculture. Different organs of human beings (and some other animals too) differ quantitatively and also in part qualitatively in their enzyme make-up. The pattern of enzymes found in serum if the cells of a particular organ are injured by a disease is characteristic and clearly different from that which occurs if another organ is diseased (188). Thus the course of disease is easy to follow by enzymatic analysis. Enzyme measurements in the food industry are important since they can indicate the degree of freshness of food, the adequacy of a particular treatment such as pasteurilization and sterilization, etc. In general, enzyme analysis is little used in agriculture as such. The presence of certain enzymes allows deductions to be made about the constituents of some food stuffs. The amount and activity of the enzyme depends on the nature of the fertilizer used and the method of cultivation during the vegetative period. The activity of the enzymes is of importance for the quality, stability on storage, germinating power, and other properties of plants.

Good assay procedures are essential for forming the basis for

acceptable analytical techniques for the analysis of substrates, activators, and inhibitors.

Among the many methods for glucose determination, the ones that employ glucose oxidase are very specific. Current status methods for determination of glucose have been reviewed by Cooper (189) and Martinek (190). An elegant method of determining glucose concentration has been reported by Wolff and Mottola (14). The method employs a closed-flow-through system which amperometrically monitors the cosubstrate oxygen in the glucose oxidase catalyzed reaction

$$\text{Glucose} + O_2 \xrightarrow{\text{GODase}} \text{Gluconic Acid} + H_2O_2$$

As many as 700 samples per hour can be analyzed by this method. This chapter describes the procedure followed and the results of applying it to determine the concentration (activity) of glucose oxidase rather than glucose. For this method to work, the enzyme had to be removed physically from the system or rendered inactive by some inhibitor after signal detection. Otherwise the baseline would continuously go down and the peak height for the same concentration of glucose oxidase would start to decrease.

Experimental Procedure

For manual assay of glucose oxidase either of two methods was followed. The first determined the gluconic acid produced in the reaction and then related that to the activity of the glucose oxidase (Sigma Chemical Co., Form N-322). The second one determined the concentration of H_2O_2 formed in the enzyme-catalyzed oxidation of glucose (191).

Manual Assay of Glucose Oxidase

Method (a)

1) Into each of two test tubes 25 mL of acetate buffer of pH 5.1 were pipetted.

2) To one tube marked "test", 0.75 g of anhydrous glucose was added and the mixture shaken and then kept for 60 minutes for equlibration. The other tube, marked "blank", receives no glucose.

3) Both tubes were placed in 35^{o}C water bath for a few minutes (temperature equilibration).

4) 1.0 mL of enzyme solution was then added (45-60 units that had been accurately weighed) to each test tube. When the enzyme was immobilized on some support, the material was inserted inside the test tube.

5) The solution was aerated for exactly 15 minutes with a sintered glass sparger.

6) 10 mL of approximately 0.1 N NaOH was added to each tube to stop reaction. The contents of each were then transferred to a 250 mL Erlenmeyer flask, with three rinses with 5 mL of water, and finally

7) titrated with 0.05 N HCl using phenolphthalein indicator.

The activity of the glucose oxidase was then calculated from the formula

$$\text{units of glucose oxidase} = 3.33 \times \text{"corrected mL"}$$

where "corrected mL" = mL for "blank" - mL for "test".

Method (b)

The following agents were pipetted successively into test tubes: Phosphate buffer, 0.02 M pH 6.1, (0.9 mL) 0.1 mL of 1% o-dianisi-

dine in anhydrous ethanol, 1 mL of soluble enzyme or immobilized enzyme, and 1 drop of peroxidase solution (activity 1.4 U/mg). The reaction was allowed to proceed at room temperature for 20 minutes. After this period it was stopped by the addition of 1 drop of concentrated hydrochloric acid and the absorbance was measured at 420 nm.

In the closed-flow method the reservoir contained 200 mL of phosphate buffer pH 7, in which 20 g/L of glucose and 20 mg/200 mL of catalase were dissolved. The glucose oxidase solutions were injected as usual with Hamilton gas-tight syringes.

Many methods were tried to remove the glucose oxidase from the stream after detection. They are described in chronological order below.

(i) Preliminary experiments (192) indicated that glucose oxidase totally lost all activity at about 80°C. Hence a heating coil was placed right after the detection zone in the closed-flow setup in order to inactivate the enzyme before it reached the reservoir. The heating coil with the other accessories used for this purpose are shown in Figure 17.

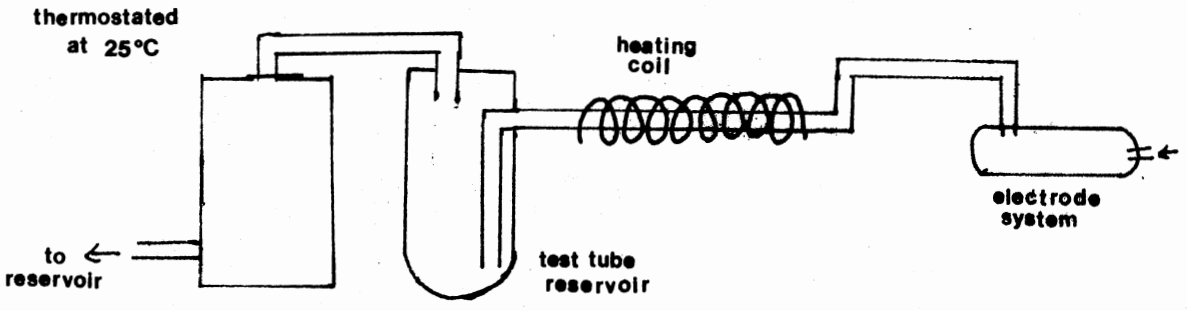


Figure 17. Heating Coil and Other Required Materials for Inactivating Glucose Oxidase

(ii) A phenoxyacetylcellulose powder (Enzorb-A) was used to remove the glucose oxidase by physical adsorption. Phenoxyacetyl cellulose is hydrophobic, and thus hydrophobic-hydrophobic interaction between this compound and the nonpolar backbone of glucose oxidase and other enzymes is favored in aqueous medium (193).

In preliminary work, a column (8 x 60 mm) was filled with a surry of 0.4 g of Enzorb-A in 1:1 ethanol/water. Glucose oxidase solution (3 g/L, 60,900 units/L) in phosphate buffer pH 7.0 was then poured through the column. One fourth mL portions of eluate were collected in a series of small test tubes. The first five eluates (total, 1.25 mL) contained no enzyme activity. Calculation using this information showed the immobilizing capacity of Enzorb-A to be at least 9.4 mg glucose oxidase per gram of Enzorb-A. This corresponds to 190 units per gram of Enzorb-A, which implies that 313 injections of 3 g/L of glucose oxidase would be required for saturation of 1 gram of Enzorb-A.

On this basis, a small column of this Enzorb-A was then inserted just after the detection zone to trap the injected glucose oxidase.

In another modification of this the inside surface of a glass coil was first coated with Apiezon-M. A suspension of Enzorb-A in 1:1 ethanol/water mixture was then passed through it. In order to fix the Enzorb-A in place better, the grease was heated nearly to its softening point and then the Enzorb-A suspension was swirled (moved) up and down the coil. The remaining suspension was finally washed out and the glucose oxidase solution passed through it. The immobilized glucose oxidase was then assayed manually by method (b).

Solid glucose oxidase mixed with Apiezon-M at 50 ^{o}C was used to coat the inside surface of a glass coil. The coil was then incorporated

into the flow system and glucose was injected to find whether signals (peaks) can result.

(iii) A commercially available hydrophobic filter paper (type MN 617WA by Duren) and derivatives of cellulose filter paper and cellulose sponges were tested (the cellulose products after derivatization to phenoxyacetylcellulose to render them hydrophobic). The general derivitizing procedure was as follows.

Pieces of the material (5 x 5 x 5 mm) were washed in hot water and then with 10^{-3} M EDTA solution of pH 7.00 to remove metallic impurities that might inactivate the enzyme. The materials were then thoroughly washed with distilled water and dried in an oven at 60 ^{o}C overnight. A 100 mL portion of a 1:1 pyridine/dimethylformamide mixture was added to the dried material, followed by addition of phenoxyacetyl chloride dropwise. The mixture was heated at 70 ^{o}C for about 10 minutes and left at room temperature overnight. Finally the product was washed thoroughly with 95% ethanol until no pyridine could be smelled in the product. It was then allowed to dry at room temperature.

Derivatization increased the weight of the cellulose sponge by about 8% on the average. Trapping of glucose oxidase on these derivatives was investigated. In one set of experiments 0.4 g of cellulose sponges (5 x 5 x 5 mm) was placed in a conical flask kept at the outlet of the detection zone in the closed-flow system. A stirring bar was included to help adsorption of the glucose oxidase on to the phenoxyacetylated cellulose sponge in the flowing stream. With this trap in place, the number of injections of glucose oxidase that could be performed without baseline deterioration was determined.

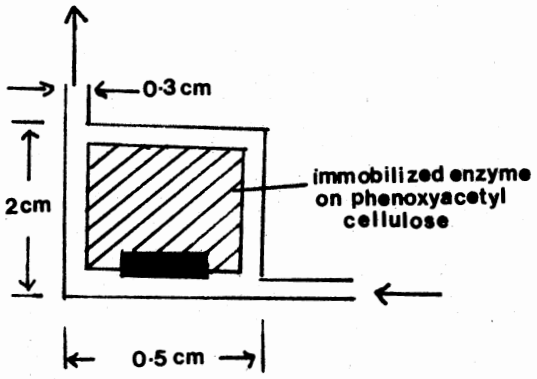


Figure 18. Configuration of Plexiglass Chamber for Containing Immobilized Glucose Oxidase

The rate of immobilization of glucose oxidase on phenoxyacetylated cellulose sponges was studied by putting five small pieces of sponge in each of three separate test tubes that contained enough solution of glucose oxidase (10×10^4 units/L, 3.0 g/L). The sponge pieces were kept for 5 minutes in the first set, 30 minutes in the second, and 1 hour in the third. The immobilized glucose oxidase in the sponges was then assayed by method (a).

In a related study immobilized glucose oxidase on phenoxyacetylated sponge was also used to determine the concentration of glucose in the unsegmented closed flow system. For this purpose a small phenoxyacetylated sponge to which a magnetic mini-stirring bar was attached (with epoxy adhesive) at the bottom was kept in a small chamber (see Figure 18) that was incorporated into the unsegmented closed-flow sytem.

Results and Discussion

In the determination of an enzyme in unsegmented closed-flow systems, removal of the injected enzyme after detection is a prerequisite for the method to be successful, as it has already been noted. The results of experiments perfromed to this end and described on previous pages can be summarized as follows.

(i) The heating coil used to deactivate the glucose oxidase worked fine except that caramelization took place inside the glass tubing when the flow was somehow discontinued. At the temperature necessary for denaturation (about 80 ^{o}C), the glucose in the buffer solution at relatively high concentration (20 g/L) soon is converted to carbon which blocks the tubing and thus creates back pressure and high resistance to flow, making the system inoperative. With proper precautions, however, i.e., turning the heating coil off for a while before discontinuing the flow, satisfactory operation is possible.

The calibration curve produced using this approach is shown in Figure 19. This portion indicates that the linear plot extends up to about 60 units per mL of glucose oxidase solution.

(ii) In practice the Enzorb-A column was found to cause undesirable backpressure.

When the Enzorb-A was attached to Apiezon-M on the inside of a glass coil, the trapping was only very limited and the immobilized enzyme lost activity fast. No better results were obtained when solid glucose oxidase was directly applied to a Apiezon-M coating of the glass tubing.

(iii) As for Enzorb-A, hydrophobic binding of proteins to phenoxyacetylated filter paper and sponges is nonspecific and essentially irreversible under most working conditions. "Hydrophobic interactions are

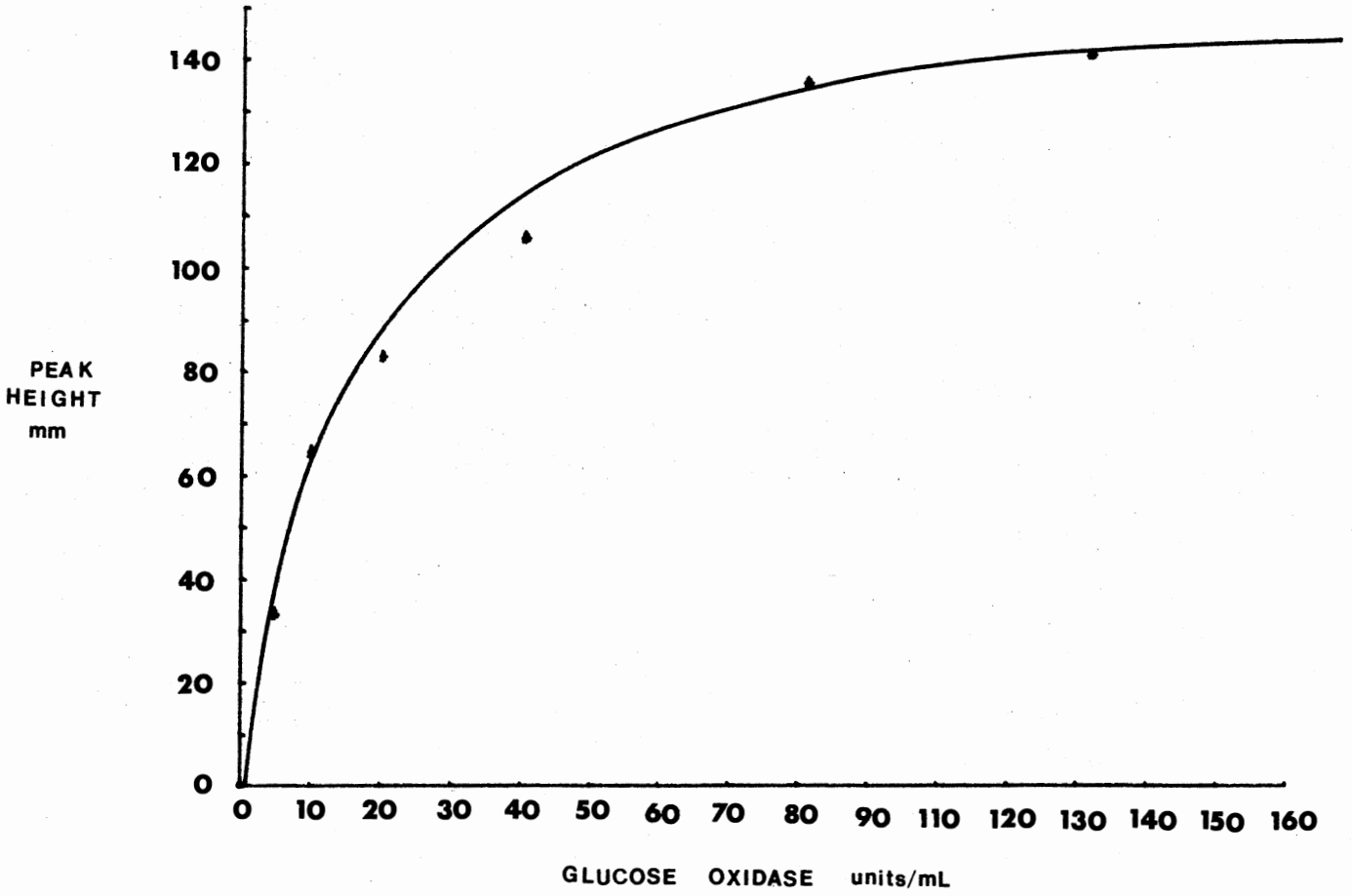


Figure 19. Calibration Curve for Determination of Glucose Oxidase

stronger at high temperatures than low, stronger at relatively high salt concentrations than low" (193). Thus the trapping was relatively insensitive to changes in pH, salt, and temperature. The phenoxyacetylcellulose filter paper and sponges were difficult to wet, except with a 1:1 solution of ethanol and water, which could first be displaced by pure water and then by the appropriate buffer.

