NEW CONFIGURATION OF AN ANALYTICAL ENZYME REACTOR: DESIGN AND PERFORMANCE

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NEW CONFIGURATION OF AN ANALYTICAL ENZYME

REACTOR: DESIGN AND PERFORMANCE

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CHAPTER I

INTRODUCTION

The catalytic activity and high selectivity of enzymes have been known to chemists for some time. The use of enzymes as analytical reagents, however, has been limited because of instability, contamination of the reaction mixture, difficulties in purification, and the high cost of certain enzymes. The use of immobilized enzymes have eliminated, or at least minimized, most of the problems associated with soluble enzyme preparations. The first immobilization of an enzyme was reported by Nelson and Griffin half a century ago (1), but most of the studies on immobilized enzymes have been performed in the last two decades. Enzymes have been immobilized on different types of support materials by using a variety of techniques.

Immobilized enzyme preparations are used in analytical chemistry in the form of enzyme reactors. Enzyme reactors generally have two parts; (a) the enzyme for catalysis and (b) the sensor for detection and measurement of the species produced or consumed as a result of enzyme catalysis. These enzyme reactors combine the selectivity of enzymes with the sensitivity of some sensors.

A large number of enzyme reactors with different configurations have been developed using various enzymes, supports, and sensors for the measurement of many types of compounds. Most of these enzyme reactors suffer from diffusional problems which result in slow

response. This is undesirable for kinetic determinations where the rate of the enzyme catalyzed reaction must be measured. Slow response is also a problem for continuous flow systems where the injected sample comes into contact with the sensor for only a short period of time. A small signal and concomitant loss of sensitivity is therefore obtained.

In order to solve this problem, a new enzyme reactor has been developed and tested which reduces diffusional problems. As a model system urea amidohydrolase (EC 3.5.1.5), commonly known as urease, immobilized on nylon shavings was used in conjunction with a flat-bottom pH electrode and a small nylon chamber.

Although the main purpose of this work was to study the stability and efficiency of this new enzyme reactor, urease has been selected as the model enzyme because (a) a large number of urease reactors have been developed in the past years facilitating comparisons, and (b) the determination of urea is important from a clinical viewpoint since the concentration of urea in biological fluids can be used as an indicator of organ performance, in particular the liver and the kidneys (2). The detailed design and description of this new enzyme reactor, as well as its response under different experimental conditions is the central subject of this thesis.

CHAPTER II

CONFIGURATIONS OF ENZYME REACTORS

Enzyme reactors with different configurations have been reported. These include:

(1) Layer-type enzyme reactor.

(2) Disc-type enzyme reactor.

(3) Gas-sensing enzyme reactor.

(4) Air-gap enzyme reactor.

(5) Stirrer-type enzyme reactor.

(6) Column or packed-bed enzyme reactor.

(7) Tubular enzyme reactor.

The first four types of enzyme reactors are commonly known as "enzyme electrodes". In none of the above cases, however, is the electrode used to directly measure the activity of an enzyme. In all cases the enzyme catalyzes a reaction to produce or consume a species to which the associated sensor responds. For this reason, it is believed to be more appropriate to generically call these devices "enzyme reactors" rather than "enzyme electrodes". Therefore, in the rest of this thesis, they will be mentioned as such.

Descriptions of different forms of enzyme reactors as well as their response characteristics will be discussed in the rest of this chapter.

Layer-type Enzyme Reactor

In this type of enzyme reactor a thin layer of immobilized enzyme is attached to the active surface of an ion selective electrode (Figure 1).

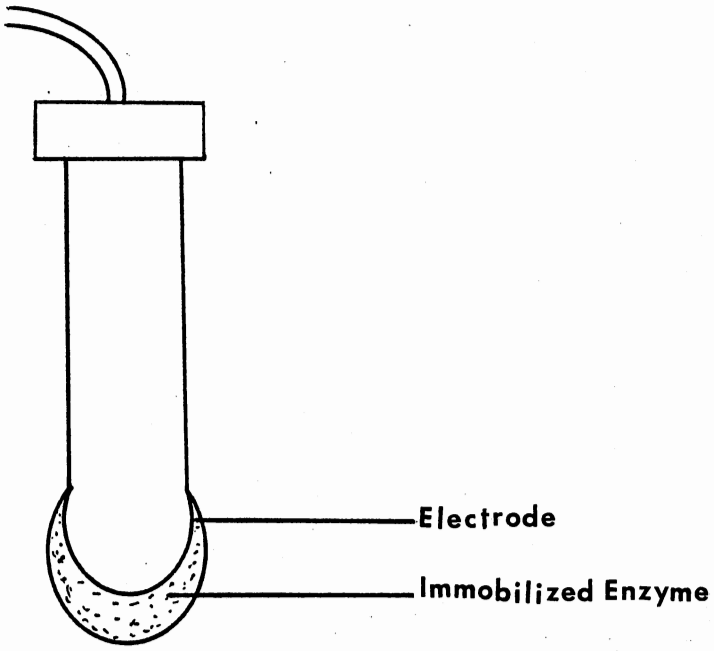


Figure 1. Layer-type Enzyme Reactor

When this enzyme reactor comes into contact with the substrate solution, the substrate diffuses into the enzyme layer where the enzyme catalyzes the reaction, and the attached electrode measures the ions produced or consumed.