The filter papers, i.e., the commercial hydrophobic filter paper and the laboratory-synthesized phenoxyacetylcellulose, were found to be of no use for our purposes. The amount of glucose oxidase immobilized on the filter paper was almost nil.

The phenoxyacetylcellulose sponge, however, fulfilled all the requirements of the experimental objective. It was preferable to break the cellulose sponge into smaller pieces before use. Its immobilization of glucose oxidase was found to be fast. Keeping the phenoxyacetylcellulose sponge in the enzyme solution for 5 minutes, 30 minutes, or 1 hour did not change the amount of glucose oxidase immobilized (see Table VII). In other words, the rate of removal of enzyme was high.

TABLE VII

IMMOBILIZATION OF GLUCOSE OXIDASE ON PHENOXYACETYL CELLULOSE SPONGE AT VARIABLE TIME

Weight of Dry Phenoxyacetyl Cellulose (g)	Time Kept in Glucose Oxidase (min)	Activity of Immobilized Glucose Oxidase (U)	Removed Activity Per Gram of Sponge
0.2936	5	11.32	38.6
0.2867	30	6.99	24.4
0.2806	60	9.66	34.4

When 0.4 g of phenoxyacetylcellulose sponge was used to trap the enzyme in the closed-flow system, 425 injections, each corresponding to 3.16 units of glucose oxidase, could be injected without deterioration of baseline. This corresponds to an adsorption capacity of 63.8 mg protein per gram of sponge. Without the enzyme trap, deterioration of baseline to the point that further use of the system became impossible occurred after 25 such injections. The typical signals are shown in Figure 20.

A secondary advantage of physically adsorbing the enzyme is that the enzyme can be washed out (i.e. desorbed) using a non-ionic surfactant such as Triton X-100 so that immobilization can proceed again. In order to permit reuse of the sponges for immobilization, however, the Triton X-100 washed materials must be rinsed with water, 2-propanol, and then water, each washing repeated 3 or 4 times.

Glucose oxidase immobilized on phenoxyacetylcellulose sponge was used to determine the concentration of glucose in the unsegmented closed-flow system by injecting the sample right into the chamber which contained the immobilized enzyme. For this to succeed, however, the flow rate had to be low (3 to 4 mL/min). Even then the peaks were broad, as can be seen in Figure 21. With some refinement of chamber design (shape, size, type of magnet, etc.), the method could be made to detect low glucose concentrations (20 g/L of glucose was used for testing purposes).

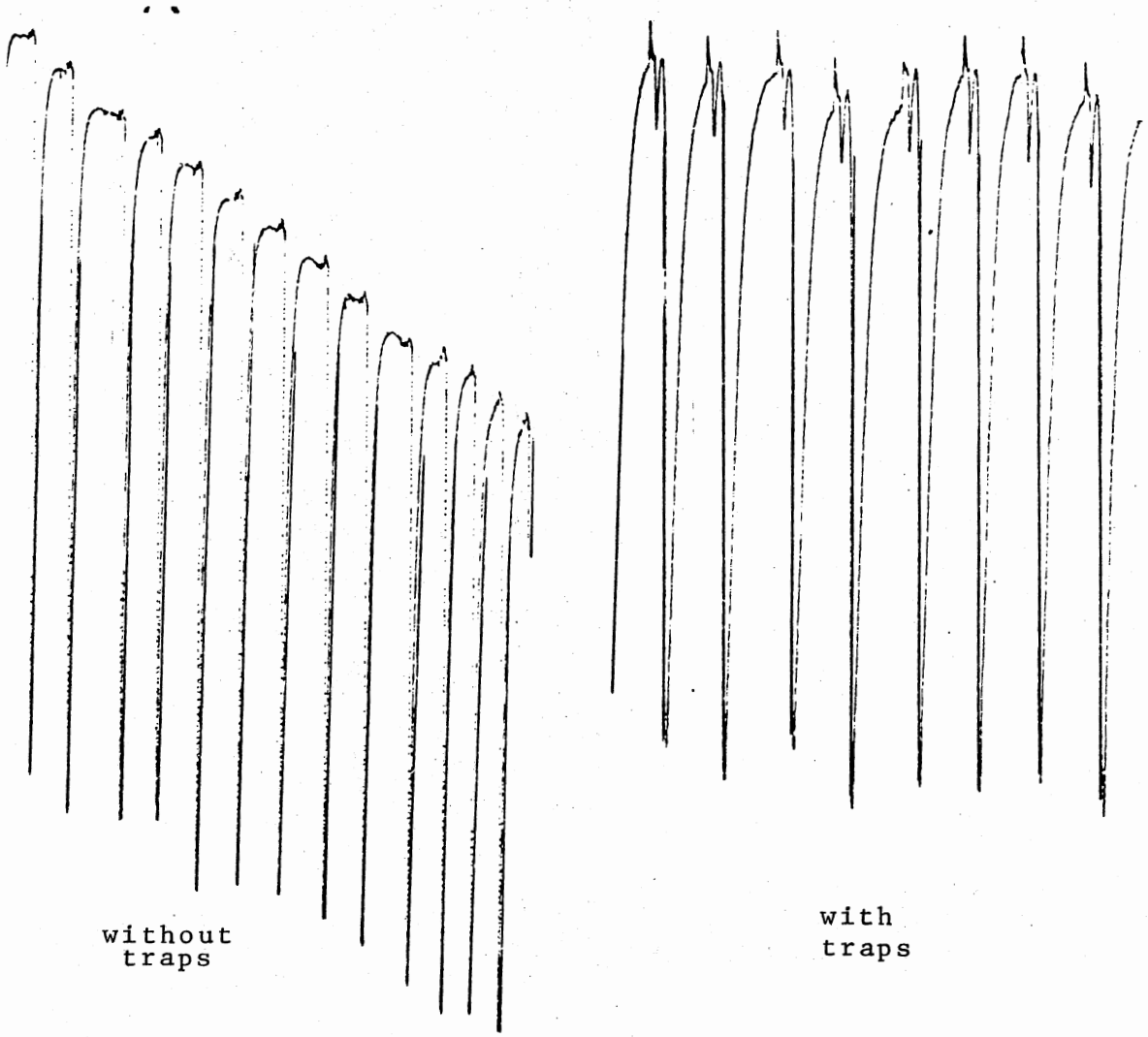


Figure 20. Typical Signals That Result With Use of Sponge Traps and Without Them

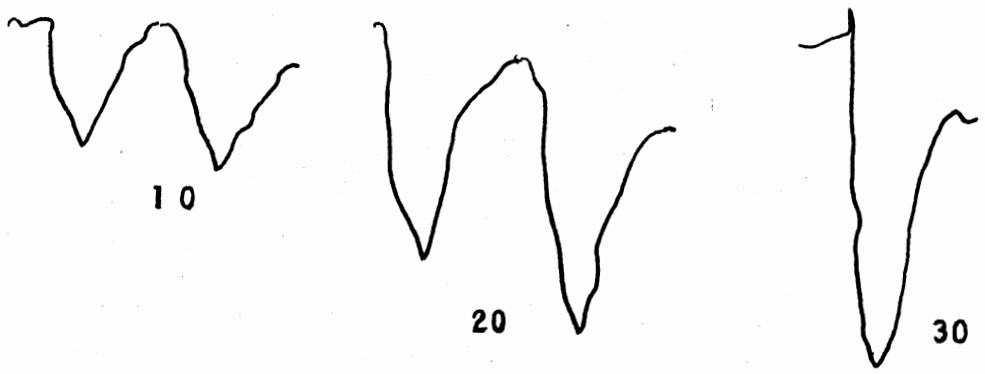


Figure 21. Peaks Produced by 10, 20 and 30 µl of Glucose (20 g/L)

CHAPTER V

KINETIC STUDIES AND ANALYSIS OF AMINO ACIDS

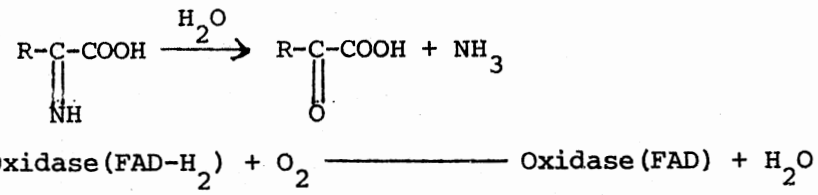
The technique of amino acid analysis becomes important because determination of the sequence of amino acids can help establish the structure of proteins and their determination in physiological fluids and tissues, and it can also give information about the metabolic degradation of some metabolites, and because their determination helps show the nutritive values of foodstuffs.

The oxidative deamination of the α-amino acids has been known since 1909. This is performed by means of the enzymes D-amino acid oxidases and L-amino acid oxidases which specifically oxidize D-amino acids and L-amino acids, respectively. The process consumes oxygen and results in the formation of ammonia and α-keto acids.

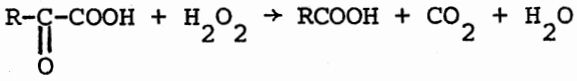
D-amino acid oxidases require flavin adenine dinucleotide (FAD) as coenzyme. Although D-amino acid oxidases are found in the kidney and liver of all mammals and some other vertebrates, they are abundant in the kidney of sheep and pigs. The reactions which appear to be involved are

$$\underset{\displaystyle NH_2}{\overset{\displaystyle |}{R\text{-}CH\text{-}COOH}} + \text{Oxidase(FAD)} \rightarrow \underset{\displaystyle NH}{\overset{\displaystyle \|}{R\text{-}C\text{-}COOH}} + \text{Oxidase(FAD-H}_2\text{)}$$

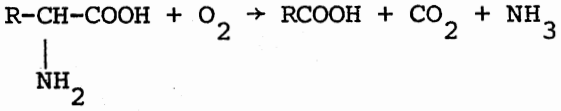
where the imino acid is hypothetical.



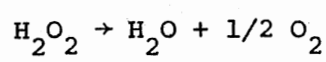
In the absence of catalase, the hydrogen peroxide so formed reacts with the α-keto acid by a decarboxylation mechanism to produce carbon dioxide and a fatty acid with one carbon atom less than the keto acid



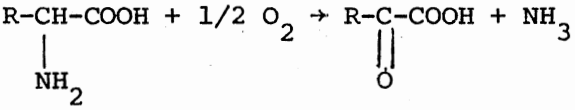
so that the overall reaction of the oxidase in the absence of catalase is written as



Renal tissues, however, possess high catalase activity and it is quite difficult to purify D-amino acid oxidase preparations from this enzyme. In the presence of catalase, the hydrogen peroxide formed by the reaction is decomposed to water and oxygen



Thus the overall reaction under these conditions is expressed as



L-amino acid oxidases are present in quite high activity in the venoms of a number of species of snakes. The highest levels of L-amino

acid oxidase are to be found in the rattlesnakes Crotalus adamanteus and Agkistrodon piscivorous. It acts exclusively on L-amino acids and exerts no measurable effect on any D-amino acids known. The mechanism of reaction is identical with that for the D-amino acid oxidase described above. Since the snake venom usually lack catalase, the reaction product is limited to formation of carbon dioxide and a fatty acid with one carbon atom less than the keto acid. Catalase must be added to the reaction mixture if the α-keto acid is to be saved.

In this study a D-amino acid oxidase was used in the soluble and immobilized form, while a L-amino acid oxidase which is relatively expensive was used only in the immobilized form.

Studies With D-Amino Acid Oxidase

Experimental Procedure

The experimental setup used is shown in Figure 14, when the soluble D-amino acid oxidase was used for determination of some D-amino acids. The mixing coil shown in the figure was, however, replaced by the slender chamber filled with the D-amino acid oxidase when the immobilized form was used.

Soluble D-amino Acid Oxidase

0.3 M of D-alanine, D-methionine, D-valine, D-lysine, D-phenylalanine, and L-proline solutions in 0.02 M pyrophosphate buffer of pH 8.3 were used for analysis.

The reservoir contained 200 units of D-amino acid oxidase/100 mL of pyrophosphate buffer (0.02 M) of pH 8.3. To this 20×10^4 units (20 mg) of catalase were added.

30 µl of the amino acid solution were injected with a gas-tight syringe.

Stability studies of the soluble D-amino acid oxidase were performed by determining the peak height that resulted when 30 µl of 0.3 M of DL-methionine was injected into the same reservoir solution (containing 200 U/100 of D-amino acid oxidase) that was kept at 4°C, on the first day of its preparation, 2 days later, and then 8 days later.

In order to determine the rate of oxidation of the D-amino acids in the presence of D-amino acid oxidase, two procedures were followed:

(i) The three electrodes, i.e., the working, counter, and reference electrodes, were inserted into a beaker with a solution containing 40 units of D-amino acid oxidase in pyrophosphate buffer (0.02 M) of pH 8.3. The rate of depletion of oxygen upon an injection of amino acid was then amperometrically detected.

Another 40 units of D-amino acid oxidase were dissolved in the buffer solution and the same procedure followed. This was continued until a saturation point resulted.

(ii) The rate of production of H_2O_2 was colorimetrically detected by interaction of the peroxide with o-dianisidine and by reading absorption at 436 nm. The procedure for this experiment was as follows:

Triethanolamine buffer (0.2 M, pH 7.6) (3 mL) containing 0.0065% o-dianisidine and some concentration of D-amino acid oxidase (4 units/3 mL) was put into two matching cuvets. Aqueous peroxidase (0.01 mL of 0.001%) was then added to each cuvet. These were then incubated at 25°C for 4-5 minutes to achieve temperature equilibrium. Then 30 µl of the D-amino acid (0.3 M) was added to the "sample" cuvet and the increase in absorbance with time was recorded.

The concentration of the enzyme was then increased to 10 units/3 mL and the same procedure repeated.

Immobilized D-Amino Acid Oxidase

D-Amino acid oxidase was immobilized on controlled-pore glass as outlined in Chapter III. Both the CPG 00500C and CPG-3000 were used as supports; in both cases 80 units/10 mL of the D-amino acid oxidase were used for immobilization. Analysis for the D-amino acids was then carried out by using the immobilized D-amino acid oxidase contained in the slender chamber (Figure 22) and amperometrically detecting oxygen.