A typical example of this type of enzyme reactor was first

provided by Guilbault and Montalvo (3). They used a monovalent cation electrode, the surface of which was covered by a layer (60-350 μm thick) of polyacrylamide gel-entrapped urease. No loss of enzyme activity was observed within 14 days and the lifetime of this urease reactor was extended to three weeks by placing a cellophane membrane over the polyacrylamide gel layer. A linear response was obtained within the range of 5×10^{-5}M to 1×10^{-3}M urea by plotting potential Vs. log [urea]. The slope of the linear portion of this calibration curve was 50 mV/decade of urea concentration. The response time of this reactor was mainly dependent on the thickness of the enzyme layer. For example, 98% of the steady-state response was obtained in 59 seconds with a 350 μm enzyme gel layer whereas about 26 seconds was required for the same response, when using 8.33×10^{-2}M urea solution. The rinsing time of the reactor after each determination also varied with gel thickness, as well as with urea concentration.

One disadvantage of using a monovalent cation electrode as part of the enzyme reactor is that other cations (most commonly Na^+ and K^+) interfere in the measurement of ammonium ion. In order to eliminate these ionic interferences, Guilbault and Hrabankova (4) prepared a similar urease reactor using polyacrylamide gel-entrapped urease. A given amount of ion-exchange resin was added to the sample solution and the response was measured after stirring the mixture. This reactor was proven to be useful and remained stable for about three weeks. Later, an ammonium ion selective electrode was used in place of the monovalent cation electrode in order to improve selectivity (5).

In 1973 Nilsson et al. (6) reported the determination of glucose,

urea, and penicillin by layer-type enzyme reactor. A pH electrode was used as the sensor. The enzyme reactors were constructed either by entrapping the enzyme within a polyacrylamide gel which surrounded the electrode (urease) or as a layer trapped within a cellophane membrane (glucose oxidase and penicillinase). The pH response was linear within the range of 5×10^{-5}M to 5×10^{-3}M urea. The response time of this reactor was about 5-7 minutes. The pH response to glucose was linear within the range of 1×10^{-3}M to 1×10^{-1}M glucose, and a linear pH response was obtained in the range of 1×10^{-3}M to 1×10^{-2}M for penicillin.

The advantages which are offered by the pH electrode when compared to the cation sensitive electrode, are (1) lack of interferences from other ions, and (2) versatility due to the fact that a large number of enzymatic reactions produce or consume protons.

Disc-type Enzyme Reactor

Cullen and coworkers reported a new configuration of an enzyme reactor in 1974 (7). They immobilized penicillinase on a fritted glass disc by adsorption. This glass disc was fixed to a flat surface pH electrode with the aid of a polyethylene sleeve. The reactor assembly has been depicted in Figure 2. This reactor showed a linear response in the concentration range of 1×10^{-5}M to 5×10^{-3}M penicillin. The slope of the calibration curve was 56-58 mV/decade change in penicillin concentration when the observed potential was plotted against log [penicillin]. The response time varied from 1 to 22 minutes depending on the thickness of the disc as well as type of penicillin used. This reactor was used

for six weeks after which replacement of the disc was necessary.

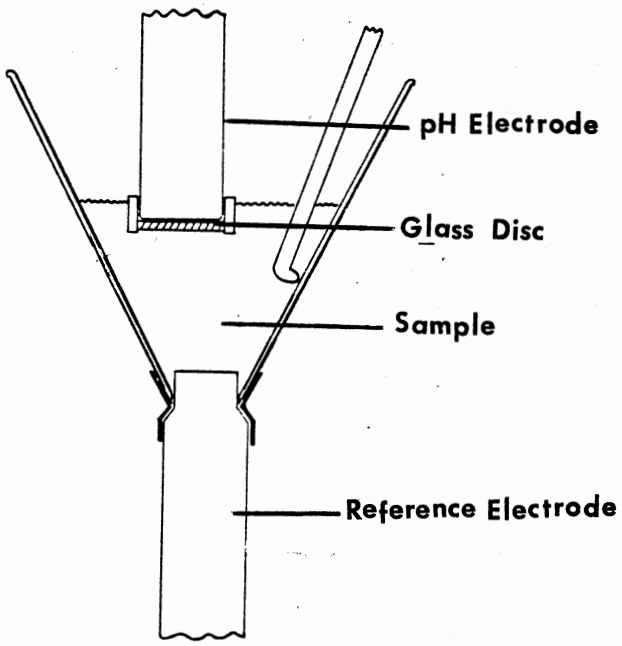


Figure 2. Disc-type Enzyme Reactor

Gas-sensing Enzyme Reactor

This type of enzyme reactor contains a gas permeable membrane placed between the enzyme layer and the surface of the sensing electrode (Figure 3). When the substrate solution comes into contact with this enzyme reactor, the enzyme catalyzes the reaction and produces at least one gaseous product which diffuses through the gas permeable membrane and comes into contact with an electrolyte solution which is adjacent to the electrode surface. In this electrolyte

solution a chemical reaction releases or consumes the chemical species to which the electrode responds. The response mechanism of the gas-sensing enzyme reactor can be explained in the following manner (8). Let us assume that CO_2 is formed as a result of enzyme catalysis, this CO_2 diffuses into the pores of the membrane and an equilibrium is established according to the following reaction:

$$CO_2\ (aq.) \rightleftharpoons CO_2\ (g)$$

External solution Membrane pores

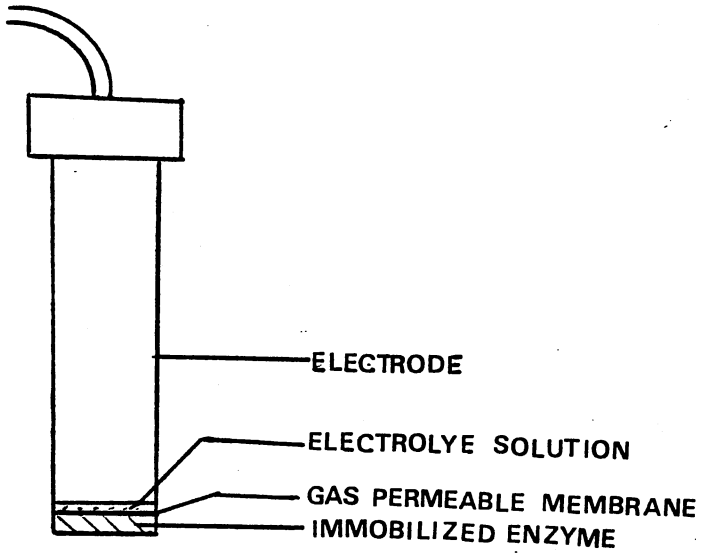


Figure 3. Gas-sensing Enzyme Reactor

Another equilibrium is also established between the CO_2 inside the pores and the attached electrolyte solution, e.g.,

$$CO_2\ (g) \rightleftharpoons CO_2\ (aq.)$$

Membrane pore Electrolyte solution

As a consequence of these two equilibrations, the external solution equilibrates with the electrolyte solution adjacent to the membrane. Here, another equilibrium is established that causes the pH change in the layer of electrolyte solution. The reaction is:

$$CO_2\ (aq.) + 2H_2O \rightleftharpoons HCO_3^- + H_3O^+$$

Electrolyte layer Electrolyte layer

The overall reaction of the above process is obtained by adding the three chemical equations to give

$$CO_2\ (aq.) + 2H_2O \rightleftharpoons HCO_3^- + H_3O^+$$

External solution Electrolyte solution

The equilibrium constant (K) is

$$K = \frac{[H_3O^+]_{elec.}\ [HCO_3^-]_{elec.}}{[(CO_2)aq.]_{ext.}}$$

If the concentration of the HCO_3^- in the electrolyte solution is made relatively high so that its concentration is not altered significantly by the CO_2 which diffuses into the electrolyte solution, then

$$\frac{[H_3O^+]_{elec.}}{[CO_2(aq.)]_{ext.}} = \frac{K}{[HCO_3^-]_{elec.}} = kg = \text{constant}$$

which may be rewritten as

$$a_1 = [H_3O^+]_{elec.} = kg[CO_2(aq.)]_{ext.} \qquad (1)$$

where a_1 is the hydrogen ion activity in the electrolyte layer.

The potential (E) of the electrode system is dependent upon a_1 as indicated by the following equation.

$$E = L + 0.0591 \log a_1 = L - 0.0591\ pH \qquad (2)$$

Here L is a constant consisting of potentials of the reference electrode, the junction potential across the salt bridge, the assymetry potential, and the logarithmic function of hydrogen ion activity of the internal solution of the electrode system. Substitution of equation (1) into equation (2) yields

$$E = L + 0.0591 \log (kg[CO_2(aq.)]_{ext.})$$

$$= L' + 0.0591 \log [CO_2(aq.)]_{ext.} \quad (3)$$

where $L' = L + 0.0591 \log kg$ = constant.

From equation (2) and (3), it is clear that the change in pH (or potential) is proportional to the amount of CO_2 formed, which in turn, is proportional to the substrate concentration.

Anfält et al. (9) succeeded in immobilizing urease onto a gas permeable membrane. An Orion 95-10 ammonium electrode was used and ammonium chloride solution was used as the internal filling solution. Urease was found to have an optimum activity at pH 6.5. By using pH 7-8, sufficient ammonia was produced to permit the urea measurement. This was in spite of the fact that about half of the enzyme activity was lost at this pH. The response time was about 2.0-2.5 minutes for urea concentrations of 1×10^{-3}M to 1×10^{-2}M and about 5 minutes for 1×10^{-4}M urea both at pH 7.4. A linear response was observed in the substrate range of 1×10^{-4}M to 5×10^{-2}M.