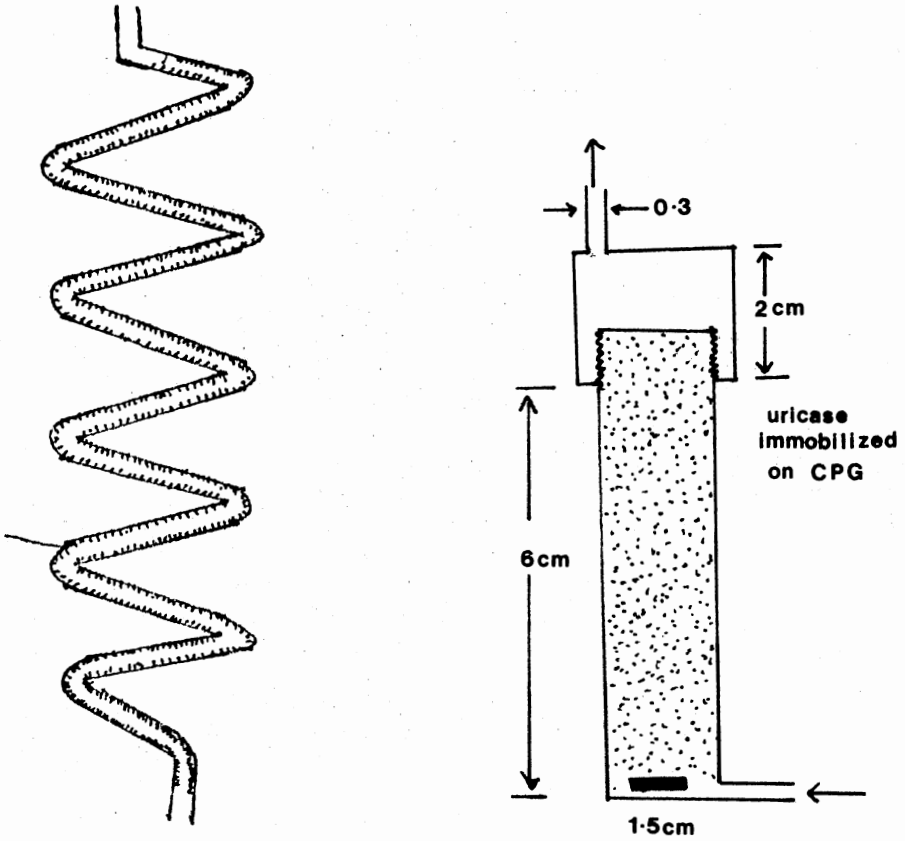


Figure 22. Design of Coil and Slender Chamber

To find whether the D-amino acid oxidase (immobilized) behaved differently toward the D-amino acids at different pH values, experiments were conducted using reservoir solutions containing pyrophosphate buffers of different pH values (6.5, 7.5 and 9.0). The same concentration (0.3 M) and amount (20 µl) of the amino acids were injected in each case.

Studies Carried Out Using L-Amino Acid Oxidase

For this particular enzyme, the study was done using the immobilized form only. L-Amino acid oxidase from Crotalus Atrox venom was immobilized on CPG-3000. The procedure followed for immobilization was exactly the same as outlined above. The 10 mL of L-amino acid oxidase used for immobilization in the final process contained 26.4 units. With this immobilized L-amino acid oxidase, in the slender chamber, some common L-amino acids were determined. The buffer solution used for this purpose was triethanolamine (0.2 M, pH 7.6).

Results and Discussion

Analytical determinations of the amino acids are based on the reactions represented by the equations

$$\text{D-Amino acid} + O_2 + H_2O \xrightarrow{\text{D-AAO}} \alpha\text{-keto acid} + NH_3 + H_2O_2$$

$$\text{L-Amino acid} + O_2 + H_2O \xrightarrow{\text{L-AAO}} \alpha\text{-keto acid} + NH_3 + H_2O_2$$

The uptake of oxygen as cosubstrate was amperometrically detected in the closed-flow system. The electrode response was proportional to the amount and concentration of the amino acid injected. The peak heights

that resulted when 30 µl of the D-amino acids were injected into the flowing stream containing D-amino acid oxidase were different for different amino acids. Some of the D-amino acids did not totally dissolve in the pyrophosphate buffer alone. Dilute HCl had to be added to them dropwise to dissolve them. The results are shown in Table VIII.

TABLE VIII

SIGNAL HEIGHTS PRODUCED BY AMINO ACIDS

Amino Acid	Peak Height (mm)
D-Methionine	186
D-Alanine	92
D-Phenylalanine	76
D-Valine	75
D-Lysine	11
L-Proline	2-6

The fact that different peak heights resulted for different amino acids of the same concentration can be attributed to different rates of the reactions.

The stability study carried out with the soluble D-amino acid oxidase indicated that it loses its activity drastically within a few days (Figure 23). A quantitative study with the immobilized D-amino acid oxidase could not be done because of the fact that packing technique and channeling were making a difference in peak heights. However, qualitatively it was found that about 3 months after immobilization the immobilized D-amino acid oxidase was still active enough to be used in

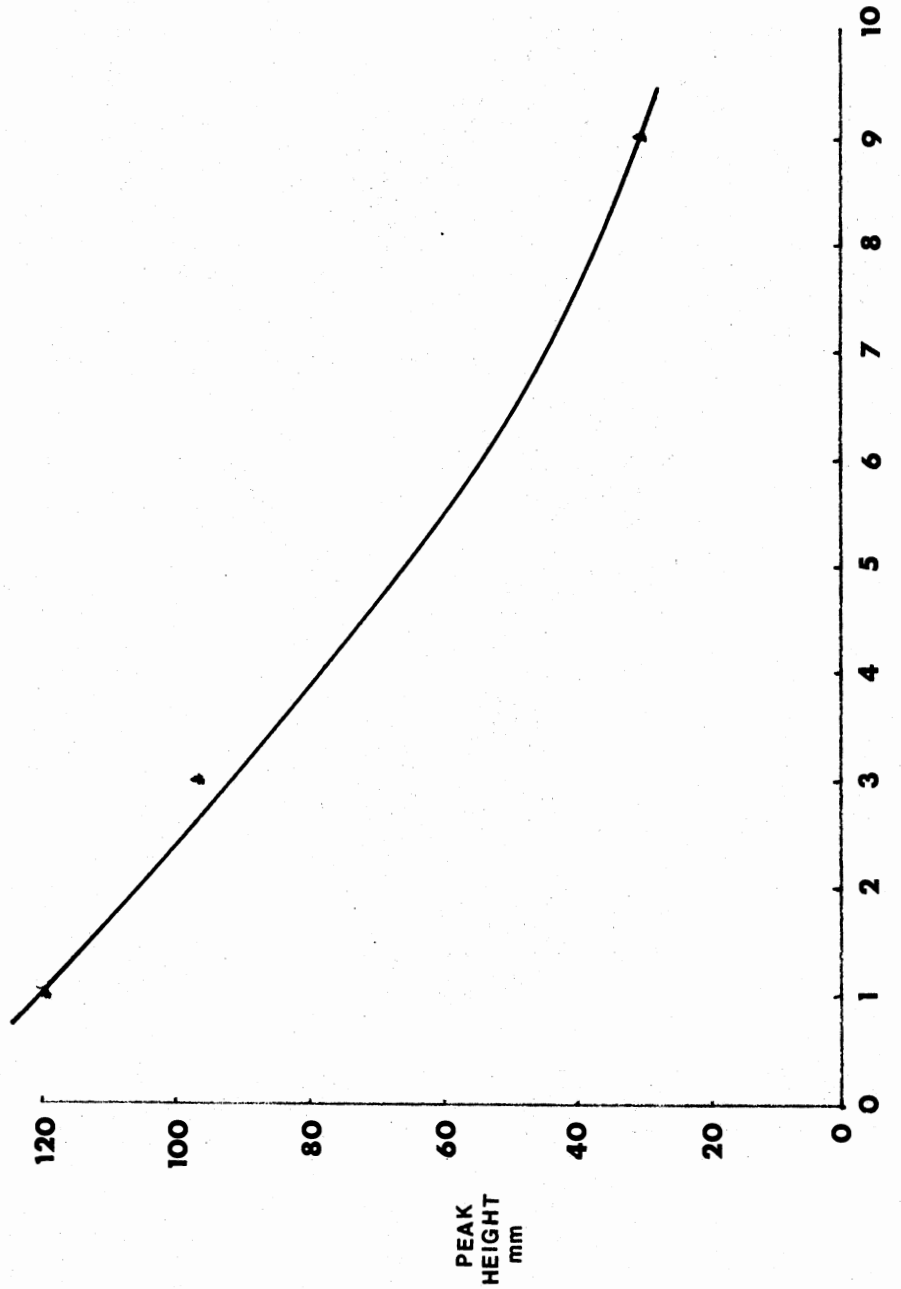


Figure 23. Long-term Stability of Soluble D-Amino Acid Oxidase

analysis for the amino acids.

The first trial of kinetic studies by the amperometric method was not successful. The large background noise that resulted prevented this analysis. This approach was then abandoned and the production rate of H_2O_2 in the reaction mixture was colorimetrically measured. At lower concentrations (4 units/3 mL) of D-amino acid oxidase, the absorbance increase with time produced by the H_2O_2 and o-dianisidine reaction in the presence of peroxidase were monitored without any difficulty. When the concentration of the D-amino acid oxidase was increased, the absorbance increase observed was small. This could be due to the interference of the yellow solution of the D-amino acid oxidase which might absorb at the monitoring wavelength (436 nm). The results found with the first run (Table IX) was good enough to indicate that the continuous amperometric detection technique cannot be used for determining D-lysine and L-proline and possibly DL-isoleucine economically. The faster the saturation absorbance (see Figure 24) is reached for a particular amino acid the higher the initial rate and thus the greater the possibility for its determination by means of continuous-flow systems with high sampling frequency.

The saturation absorbance in this respect is defined as the highest absorbance reached and remaining constant for some time. The time required to reach 1/2 the saturation absorbance plus the extent of the saturation absorbance can indicate the sensitivity of that amino acid determination. The shorter the half-time to saturation absorbance and the higher the saturation absorbance the better the sensitivity. Thus D-methionine, D-alanine, D-Valine and D-Phenylalanine were determined successfully by the closed-flow system.

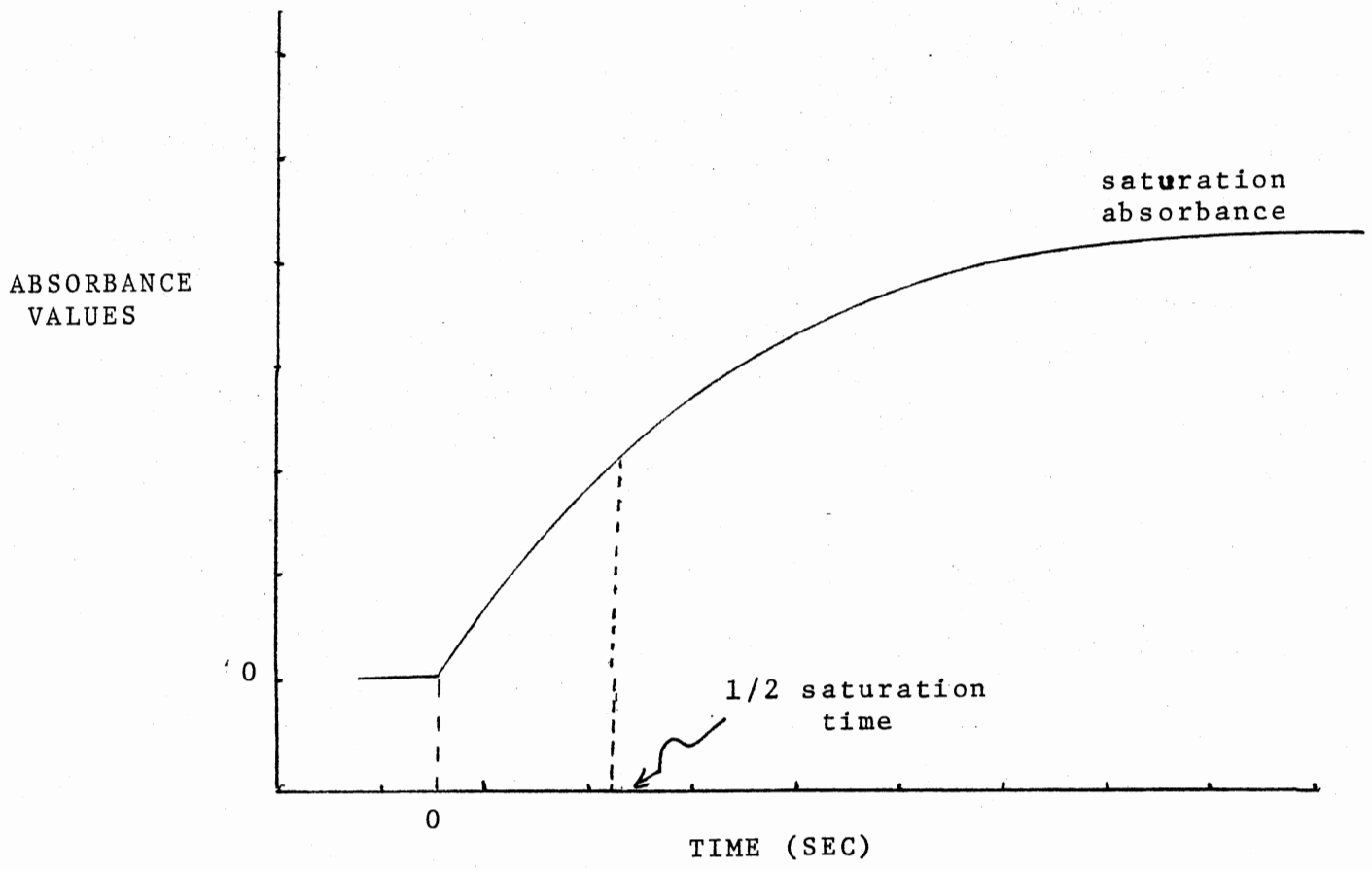


Figure 24. Curve That Defines Terms Used in Discussion the Kinetics of Reactions of Amino Acis

TABLE IX

RATE OF REACTION OF THE D-AMINO ACIDS WITH OXYGEN IN THE PRESENCE OF D-AMINO ACID OXIDASE

Amino Acid	Half-time to Saturation Absorbance (sec)	Saturation Absorbance
D-Methionine	25	0.17
D-Valine	55	0.16
D-Alanine	50	0.14
D-Phenylalanine	75	0.06
D-Lysine	$> 12 \times 10^2$	0.00
DL-Isoleucine	$\sim 6 \times 10^2$	0.15
L-Proline	$\sim 9 \times 10^2$	0.09

Column packing difficulties and channeling problems that resulted when the CPG-00500C immobilized D-amino acid oxidase was used in the slender chamber made analysis of the D-amino acids erratic. As a result quantitative analysis was not possible. The peaks that resulted in any case were very broad. With the D-amino acid oxidase immobilized on CPG-3000 the above effects were minimized. The peak heights that resulted when 30 µl of 0.3 M D-amino acid solutions were injected are shown in Table X.

All the analyses of D-amino acids conducted so far using the unsegmented continuous-flow system were at pH 8.3 in pyrophosphate buffer. When the pH of the pyrophosphate buffer was varied, however, the signal heights of the different amino acids were found to be different. The results found are shown in Table XI.

With the exception of D-methionine, the peak height of which remains almost constant, the D-amino acids gave larger peak heights with increase

TABLE X

SIGNAL HEIGHTS PRODUCED BY 30 NL OF 0.3 M D-AMINO ACID OXIDASE IMMOBILIZED ON CPG-3000

D-Amino Acid	Peak Height (mm)
D-Methionine	102
D-Alanine	78
D-Phenylalanine	56
D-Valine	55
DL-Isoleucine	17
L-Proline	5
D-Lysine	Insignificant

TABLE XI

SIGNAL HEIGHTS FOR OXIDATION OF AMINO ACIDS AT DIFFERENT PH VALUES

Amino Acid	Peak Height (mm) at pH 6.5	pH 7.5	pH 9.0
D-Methionine	85	109	108
D-Alanine	15	58	94
D-Phenylalanine	15	38	89
D-Valine	19	56	86
D-Lysine	3	15	5

in pH of buffer. This trend was especially marked for D-phenylalanine. D-Lysine which produced only tiny peaks (less than 5 mm) at other pH values gave quite a significant peak at pH 7.5.