Another example of a gas-sensing enzyme reactor was given by Mascini and Guilbault in 1977 (10). A thin Teflon ammonia permeable membrane (10-35 μm thick) containing immobilized urease was used in conjunction with a Radiometer type E 5036/0 electrode. Ammonium chloride was used as the internal electrolyte solution. The response time of this reactor was about 3-4 minutes (at pH 8.5) with 1.0×10^{-4}M

to 1.0 x 10^{-3}M urea solutions. The slope of the calibration curve was 55 mV/decade of urea concentration in the range of 1.0 x 10^{-4}M to 1.0 x 10^{-3}M urea. This reactor was stable for about two months. Similar gas-sensing enzyme reactors with Teflon membranes were reported for the determination of phenylalanine and lysine (11).

Gas sensing enzyme reactors generally exhibit good selectivity and relatively fast response. However, some practical limitations are present in the use of these reactors due to several factors which include:

(1) The enzyme catalyzed reaction must produce at least one gaseous product.

(2) Diffusion across the membrane controls the response time.

(3) In order to obrain a fast response the membrane must be very thin, and, at the same time, retain a suitable mechanical strength in order to allow reliable mounting and durability. Often these requirements are mutually exclusive.

(4) Possible clogging of the pores of the membrane can often limit the usefulness of these sensors in samples such as biological fluids.

Air-gap Enzyme Reactor

This type of enzyme reactor consists of a gas-sensing electrode, a small reaction chamber, and an air-gap which separates the surface of the indicator electrode from the sample solution (Figure 4).

In 1974 Guilbault and Tarp (12) reported an air-gap enzyme reactor. Immobilized urease was placed at the bottom of the reaction

chamber and was covered with a piece of nylon net. The sample solution (urea in pH 8.5 buffer) was placed at the bottom of the chamber which was then sealed by inserting the electrode. A well-soaked polyurethane sponge provided a fresh film of electrolyte solution (e.g. ammonium chloride solution) to the electrode surface each time the electrode was placed in the electrode holder. Gaseous ammonia, generated at the bottom of the chamber, diffused to the electrode surface where it reacted with the electrolyte layer.

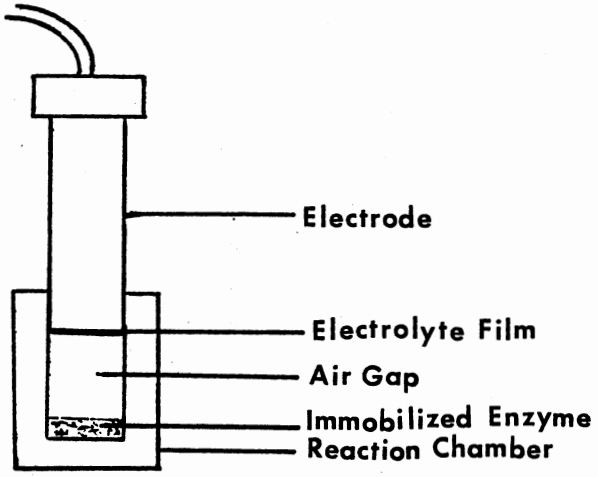


Figure 4. Air-gap Enzyme Reactor

The response time of this reactor was about 2.0 minutes at 1.0 x 10^{-1}M urea and about 3-4 minutes at lower urea concentrations. A linear response was obtained in the range of 5.0 x 10^{-5}M to 1.0 x 10^{-1}M urea at pH 8.5. This reactor showed no loss in activity during a

period of three weeks.

The use of an air-gap enzyme reactor offers several possible advantages over the three previously discussed types of enzyme reactors. Advantages include:

(1) As the electrode never comes in direct contact with the sample, the chance of sample contamination is reduced.

(2) Since the diffusion of gases in air is faster than in solid, aqueous, or even porous media, this type of reactor generally exhibits a faster response.

(3) The electrolyte layer can be easily renewed or changed and one electrode can be used for the determination of different gaseous products.

Stirrer-type Enzyme Reactor

In all the four previously discussed enzyme reactors, the immobilized enzyme preparation has been stationary, either in a layer attached to the electrochemical sensor or fixed at the bottom of the reaction chamber. In 1975 Guilbault and Stokbro (13) reported a new form of enzyme reactor, in which the enzyme was placed on the surface of a Teflon coated magnetic stirring bar.

The enzyme stirring bar was constructed by placing a layer of commercially immobilized urease on the magnetic stirring bars with the aid of a nylon net. The nylon net containing immobilized urease was held tightly on the magnetic bar with a thin rubber band. The immobilized enzyme layer covered about two-thirds of the stirring bar. The stirrer was placed at the bottom of a small chamber which contained the sample solution. A flat surface pH electrode, a thin layer of electro-

lyte solution, and an air-gap were used as the sensor (Figure 5). Although the enzyme stirring bar has been used with an air-gap electrode, it can, in principle, be used with other types of sensors. The calibration curve obtained using the above reactor was linear within the range of 1 x 10^{-2}M to 4 x $1-^{-4}$M urea (in pH 8.5 buffer solution) and had a slope of about 0.95 pH unit/decade of urea concentration. The response time of this reactor in blood sera was about 2.0 minutes and the reactor was stable for over 60 days.

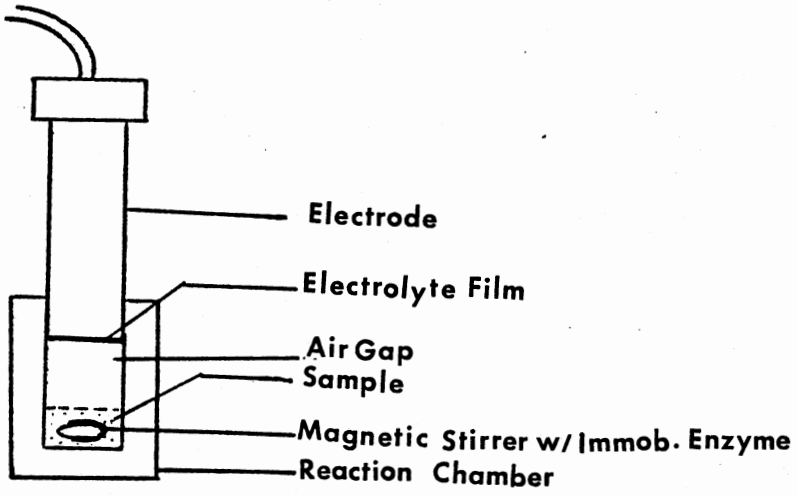


Figure 5. Stirrer-type Enzyme Reactor

Column or Packed Bed Enzyme Reactor

In column or packed bed enzyme reactors, the enzyme remains attached to a solid support, most commonly glass beads. These beads

(or other solid matrices) containing immobilized enzyme are then packed into a column. This type of enzyme reactor is generally used with continuous flow systems.

A typical example of a column or packed bed enzyme reactor was provided by Johansson and coworkers (14). The enzyme urease was covalently bound on controlled pore glass. A PVC tube (i.d. 3.4 mm, length 45 mm) was used as the enzyme reactor (Figure 6).

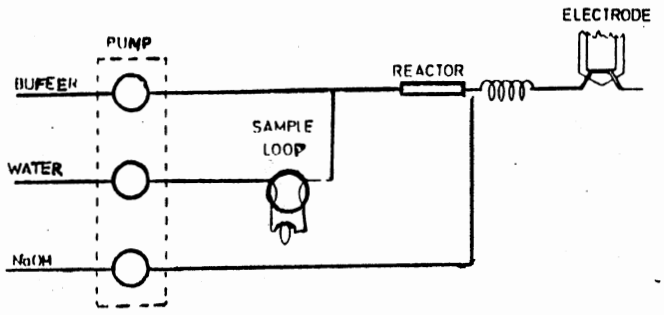


Figure 6. Packed Bed Enzyme Reaction Implemented as Part of a Flow System.

A sample of urea was injected into the sample loop in the by-pass position. In this position water was mixed with the buffer and the diluted buffer then passed through the enzyme reactor via a T-joint, where it was mixed with 0.5M NaOH solution. The alkaline solution then passed through the detector giving a baseline. When the flow was switched to pass through the sample loop, the sample was mixed with the buffer and passed over the enzyme reactor. The response of this packed

urease reactor was linear from 5 x 10^{-5}M to 3 x 10^{-2}M urea solution, and about 8 samples were analyzed per hour. The reactor showed no significant loss of activity after a period of one month. A similar packed bed enzyme reactor has been reported for the determination of L-amino acid (15).

A coiled glass tube, i.d. 1.5 cm, length 40 cm (12 turns, 4 mm o.d.) packed with glass beads containing immobilized uricase was used by Iob and Mottola (16) for the determination of uric acid by using a continuous flow system (Figure 7). The overall reaction is as follows:

$$\text{Urate} + \tfrac{1}{2}O_2 \xrightarrow{\text{uricase + catalase}} \text{allantoin} + CO_2$$

Decrease in oxygen level was monitored amperometrically.

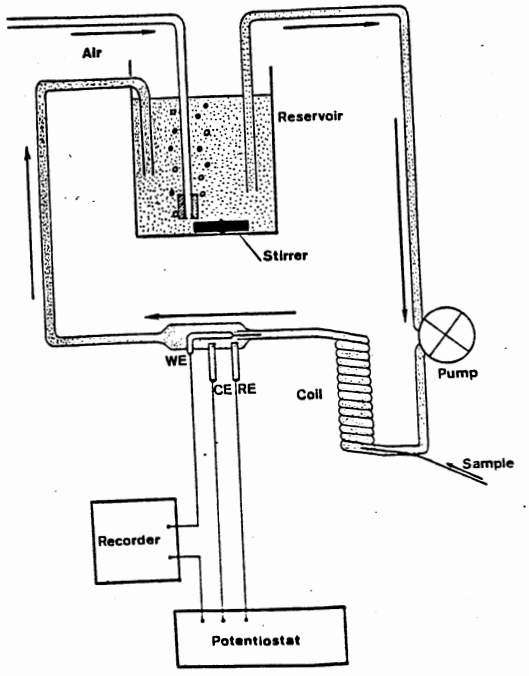


Figure 7. Packed Bed Enzyme Reaction Implemented in a Continuous Flow System.