L-Methionine, L-norvaline, L-citrulline, and to some extent L-isoleucine were the only amino acids, among those tested, that were soluble enough to make 0.3 M solution in 0.2 M triethanolamine buffer of pH 7.6. The peak heights that resulted when 5 µl of each solution were injected into the continuous-flow system whose reactor was the slender chamber filled with the CPG-3000 immobilized L-amino acid oxidase are shown in Table XII.

TABLE XII

SIGNAL HEIGHTS FOR OXIDATION OF SOME L-AMINO ACIDS (5 µL OF 0.3 M)

Amino Acid	Peak Height (mm)
L-Methionine	179
L-Norvaline	144
L-Citrulline	82
L-Isoleucine (10 µl of ≈ 0.3 M)	60

0.5% of these L-amino acids and others were prepared and analyzed in the identical manner explained above. The results found are shown in Table XIII.

TABLE XIII

SIGNAL HEIGHTS OF 0.5% SOLUTIONS OF SOME COMMON L-AMINO ACIDS

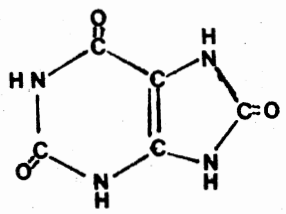
Amino Acid	Peak Height (mm)
L-Methionine	33
L-Norvaline	58
L-Citrulline	30
L-Isoleucine	30
L-Tryptophane	33
L-Leucine	56
L-Norleucine	48
L-Phenylanine	30
L-Histidine	13

It can easily be seen then that unsegmented closely continuous-flow system can be used to determine the concentrations of the common amino acids. Both the soluble and the immobilized form of the rather cheap D-amino acid oxidase can be utilized in the closed-flow system of the analysis for the D-amino acids. However, the L-amino acid oxidase can be utilized better in the immobilized form since it is not economically feasible to use it in the soluble form. Immobilization of the amino acid oxidases on CPG-3000 rather than CPG-00500C gives better flow and avoids the problem of packing since the particle sizes of the CPG-3000 are larger than those of the CPG-00500C. Optimization of amino acid analysis by this technique can be done by effecting immobilization on CPG-3000 that is already attached to the inside surface of a glass coil as in the case of uricase. As can be seen in Table XI, the optimum pH for the analysis of individual amino acids can differ from one to another and thus the right choice can be made.

CHAPTER VI

THE USE OF IMMOBILIZED URICASE IN UNSEGMENTED CLOSED-LOOP CONTINUOUS-FLOW ANALYSES OF URIC ACID

The structure of uric acid is



Uric acid is metabolically derived from purines from the diet and from those synthesized in the body. About one sixth of the 1.1 g of uric acid in an average healthy adult human being is present in the blood. Uric acid is the end product of purine metabolism in man, in the anthropoid ape, and in the Dalmatian dog (194).

Determination of serum uric acid levels is most helpful in the diagnosis of gout, in which serum levels are frequently between 6.5 and 10 mg/100 mL. Serum uric acid levels are also increased by increased metabolism of nucleoproteins, as in leukemia, polycythemia, and familial idiopathic hyperuricemia. Uric acid levels are also elevated in decreased renal function.

The normal ranges of uric acid concentration in some human fluids are indicated in Table XIV.

TABLE XIV

NORMAL RANGES OF URIC ACID IN HUMAN BEINGS

Serum	Concentration (mg %)
Males	3.8 - 7.1
Females	2.6 - 5.6
Children	2.0 - 5.5
Urine	**Concentration**
Adults	250 - 750 mg/24 hours

Source: (195).

Uric acid levels in urine generaly reflect endogeneous nucleic acid breakdown and the amount of dietary purines.

In 1912 Folin and Denis (196) introduced a method for determining uric acid based on the formation of a blue color from its reduction of phosphotungstic acid in alkaline solution. The uric acid was first isolated as the insoluble silver salt from blood. This method has been modified in subsequent years and is presently one of the two in use in clinical laboratories for the determination of blood uric acid. In this reaction uric acid is oxidized to allantoin and carbon dioxide:

$$\text{Uric Acid} + O_2 \rightarrow \text{Allantoin} + CO_2$$

One modification of this phosphotungstic acid method has been auotmated with success (197).

The second method used was introduced by Praetorius (198, 199) and

involves the oxidation of uric acid in the presence of uricase. Uric acid has an absorption peak in the region between 290 and 293 nm whereas the products of uric acid oxidation (i.e., allantoin and CO_2) have no absorption in this wavelength range. The decrease in absorbance in a fixed time is proportional to the uric acid originally present in the sample.

The specificity of this reaction has led others to modify it into a colorimetric determination by the addition of a second reaction (200) (using peroxidase to catalyze the oxidation of leuco dyes by hydrogen peroxide). With the possible exception of method using uricase, most have approximately the same inadequacies: nonspecificity of the color reaction, lack of constant proportionality between the concentration of uric acid and optical density (most methods are linear up to 6 mg % only), high serum blanks, turbidity, potential danger from cyanide formation, and loss of uric acid during the preparation of protein-free filtrate.

Uricase is, however, rather costly (Table XV) and has low stability in soluble form, and thus clinical analysis of uric acid is presently expensive. The cost of this method can be reduced by using closed-flow systems in which the enzyme can be reused many times before it is discarded. The greatest decrease in analysis cost, however, can come by immobilizing the enzyme. The experimental work carried out to accomplish these purposes is described in this chapter.

Experimental Procedure

The method of immobilizing uricase attempted in this work and on different supports can be discussed under three headings, depending on

the physical make of the support:

(a) On sponge or jelly-like materials that can be contained within a chamber

--- on phenoxyacetylcellulose sponge

--- on polyglutaraldehyde gel

(b) On particles of variable sizes that can be used in packed columns and coils

--- on CPG/CDI-glycophase

--- on silica gel

--- on controlled pore glass (CPG)

(c) On open tubes

--- on etched glass and whiskers

TABLE XV

RELATIVE COST OF SOME COMMON ENZYMES

Enzyme	Cost Per U, ¢	Relative Cost
Urease	0.2	1.0
Glucose Oxidase	0.3	1.5
Creatine Kinase	0.7	3.5
Lactate Dehydrogenase (LDH)	1	5.0
LDH-Isoenzymes	18	90
Pyruvate Kinase	0.3	1.5
Uricase	150	750
Alcohol Dehydrogenase	0.06	0.03
Hydroxy Steroid Dehydrogenases		
Hydroxy Steroid	90	450
Hydroxy Steroid	150	750

The uric acid assay was done according to the Sigma Manual (Sigma Technical Bulletin No. 292-UV). The procedure is as follows:

(1) A mixture of 0.2 mL of uric acid containing sample, 1 mL of glycine buffer (0.1 M, pH 9.4), and 6.0 mL of water were pipetted into a test tube.

(2) Into each of 2 test tubes 3.0 mL of same mixture were pipetted. One was labeled as "blank" and the other as "test".

(3) a. A 0.05-mL portion of distilled water was added to the "blank" with mixing.

b. A 0.05-mL portion of uricase enzyme (0.2 - 0.4 units/mL) was pipetted into "test" with mixing.

c. Both test tubes were allowed to stand at room temperature for approximately 15 minutes.

(4) The solutions were then transferred to quartz cuvets and the absorbance measured at 292 nm. The absorbance obtained by reading the "blank" (which was inserted in the reference position) vs the "test" was used in the calculations to derive the uric acid concentration.

(a) Immobilized Uricase for use in chambers, i.e., on phenoxyacetyl cellulose sponge and polyglutaraldehyde.

The method of immobilization of uricase on the phenoxyacetylcellulose sponges was the same as in the glucose oxidase method discussed in Chapter IV. The preparation of the phenoxyacetyl cellulose was explained in Chapter III. Immobilization of uricase on polyglutaraldehyde was carried in the following way:

Uricase (200 mg, 20 units/g) was dissolved in 5 mL of borate buffer ($Na_2B_4O_7 \cdot 10H_2O$, $(NH_4)_2SO_4$, 0.1 M, pH 9.2). To this 6 drops of glutaraldehyde were added and the mixture stirred. The solution was then

frozen in a dry ice-acetone (1:1) coolant, and left in a refrigerator for a day.

The relative activities of the uricase immobilized on phenoxyacetylcellulose and polyglutarldehyde were measured. For comparison, a native uricase (≈ 0.3 units) was tested too.

Long term stability studies of uricase immobilized on phenoxyacetylcellulose were performed. For this purpose one set of sample was stored in the refrigerator at 4°C while the other set was kept at room temperature.

To ascertain whether this immobilization of uricase on phenoxyacetylcellulose sponges is fast or slow, pieces of the sponges were immersed in 2 mL of solution containing 0.4 units of uricase and kept there for 10 minutes in one case and 140 minutes in the second case.

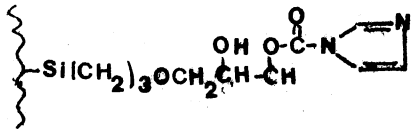
As for glucose oxidase, an experiment was carried out to find out if description of and then reimmobilization of uricase on phenoxyacetylcellulose could be done.

Immobilized uricase on phenoxyacetylcellulose sponge was incorporated into the flow system and the uric acid assay performed by injecting 100 mg% uric acid.

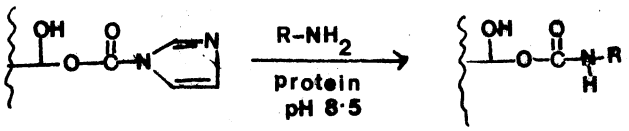
(b) Immobilized Uricase for use in packed columns or coils.

(i) Immobilization of uricase on CPG/CDI- activated glycophase was effected. CDI-Glycophase is simply controlled-pore glass that has been modified (Pierce Chemical Co.) specifically for use with enzymes and affinity ligands. It is commercially produced by treating CPG (250 Å pore, 120/200 mesh) in such a way that it covalently incorporates a hydrophilic nonionic carbohydrate type layer and thus loses the ionic and denaturing properties of glass. The resulting "glycerol" coating,

hence "Glycophase", is then activated by using the carbonyldiimidazole, CDI, method of Bethell, et al. (185) to achieve CDI-activated Glycophase.



For uricase immobilization 2 g of CDI-Glycophase was added to a solution containing 2 mg uricase (≈ 8 units) in 15 mL of 0.1 M borate buffer, pH 8.5, containing 1.5 mg benzamidine. This was agitated in the shaker for 24 hours. It was then filtered, and the solid washed with buffer, water, 0.2% NaCl, and finally water. The reaction is expected to be:



About 1 g of this material was then loaded into a glass coil (see Figure 22) and used in the usual way to determine uric acid in the closed-flow system.

(ii) Silica Gel

The glutaraldehyde method of immobilization is one of the simplest and commonest techniques used and has been applied for immobilizing uricase on silica gel, CPG, and glass whiskers. In all three supports the immobilization technique is the same and has been discussed before in Chapter III.

A column of uricase immobilized on the modified silica gel was prepared and used to determine uric acid; it was positioned just in front

of the electrode system of the closed-flow system.

(iii) Controlled-Pore Glass

The controlled-pore glass (CPG) samples used for immobilization of uricase were of two types: CPG 00500C and CPG-3000; the main difference between the two was the particle diameter, the former being smaller.

Long term stability studies of the immobilized uricase on the two supports was studied. The native enzyme was similarly treated. Since flow characteristics through the CPG-3000 were found to be better than with CPG-00500C, the remaining part of the experiments was carried out with the CPG-3000 immobilized uricase.

The apparent K_m of uricase and CPG-3000 immobilized uricase was determined from the initial rates of reaction. Uric acid assay was done according to Sigma's method. The uric acid solutions prepared for this purpose were 0.01, 0.02, 0.05, and 0.08 mM, and the changes in absorbance resulting in the first minute (i.e., 10 sec to 70 sec after the reaction was started) were measured. The [S] at 1/2 V_{max} in the plot of V vs [S] gave the apparent K_m.

Uric acid was determined by positioning the slender chamber (see Figure 22) or glass coil filled with CPG-immobilized uricase just at the inlet of the electrode system in the unsegmented closed-flow system. Similarly uric acid determination was performed with immobilized uricase on the modified CPG which was fused to the inside of a glass coil.

Every enzyme has a pH at which reaction of substrate proceeds at a maximum rate. The optimum pH for an enzyme can change on immobilization. This was studied for the immobilized enzymes under discussion.

Once the instrument was optimized (optimum flow of buffer and needed amplification setup for signals), both standards and serum samples

were analyzed for uric acid. Furthermore, the effect of flow rate and variation of sample size on peak height was studied. Experiments were also done to see if high concentrations of allantoin, the product of the uric acid oxidation, could produce an interference [significant change in the signal (peak height)].

(c) Immobilized Uricase for open tube.

In the initial studies the inside surface of the glass coil was roughened by heating. As suggested by Corning (201), the glass coil was heated at 700 ^{o}C for 7 days. Phase separation is expected to occur in this treatment. The coil was cooled and treated with hot 3 N hydrochloric acid solution. The etched surface was then treated in the same way as the CPG was treated when uricase was immobilized on it.

The technique of growing whiskers developed from these initial studies was that of Onuska et al. (186). For this purpose concentrated hydrochloric acid was added to wise-bore Pyrex glass tubing made into a coil. The coil was then sealed and heated overnight at 80 ^{o}C. When cool, the coil was opened, the acid emptied and washed sequentially with distilled water, acetone, and diethyl ether, and then blown dry with nitrogen. A saturated solution ($\approx$ 5% W/V) of ammonium hydrogen fluoride in methanol was used to fill the coil and allowed to stand there for 1 hour. The solvent was then removed by a uniform nitrogen flow which was continued until a milky film remained on the walls. The coil was then sealed and heated in a muffle furnace at 450 ^{o}C for 3 hours. After cooling the coil was opened in a hood, washed with methanol several times, and then blown dry with nitrogen. At this stage the whiskers produced were ready for treatments leading to immobilizing the enzyme as in the case of CPG. In one case the alkylation (with 3-aminopropyl

triethoxysilane) of the silica moity was done after heating at 500 °C for 6 hours (just as in the CPG case), while in the others alkylation was carried without such heating.

The uricase immobilized on whiskers was then used to analyze uric acid standard solutions of concentrations found in normal human blood serum.

Results and Discussion

(a) The relative activities of the immobilized uricase on the different supports can be stated in terms of absorbance values found at 292 nm: the higher the absorbance the higher the activity of the enzyme. For enzyme on about 0.1 g of phenoxyacetylcellulose, polyglutaraldehyde, silica gel, and the different CPGs and for native uricase (≈ 0.3 units/mL), the values found are shown in Table XVI. As the values found indicate, the controlled-pore glasses immobilize uricase to a larger extent in comparison with the other supports.

TABLE XVI

RELATIVE ACTIVITY OF IMMOBILIZED URICASE ON DIFFERENT SUPPORTS

Support	Absorbance at 292 nm
None (native uricase)	0.12
Phenoxyacetylcellulose (0.03 g)	0.235
Polyglutaraldehyde (0.5 g)	0.04
Silica gel	0.19
CPG 00500C	0.76
CPG-3000	1.29
Enzorb-A	0.37

Long term stability studies of soluble uricase (Figure 25) indicate that it loses almost all its activity in about 12 days. When immobilized, however, most of the activity is retained for rather long time, sometimes for as long as 10 months (or maybe more--further studies were not carried out).

Table XVII shows the results of storing uricase immobilized on phenoxyacetylcellulose sponge at room temperature versus at 4°C. In both cases most of the activity was still retained after 17 days. Furthermore Table XVII shows that the amount of uricase used for immobilization does not affect the amount that is immobilized; i.e., when the quantity required to saturate the support is available, no more of enzyme will be attached. Thus only a very small quantity of uricase is needed for immobilization.

TABLE XVII

LONG TERM STABILITY OF URICASE IMMOBILIZED ON PHENOXYACETYLCELLULOSE SPONGE

	Amount of Phenoxyacetylcellulose (g)	Amount of Uricase (Units)	Absorbance				
			Days				
			1	2	3	4	5
a.	0.075	0.04	0.65	0.52	0.50	0.52	0.49
b.	0.086	0.2	0.68	0.59	0.52	0.55	0.46
c.	0.086	0.4	0.67	0.50	0.53	0.43	0.45
d.	0.070	1.0	0.61	0.47	0.39	0.36	0.37
e.	0.082	0.04	0.73	0.61	0.46	0.52	0.51
f.	0.063	0.2	0.62	0.48	0.43	0.43	0.41
g.	0.074	0.4	0.76	0.53	0.41	0.48	0.46
h.	0.083	1.0	0.84	0.66	0.54	0.52	0.48

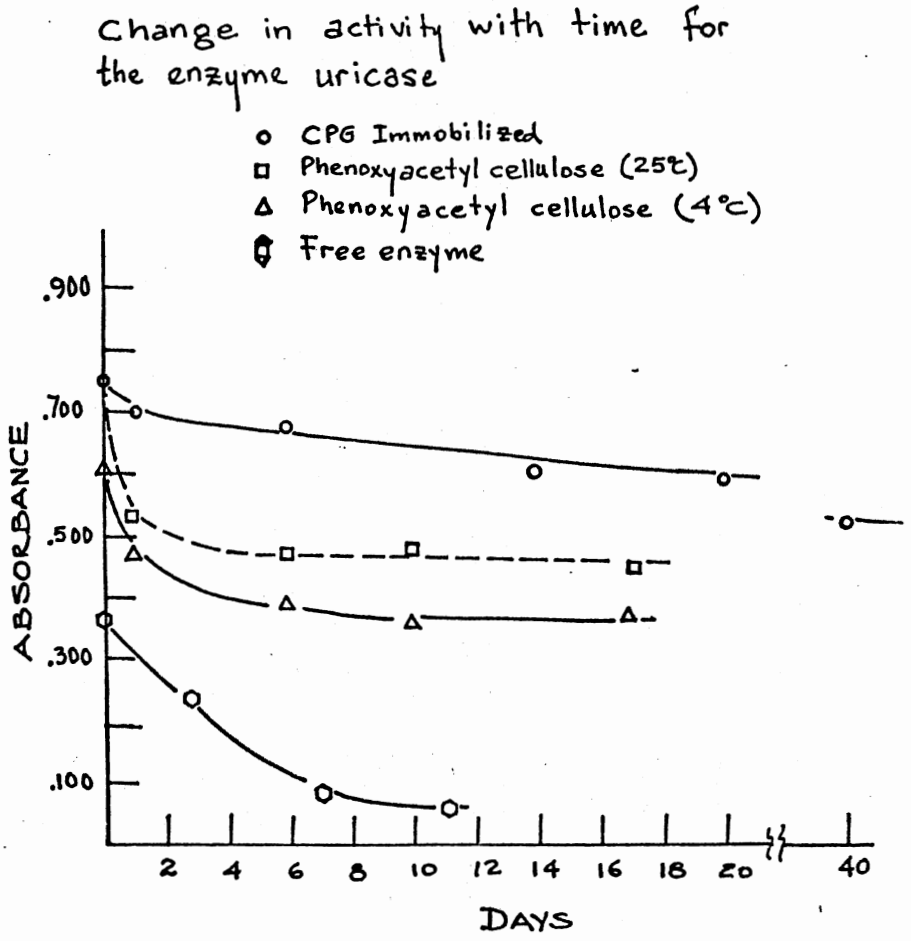


Figure 25. Long Term Stability of Uricase

The rate of immobilization of uricase on the phenoxyacetylcellulose sponges was found to be fast. Two sponges of about the same weight were wetted and kept in 2 mL beakers that contained 2 mL of 0.4 units of uricase each. One sponge was kept in the beaker for 10 minutes and the other one for 40 minutes. The activity of immobilized uricase was found to be the same in both cases (absorbance of 0.65), indicating that a short period of time, 10 minutes or less, is enough to adsorb uricase on the support.

Uricase immobilized on phenoxyacetylcellulose sponge was found to be easily desorbed with the surfactant Triton X-100. Reimmobilization of uricase on the washed phenoxyacetylcellulose sponge was also found to be possible. (0.13 g of phenoxyacetylcellulose sponge gave an absorbance of 0.76 on initial immobilization and 0.69 on reimmobilization.)

When the uricase immobilized on phenoxyacetylcellulose sponge was incorporated into the flow system to analyze uric acid, small and broad peaks resulted in the opposite direction of the expected one. Injection of water gave the same result. This could be due to the fact that the injected sample was getting in contact with the surface of the sponge which had a very small activity of the enzyme.

(bi) The back pressure that resulted when uricase immobilized on CDI-activated glycophase loaded into a glass coil and used in the flow system was too much to permit use of this enzyme for our purposes. In such cases where back pressure became significant, the flow of fluid continuously decreased (thus lowering the baseline) and sometimes interrupted the flow. Small signals could still be detected, however, when uric acid was injected.

(bii) The CPG and glass whiskers were found to have high immobil-

ization capacity. The flow characteristics in all three cases are good, the order of excellence being glass whiskers, silica gel, and the CPG (CPG-3000).

The flow characteristics of the buffer solution through the column made of uricase immobilized on silica gel in the closed-flow system was good. No problem of back pressure arose from packing of the silica gel in the tubing. However, when a high concentration of uric acid (100 mg%) was injected, the signal that resulted (peak height) was too small to serve our purpose.

(biii) Controlled pore glass is used as a support material after the proper modification in liquid chromatography because it possesses high mechanical strength which makes it operable at the high pressure used in the instrument. Furthermore, controlled-pore glass can be obtained in different pore surface areas and particle sizes, it is easy to handle, it is thermally stable, and it does not swell with addition of solvents.

The controlled-pore glasses used for immobilization of uricase were CPG-00500C and CPG-3000.

Uricase retains most of its activity for many months (> 9 months) when fixed on CPG-3000. This means that the immobilized uricase can be used for the determination of uric acid for quite a long period of time. Figure 25 shows the extent of decrease of activity of the CPG-3000-immobilized uricase with time.

The apparent K_m values of native uricase and CPG-immobilized uricase were found to be 1.4×10^{-5} M and 6.0×10^{-6} M, respectively (see Figure 26). The literature value for native uricase using tris buffer pH of 8.5 has been reported as 1.7×10^{-5} M (202). This decrease in K_m

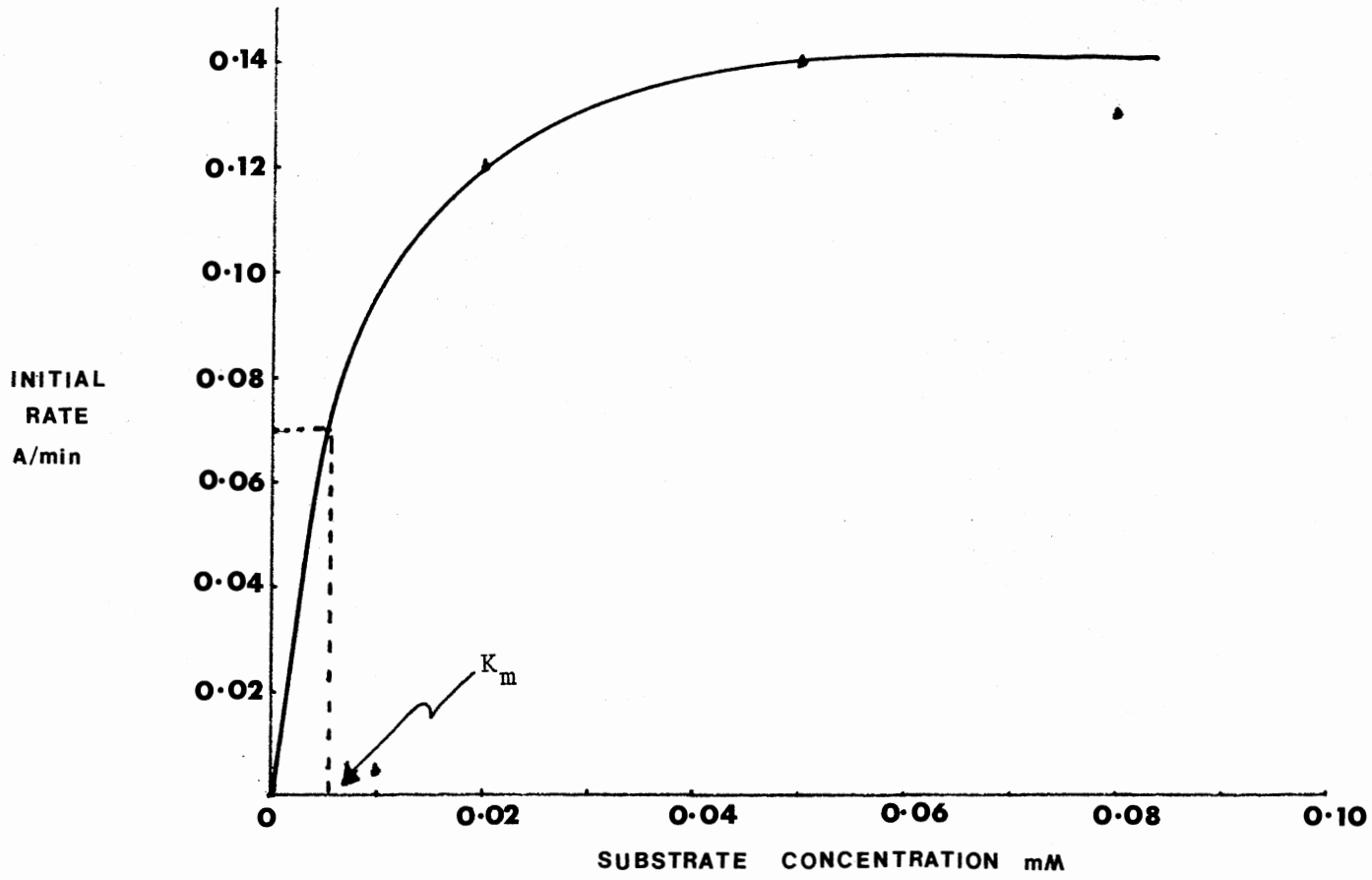


Figure 26. Apparent K_m Value Determination for Native and Immobilized Uricase

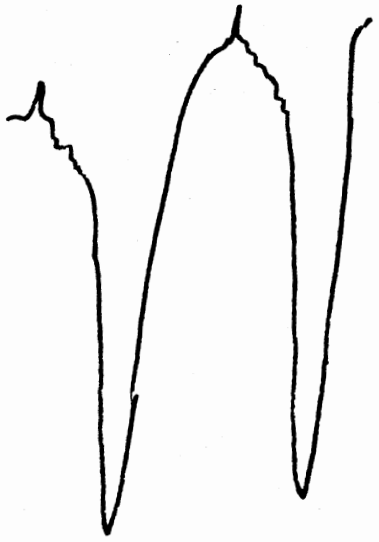
when an enzyme is immobilized does not conform with the behavior of most other enzymes, although there are a few exceptions, of which uricase seems to be a case.

The fourfold lower value of this constant for immobilized preparations indicates that saturation (when the rate becomes independent of uric acid concentration) occurs at a smaller concentration of uric acid, thus limiting the concentration range amenable to determination. On the other hand, initial rates are larger with smaller values for the constant, allowing one to obtain larger signals and detect them at earlier stages of the reaction.

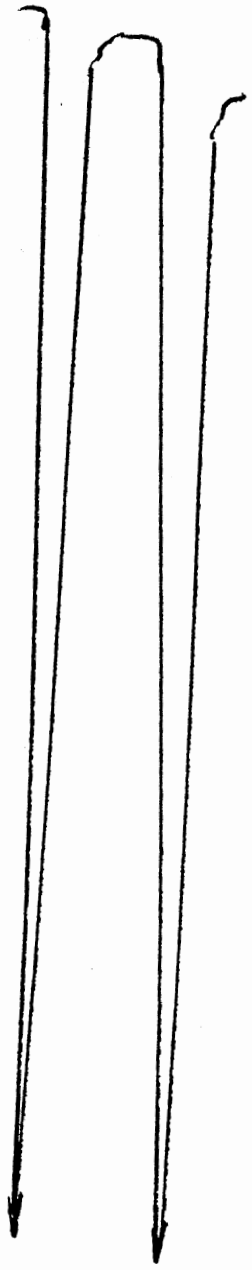
As shown in Figure 27, the signals resulting when uric acid (30 µL of 100 mg%) is injected into the flowing stream of the closed-flow system are better when the immobilized uricase is filled in the glass coil rather than in the slender chamber. The amounts of immobilized uricase used for both cases were comparable. As can be seen from the figure, not only the sizes but also the shapes of the peaks were better when the glass coil was used. It has been found better still to use the immobilized uricase on CPG fused to the inside surface of glass coil.

The flow rate in this particular case was found to be excellent since the CPG does not become compacted or compressed by the pump. The fusion of the CPG-3000 in the inside surface of glass coil was carried out by first filling a glass tubing which was closed on one end with the CPG-3000 and then making a coil out of it using the high temperatures of the oxy-acetylene flame.

As Figure 28 shows, the optimum pH of uricase under flow conditions was found to be 9.5. The literature value is found to be 9.0 (203).



CPG. IMMOBILIZED URICASE
FILLED IN THE SLENDER CHAMBER.