A flow rate of 7 ml/minute was recommended in order to obtain good sensitivity and maximum determination rate (about 100 determinations per hour). The calibration curve had two linear portions from 0 to 10 and from 10 to 100 mg of urate/100 ml of solution. This reactor retained over 70% of its initial activity after 10 months.

Tubular Enzyme Reactor

In this type of enzyme reactor, the enzyme is immobilized directly onto the wall of a suitable tube. This type of enzyme reactor is generally used with continuous flow systems. A typical example of a tubular enzyme reactor was provided by Iob and Mottola (17). A 31-turn coiled glass (2 mm i.d., 9 mm o.d.) tube containing immobilized uricase was used for the determination of uric acid in human blood serum. The novelty of this reactor lies in the fact that, before immobilization of the enzyme, the walls of the reactor were treated with ammonium hydrogen fluoride at high temperature. This treatment resulted in the formation of filamentary protrusion (whiskers) on the wall surface. Formation of these whiskers greatly increased the surface area available for enzyme immobilization.

This tabular reactor was used with a continuous flow system (instrumental set-up is similar to that shown in Figure 7). This system allowed the processing of over 200 samples/hr at a gravitationally-controlling flow rate of 21 ml/minute in the concentration range of 1-100 mg of urate/100 ml of solution. The reaction retained about 70% of the initial activity after 10 months.

Another example of this type enzyme reactor was provided by Sundaram and Hornby (18). They immobilized urease on the wall of a 2 cm long

nylon tube by covalent attachment. This reactor retained about 60% of the initial activity after 70 days.

CHAPTER III

IMMOBILIZED UREASE AS AN ANALYTICAL REAGENT

Properties of Urease

Urease, the enzyme which catalyzes the hydrolysis of urea, was the first enzyme to be obtained in crystallized form. This was done by Sumner in 1926 who obtained the enzyme from Jack bean, Canavalia ensiformis (19). The molecular weight of urease is 473,000 (20). It is moderately soluble in water. Like all enzymes, urease is a protein and is composed of 51.6% C, 7.1% H, 16% N and 1.2% S (21). The mercapto groups of urease have been shown to be essential for catalytic activity (22), and each urease molecule contains 47 mercapto groups (23). Like other enzymes, it is also very sensitive to metal ions (24). Ethylenediamine tetra acetate (EDTA) is the reagent most widely used for the protection of the enzymes against inactivation by metal ions. Urease also becomes inactivated by other chemicals such as polyhydric phenols (25), ascorbic acid (26), and penicillin (27). The pH optimum for soluble urease has been determined by several workers who observed that the optimum pH for urease varied between 6.4-7.6 depending on the buffer (28, 29). It has been reported that the activity of urease was reversibly increased by moderate heating. However, a complete loss of enzyme activity was observed after heating at 90^{o}C for 5 minutes (30). Enzymes become

inactivated at high temperature due to denaturation of protein (31). Urease exhibits specificity for urea. It catalyzes the hydrolysis of urea as shown below.

$$\underset{\text{urea}}{\overset{\overset{O}{\|}}{H_2N-C-NH_2}} + 3H_2O \xrightarrow{\text{urease}} 2NH_3 + CO_2 + 2H_2O \rightleftharpoons 2NH_4^+ + HCO_3^- + OH^- \quad (4)$$

By examining the above reaction it is apparent that the substrate concentration (or enzyme activity) can be determined by simply monitoring the pH (or potential) change or by measuring the change due to the formation of the gaseous products CO_2 and NH_3.

The importance of determining urea has already been mentioned in Chapter I. Several methods have been proposed for direct estimation of urea, but almost all of these procedures suffer from some limitations which have restricted their applicability (32). Enzymatic methods for the determination of urea enjoy popularity primarily because of the ease, rapidity, and reliability with which urea can be determined.

Immobilization of the Enzyme Urease on Nylon Support

In most cases, urease, in immobilized form, has been used for the determination of urea. Immobilization refers to the localization or confinement of an enzyme by a suitable method which allows the enzyme to be physically separated from the substrate and the products.

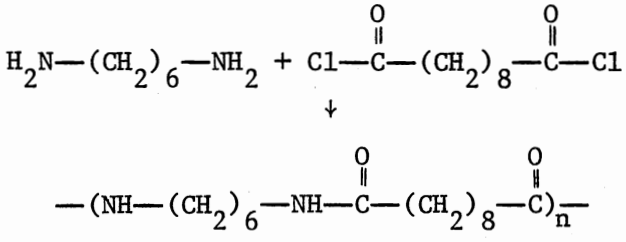
Various methods have been developed for immobilization of enzymes. These can be broadly classified into two groups (33). (a) Physical

methods which include any technique that involves localizing an enzyme in any manner which is not dependent on covalent bond formation between the enzyme and the support, e.g., adsorption, entrapment, or micro-encapsulation. (b) Chemical methods involve the formation of covalent bonds between some nonessential amino acid residues of the enzyme and some reactive groups of the support material.