CPG. IMMOBILIZED URICASE
FILLED INSIDE GLASS COIL

Figure 27. Typical Signals That Result When Immobilized Uricase was Used in the Chamber and Coil Configurations

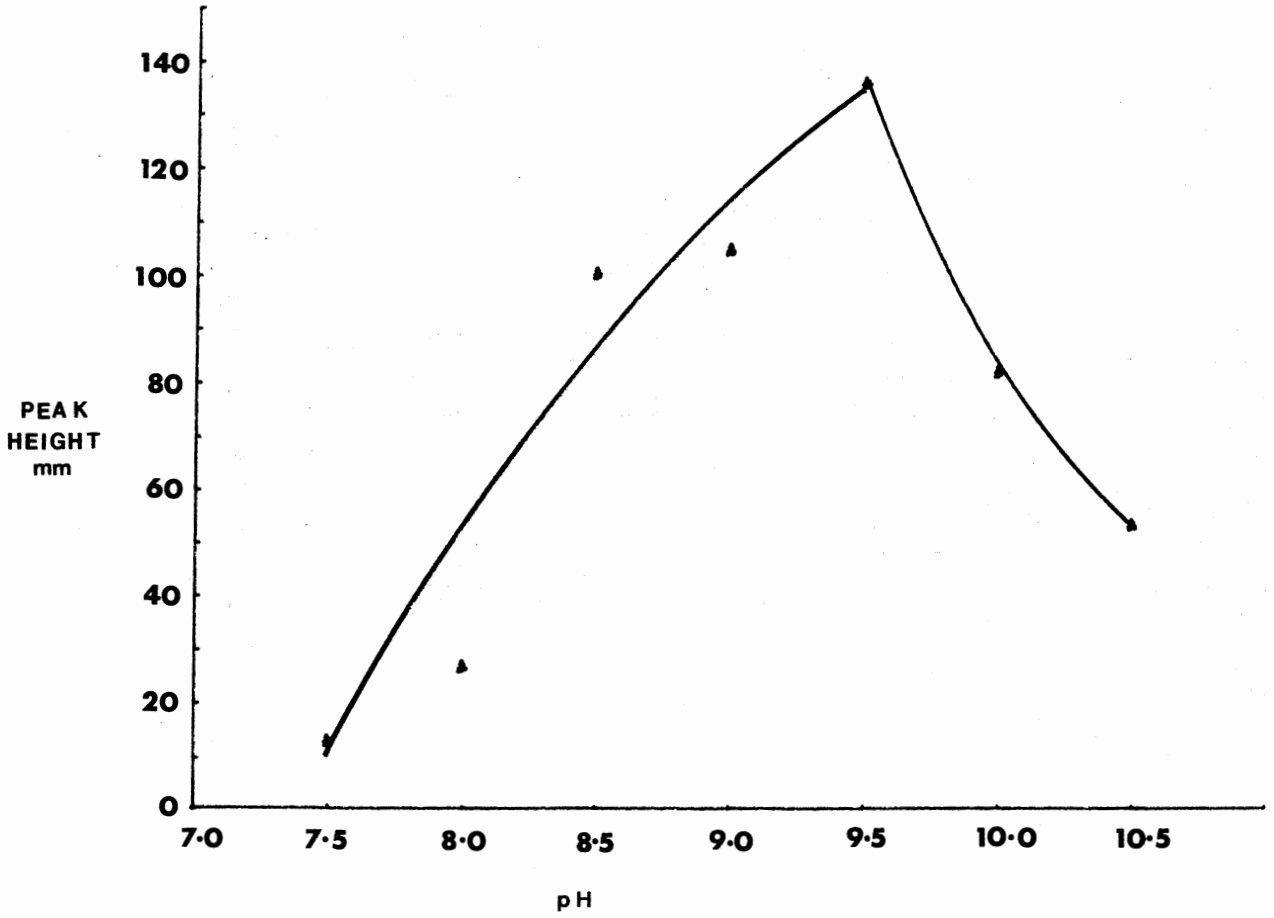


Figure 28. pH Effect on Activity of Immobilized Uricase

Return of signal to baseline was unaffected by pH changes since it mainly depends on flow rate of the flowing stream. The borate buffer used for the analysis of uric acid had a pH of 9.5 throughout all the work reported here.

Enzymes are increased in activity by increase in temperature up to a certain level, and beyond that they start denaturing. In analytical methods the optimum temperature can be used in order to increase the sensitivity. A temperature study carried on uricase under flow conditions as shown in Figure 29 gives 30 ^{o}C as the optimum temperature. Even though temperature is found to be critical in the evaluation of some kinetic parameters, the optimum temperature value for uricase is not recorded in the literature.

One of the objectives of these experiments was to be able to assay uric acid in blood serums. The normal range of uric acid in blood serum of human beings has been reported earlier (page 128).

An acceptable method should be able to determine uric acid concentration in the range of about 1 mg% to 10 mg%. In addition, the calibration curve within this range should be linear. Figure 30 shows a typical calibration curve obtained with uric acid standards from "Calibrate" serum calibration references (General Diagnostics). The "Calibrate" serum standards are commercially produced samples that simulate human serum. This is well within the range required for uric acid determination in blood.

Typical signal profiles for standards that fall within this range are shown in Figure 31.

In present day clinical analysis using automated methods, one requirement is to attain high frequency of sample analysis. In order to

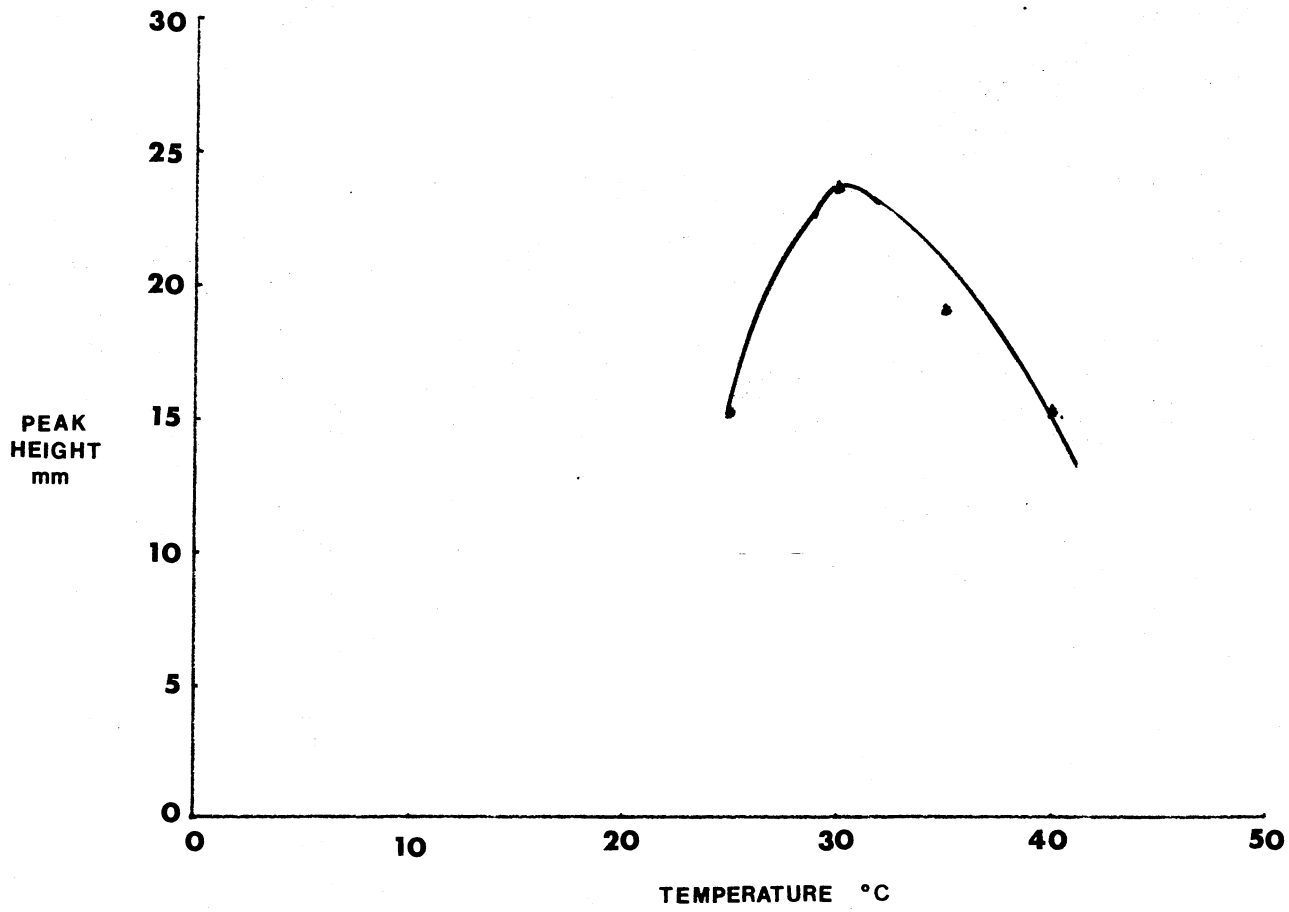


Figure 29. Temperature Effect on Activity of Immobilized Uricase

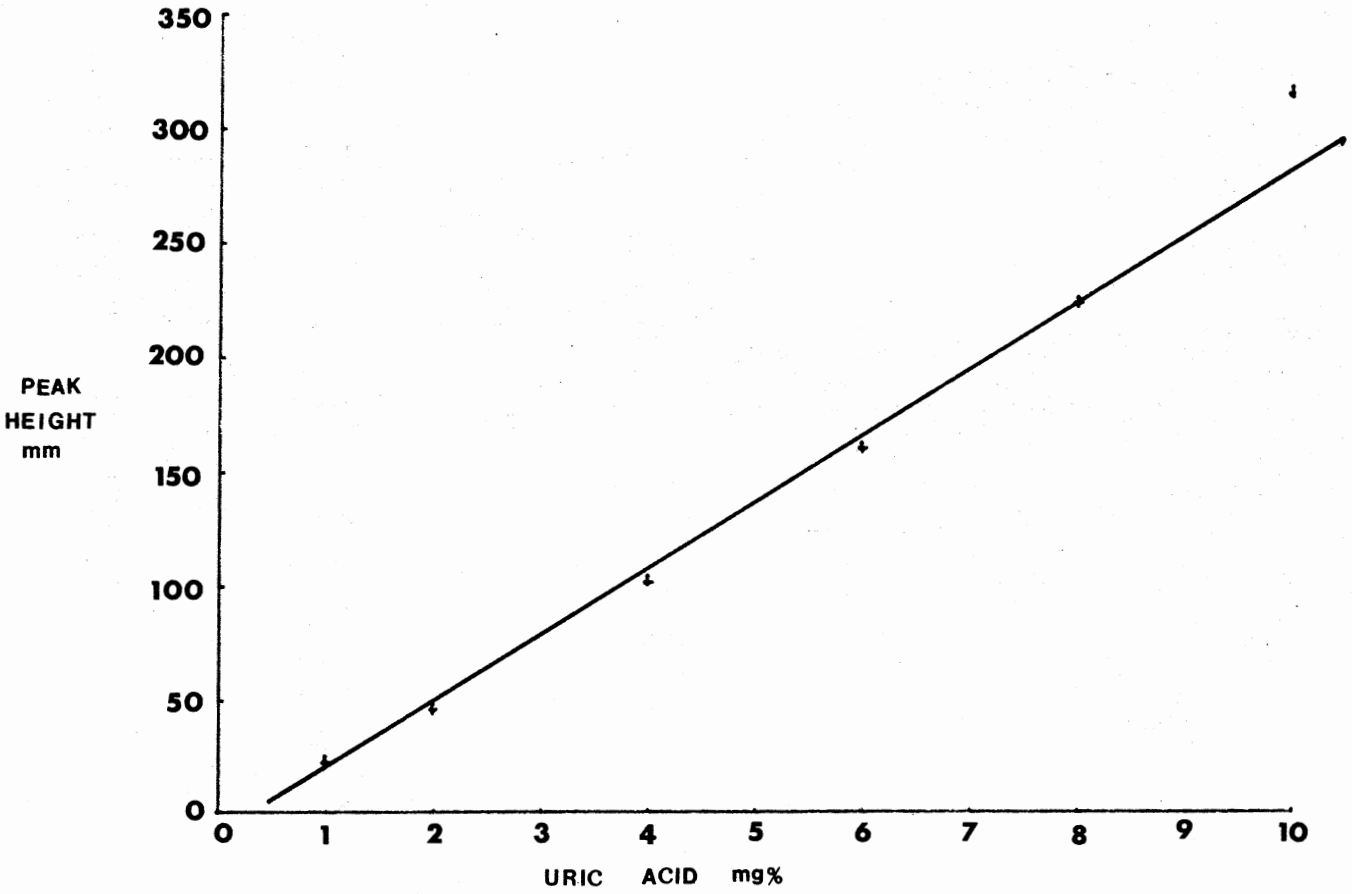


Figure 30. Calibration Curve for Uric Acid Determination

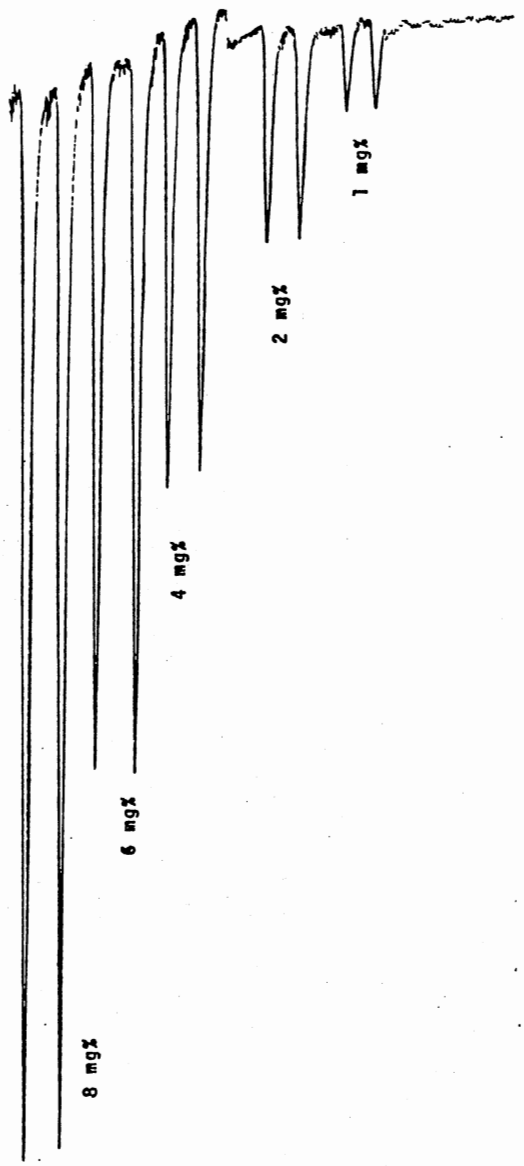


Figure 31. Typical Signal Profiles in Uric Acid Determinations

study the sampling frequency, the effect of flow rate on peak height and on the rate of return to baseline (t_{bas}) was performed. The result is shown in Figure 32. A flow rate of 7 mL/min is recommended to realize good sensitivity and maximum affordable determination rate (about 100 determinations/hour under these conditions).

The typical sample size used for determination was 80 µl since it provides reasonable sensitivity without much sacrifice in the time for return to baseline that dictates the determination rate. Table XVIII shows data obtained with different sizes of loops used with the rotary valve. If the sample size is increased to a significatnly high value, dispersion can occur and increase the time for return to baseline, which further implies that the sampling frequency becomes small.

Reproducibility of the results was tested by repetitive injection of 80 µl samples of 4 and 9 mg% uric acid solutions at a frequency of 110 samples/hour. The relative standard deviations for sets of 30 injections were 4.17 and 2.68, respectively.

TABLE XVIII

EFFECT OF SAMPLE SIZE ON URIC ACID SIGNAL PEAK HEIGHTS

Sample Size (L)	Peak Height (mm)	Return to Baseline (min)
30	64.3	0.7
35	70.2	0.7
40	84.6	0.7
60	89.7	0.8
80	101.6	1.0
100	121.6	1.2

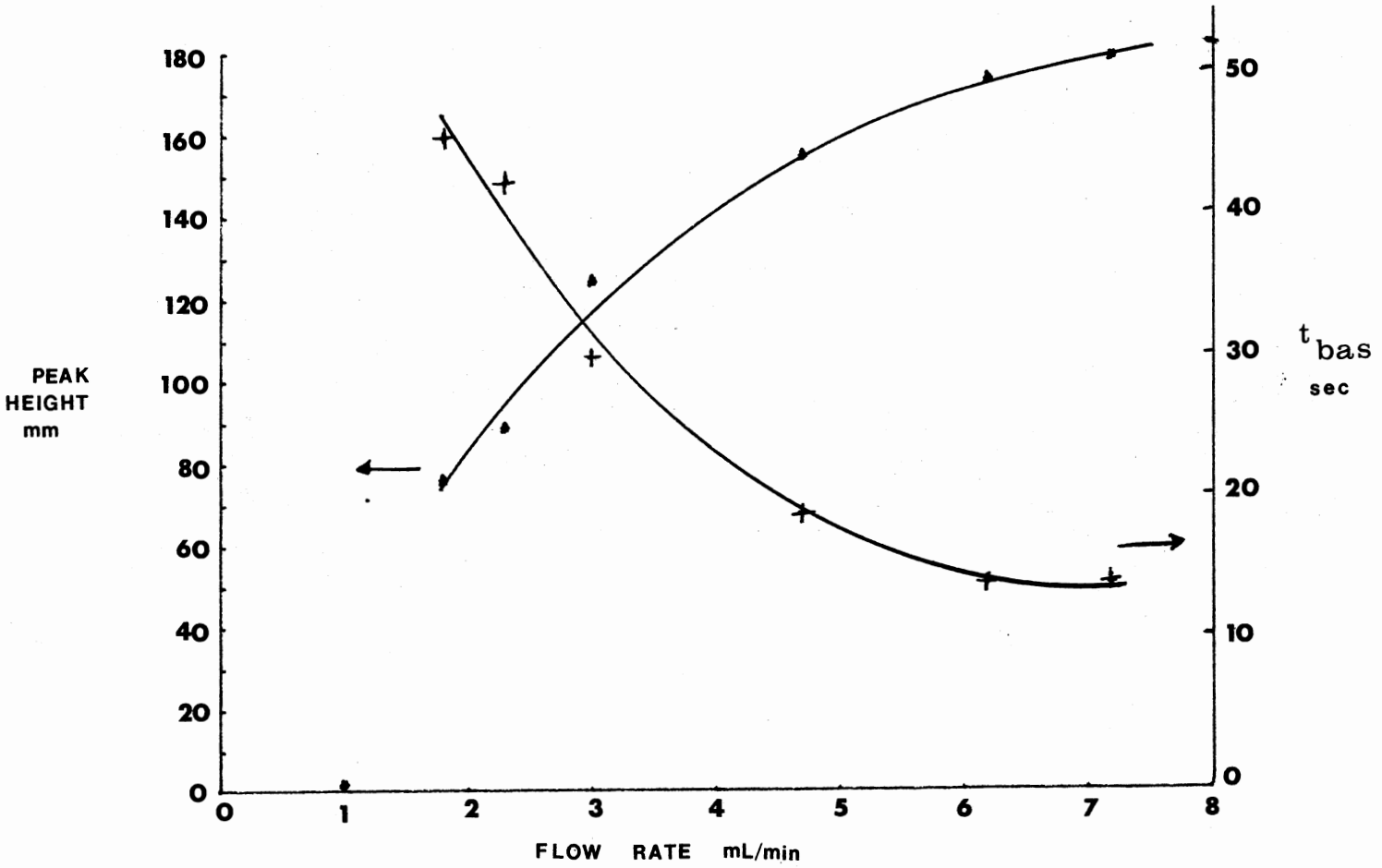


Figure 32. Effect of Flow Rate on Peak Height and on Return to Baseline

Serum uric acid analysis was carried out with the closed-flow system and the results compared with values found with the Centrifichem (Union Carbide) and the SMA 18/90 (Technicon Instrument Corp.). The Centrifichem uses uricase and measures the decrease in absorbance at 292 nm, while the SMA 18/90 utilizes the phosphotungstic acid method and measures the increase in absorbance due to tungsten blue at 710 nm. Figures 33 and 34 graphically illustrate the results for 33 and 22 samples, respectively. The Pearson correlation coefficients of results from the closed-flow systems and the Centrifichem and SMA methods were found to be $r = 0.92$ and $r = 0.98$, respectively. An $r > 0.95$ is considered to be satisfactory for most practical purposes. Accordingly the results found with our method and with the SMA 18/90 method are in good enough agreement to be used for experimental purposes. Correlation with the Centrifichem, however, was only fair.

High concentrations of allantoin (0.01 M) does not affect the signal height. For this 10 mg% uric acid in 0.01 M allantoin solution was used in one case (signal height 58 mm) and 10 mg% uric acid standard without allantoin was used for the second case (signal height 61 mm).

(c) Several techniques exist for modifying inner glass surfaces of capillary column and wide-bore tubings. Among these hydrogen fluoride has proven to be the most efficient roughening agent by formation of silica whiskers. These whiskers exhibit high mechanical strength and an increase in the glass surface area up to 1000 fold. The methods that use hydrogen fluoride (204, 205), however, have the disadvantage of not giving uniform whiskers and being very hazardous. The method used for our purposes was ammonium hydrogen fluoride. This was expected to overcome these problems.

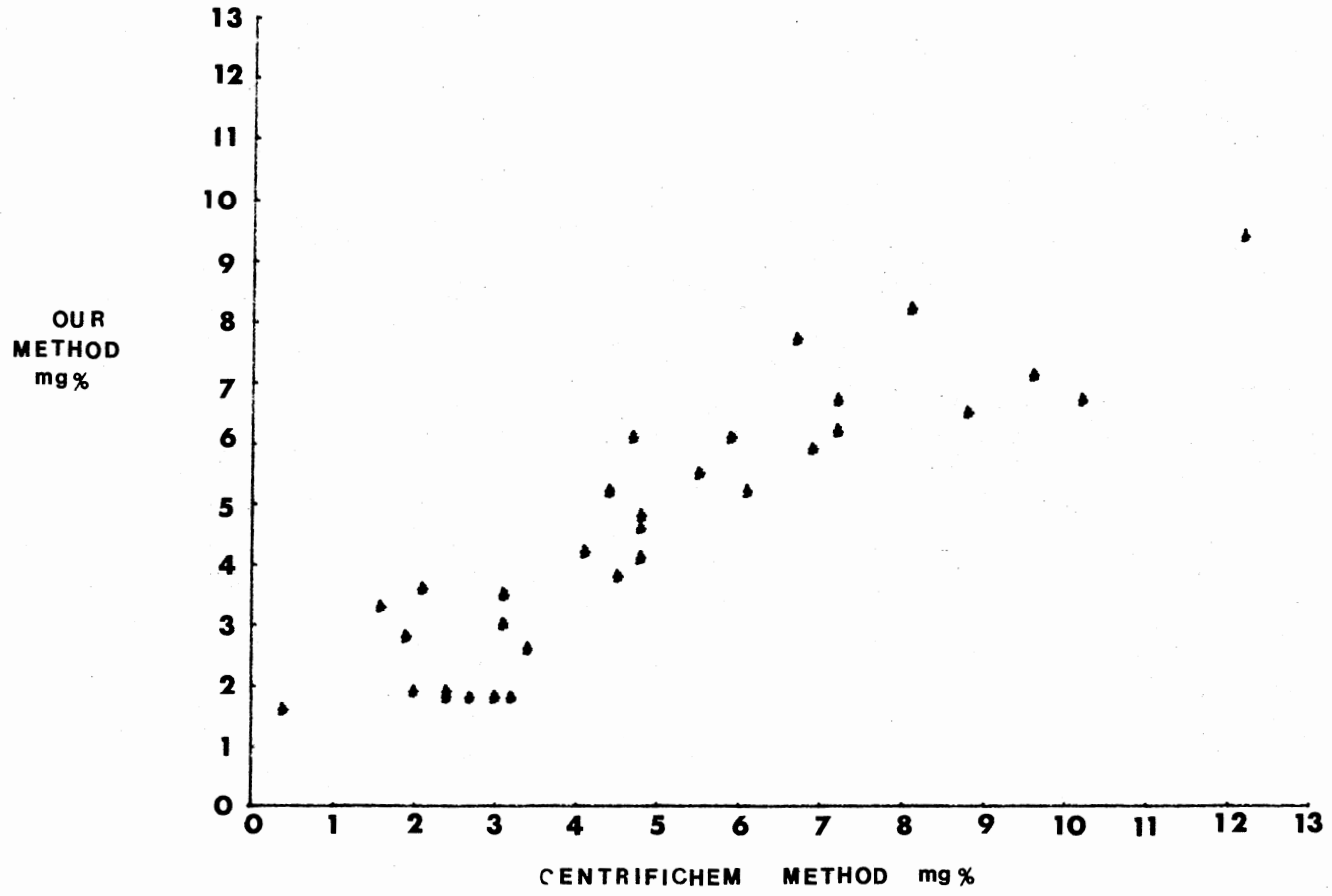


Figure 33. Comparison of Uric Acid Values Obtained by our Method and by the Centrifichem Method

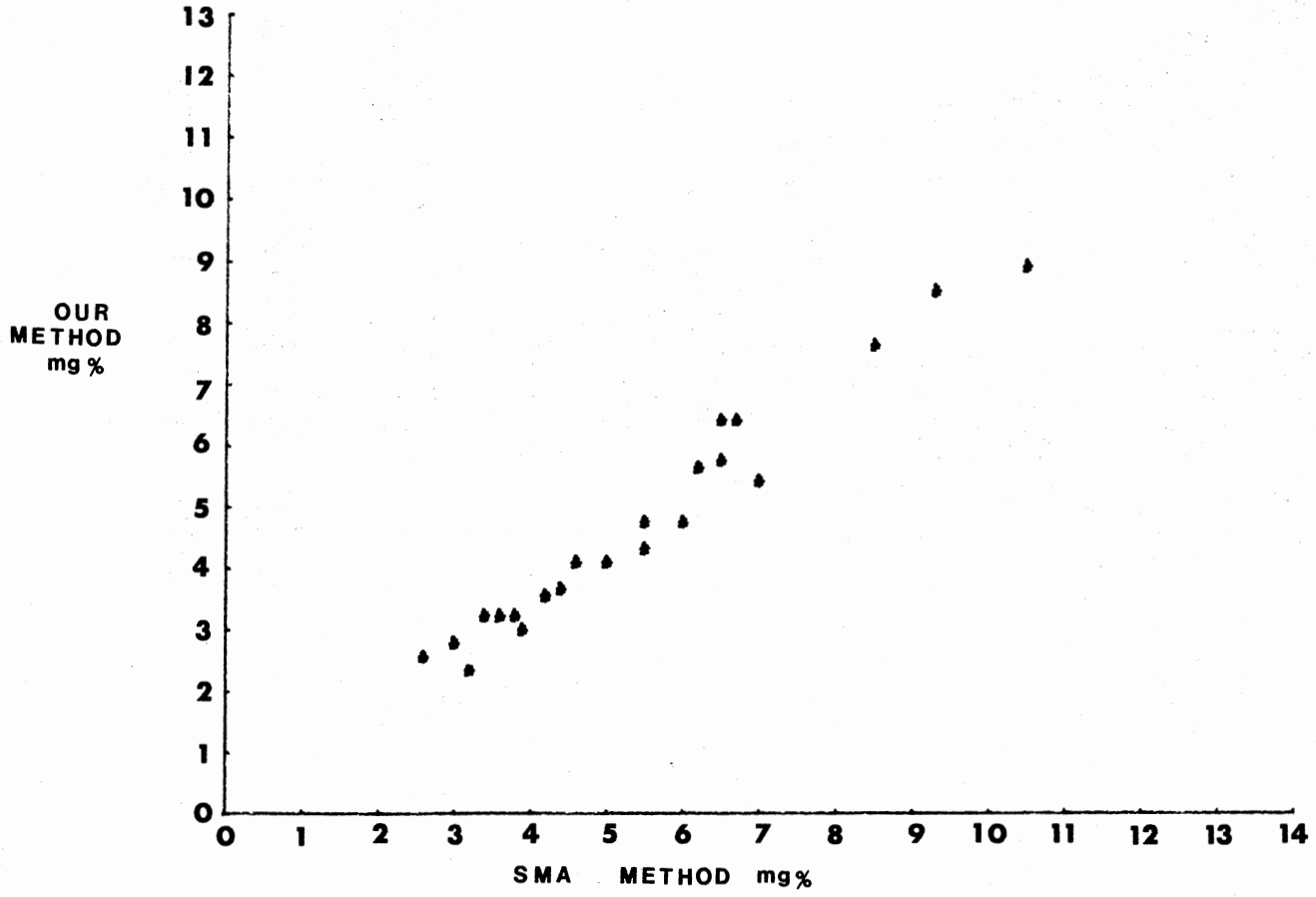


Figure 34. Comparison of Uric Acid Values Obtained by our Method and SMA 18/90 Method

The etching the inner surface of pyrex glass coil with hot 3 N HCl after heating at 700 °C for 7 days was not sufficient to permit immobilization of uricase. As can be seen from the electron microscopy scanning micrograph (Figure 35), the etching is not significant.

Better results were obtained when whisker growth was accomplished in a glass coil by the procedure of Onuska et al. (186) and then immobilization of uricase was carried out as for CPG, including heating of the whiskers to 500 °C for 6 hours.

It was found best, however, to use the developed whiskers to immobilize uricase without further heat treatment, i.e., by excluding the stage of heating to 500°C.

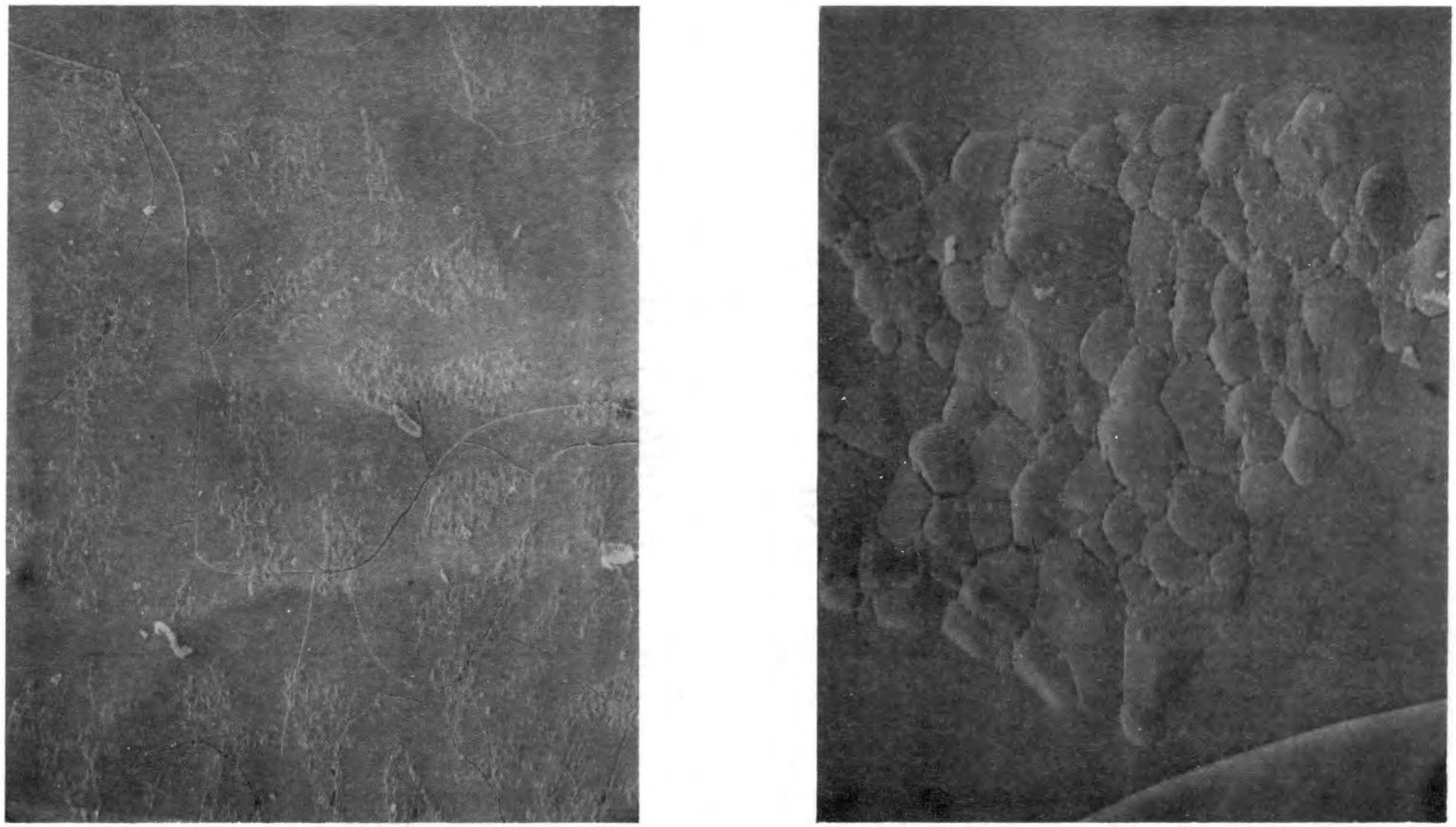
Table XIX shows the amount of uricase immobilized on the different types of etched glass surfaces including the whiskers.