Urease has been immobilized by both physical and chemical methods using a variety of supports (3, 18, 34). Immobilization of enzymes by covalent bonding to the nylon support will be the subject of discussion in the rest of this chapter.

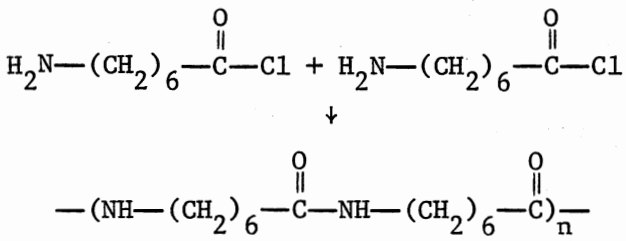
Nylons are a family of linear polymers consisting of repeating assemblies of methylene groups joining together by amide linkages. Several types of nylon are available commercially, differing only in the number of methylene groups in the repeating alkane segments. These different types of nylon are designated according to the number of carbon atoms in their component monomeric units, e.g.,

a)

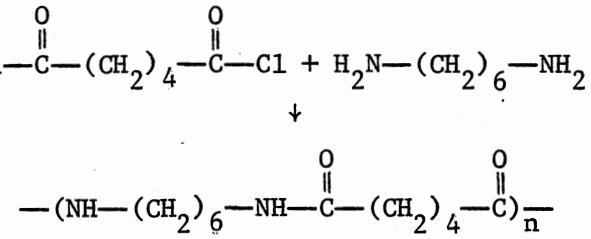


Nylon 6.10

b)



Nylon$_6$

c) 

Nylon 6.6

Nylons have been used as supports for several enzyme immobilization methods (35).

(1) Activation of nylon by peptide bond cleavage: In essence this method involves three separate steps. (i) The nylon is partially depolymerized by cleaving some of the amide linkages. (ii) Either the aliphatic amino group or the carboxyl group (which are both released in the first step) is activated. (iii) The enzyme is allowed to react with the activated nylon derived from the second step. The reactions which are involved in this method are shown in Figure 8. In most cases, the covalent bond formation between the enzymes and the nylon supports have been performed quite satisfactorily by coupling through the amino groups (Figure 8a) or the carboxyl groups (Figure 8b) of the support leaving the ionized carboxyl or amino groups free on the surface respectively (36, 37). However, it has been reported that residual ionized carboxyl groups on the surface are intolerable for certain enzymes (38). This problem has been eliminated by breaking the peptide bond using N,N-dimethyl-1,3-propanediamine which liberates the free amino group and at the same time, amidate the free carboxyl groups (Figure 8c).

(2) Covalent binding of enzymes to nylon by a method involving N-alkylation: This method is based on (i) hydrolysis of nylon to generate free amino and carboxyl groups and (ii) resealing the free