TABLE XIX

RELATIVE ABILITY OF DIFFERENT TYPES OF ETCHED GLASSES FOR IMMOBILIZING URICASE

Procedure Followed for Etching	Absorbance
Hot 6 N HCl after heating at 700 °C	0.18
Ammonium hydrogen fluoride after heating at 700 °C	0.34
Ammonium hydrogen fluoride - heating excluded	0.89

Figure 36 and 37 show electron micrographs of the whiskers that gave best results. The whisker growth is quite uniform near the ends



160X

1400 X

Figure 35. Electron Scanning Micrograph Picture of the HCl Etched Glass Surface

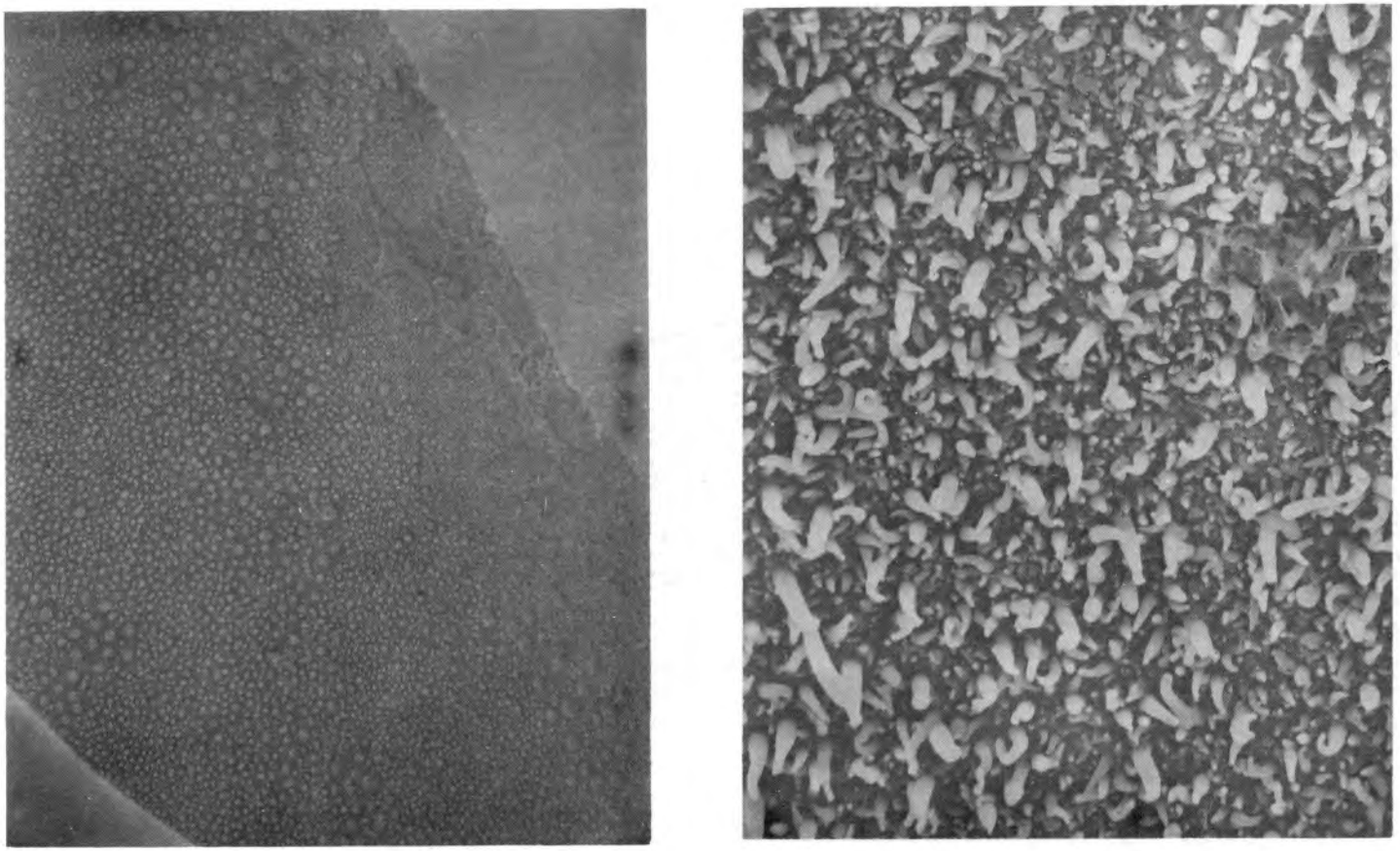
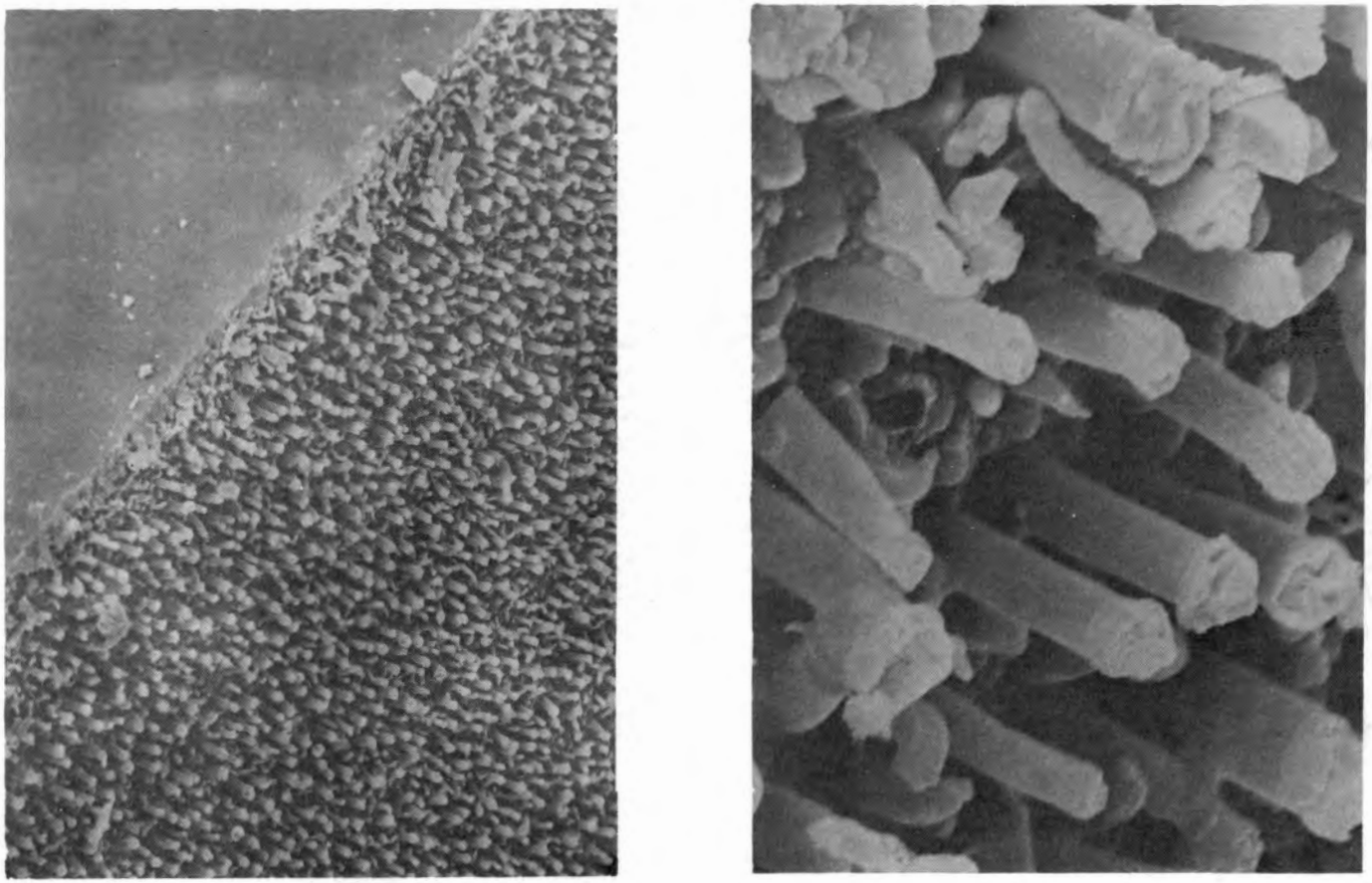


Figure 36. Electron Micrograph Pictures of Whiskers That Gave Best Results



near the ends of the coil

1400X

5000X

Figure 37. Electron Micrograph Pictures of Whiskers That Gave Best Results

of the coil, while in the middle part non-uniform distribution could be seen.

When uricase (1.05 units) immobilized on whiskers was incorporated into the flow system in order to determine uric acid samples, good results were found. Just as in the CPG-3000 case uric acid samples of 2 mg% and below could be easily determined. The peak height profiles that resulted in this experiment are seen in Figure 38.

A trial experiment carried out to determine uric acid in urine indicated that such high concentrations of uric acid could be analyzed without preliminary dilution (as required in all other techniques) of urine samples.

Summary

The principal objective of the project undertaken was to observe if some soluble and immobilized enzymes could be utilized in unsegmented continuous closed-flow systems. The enzymes used for these studies were oxidases, i.e., enzymes that catalyse reactions that involve a substrate and oxygen to form some oxidation products:

$$\text{Substrate} + O_2 \xrightarrow{\text{Enzyme}} \text{Products} + H_2O_2$$

The system of detection was amperometry in which the depletion of oxygen was monitored at -0.6 V Vs SCE.

Chapter IV describes the application of a closed-flow system for the determination of glucose oxidase. Removal of the injected glucose oxidase after detection was an inevitable necessity for initiating a successful method. Among the methods utilized to remove the injected glucose oxidase, which included heating to about 80 ^{o}C (to denature the

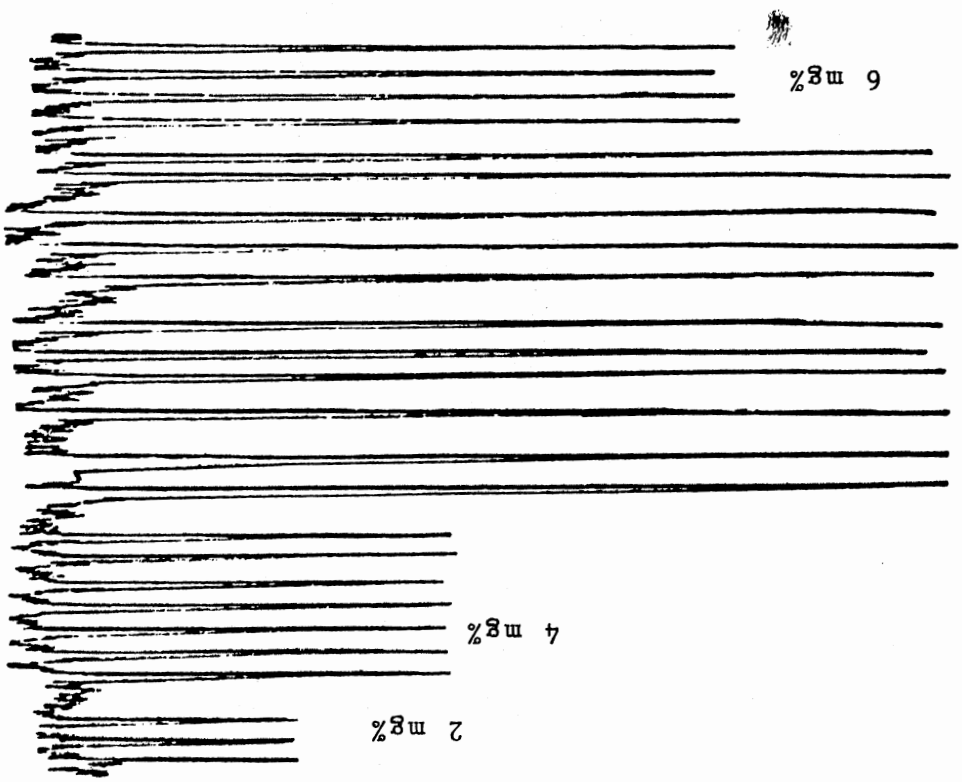


Figure 38. Peak Profiles of Uric Acid Samples Found Using Whiskers

glucose oxidase), adsorption on Enzorb-A, adsorption on filter papers (commercial hydrophobic filter paper and laboratory synthesized phenoxyacetylcellulose), and adsorption on phenoxyacetylcellulose sponges, the last one was found to give the best results. The phenoxyacetylcellulose sponges which were synthesized by reaction of phenoxyacetyl chloride with cellulose sponges were found to trap successfully (immobilize) the injected glucose oxidase at the normal flow rate of the buffer solution.

As described in Chapter V, different types of amino acids were analyzed in closed-flow systems by utilizing the relevant amino acid oxidases. The relatively expensive L-amino acid oxidase was used only in the immobilized form (on CPG-3000), while the D-amino acid oxidase was used in both soluble and immobilized forms (on CPG 00500C and CPG-3000). D-methionine, D-valine, D-alanine, D-phenylalanine, D-lysine, DL-isoleucine, and L-proline were tested with D-amino acid oxidase while L-methionine, L-norvaline, L-citrulline, L-isoleucine, L-tryptophane, L-leucine, L-norleucine, L-phenylalanine, and L-histidine were analyzed with L-amino acid oxidase. The sensitivity of the various amino acids were found to be different as was observed by the different signal heights on injection of the same volume of the same concentration of the amino acids and by the different values of saturation absorbances. Accordingly the D-amino acids with the exception of D-lysine gave significant signal heights (but of different values). The immobilized amino acid oxidases used for this purpose were filled in a slender chamber. Column packing difficulties and channeling problems gave erratic results when the CPG 00500C was used for immobilization. pH studies carried out for the D-amino acid oxidase indicated that larger peak heights resulted

with increase in pH of buffer.

Special attention was given to the study of uricase which in soluble form is too unstable and expensive for routine use in uric acid analysis.

As described in Chapter VI uricase was immobilized on different supports and tested for uric acid analysis. Uricase was immobilized on (a) sponges or jelly-like materials that can be contained within a chamber--phenoxyacetylcellulose sponge and polyglutaraldehyde gel, (b) particles of variable sizes that can be used in packed columns and coils--CPG/DCI-glycophase, silica gel and controlled pore glass, (c) on open tubes--etched glass and whiskers.

Uricase immobilized on controlled pore glass (CPG-3000) and on whiskers gave the best results. The CPG and whisker-immobilized uricase was found to have a long term stability of more than 9 months. Both standards and serum samples were analyzed for uric acid at the observed optimum pH of 9.4. Comparison of the values obtained by this technique with those found by already known standard automated techniques (the Centrifichem method of Union Carbide and SMA 18/90 of Technicon Corporation) gave satisfactory results.

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