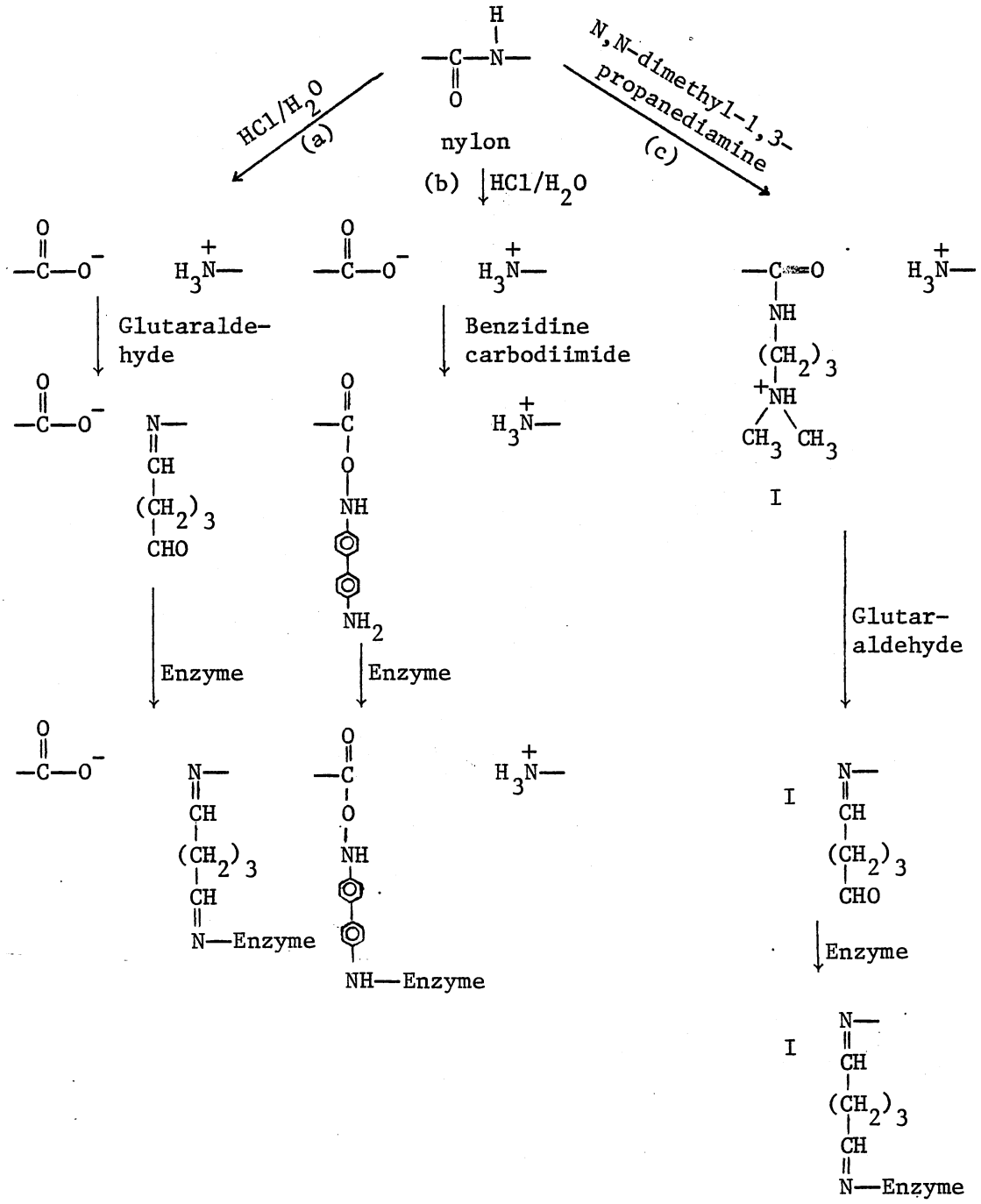


Figure 8. Covalent Binding of Enzyme on Nylon Support by Cleavage of Peptide Bonds.

amino and carboxyl groups by a four-component condensation reaction which involves the neighboring carboxyl and amino groups on the nylon, an aldehyde, and an isocyanide. In such a reaction (Figure 9), the carboxyl and amino components (R_1 and R_2) combine to form an N-substituted amide, the aldehyde and isocyanide components (R_3 and R_4) appearing as the side chain on the amide nitrogen. This procedure utilized acetaldehyde and 1,6-diisocyanohexane and polyisonitrile-nylon was obtained (Figure 10).

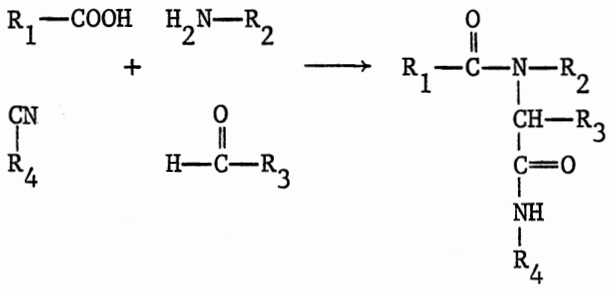


Figure 9. Four Component Condensation Reaction Used for Enzyme Immobilization.

Enzymes can be bound to polyisonitrile-nylon through their amino groups by four component reactions in the presence of acetaldehyde and acetate. Conversely, enzymes can be bound through their carboxyl groups in the presence of acetaldehyde and an amine.

(3) Covalent binding of enzymes to nylon by methods involving O-alkylation of the nylon: All the previously discussed methods involve the partial depolymerization of the nylon in order to generate

the reactive groups necessary for enzyme coupling. However, cleavage of the amide linkage also impairs the mechanical strength of the support. Enzyme immobilization by O-alkylation of the nylon generates sufficient reactive centers without necessitating any depolymerization of the support. The treatment of nylon with an alkylating agent, such as dimethyl sulfate for instance, results in the O-alkylation of some of the peptide bonds of the support. The imidate salt of nylon thus formed can be used directly for enzyme coupling (Figure 11a) or can be converted to hydrazide- or amide-substituted nylon (Figure 11b, 11c).

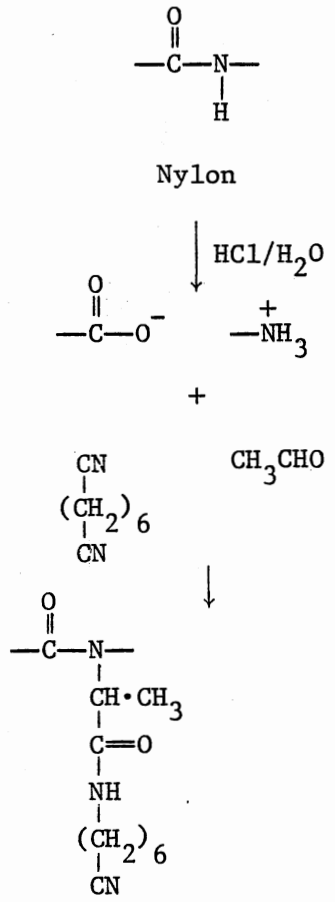


Figure 10. Synthesis of Polynitrile-nylon.

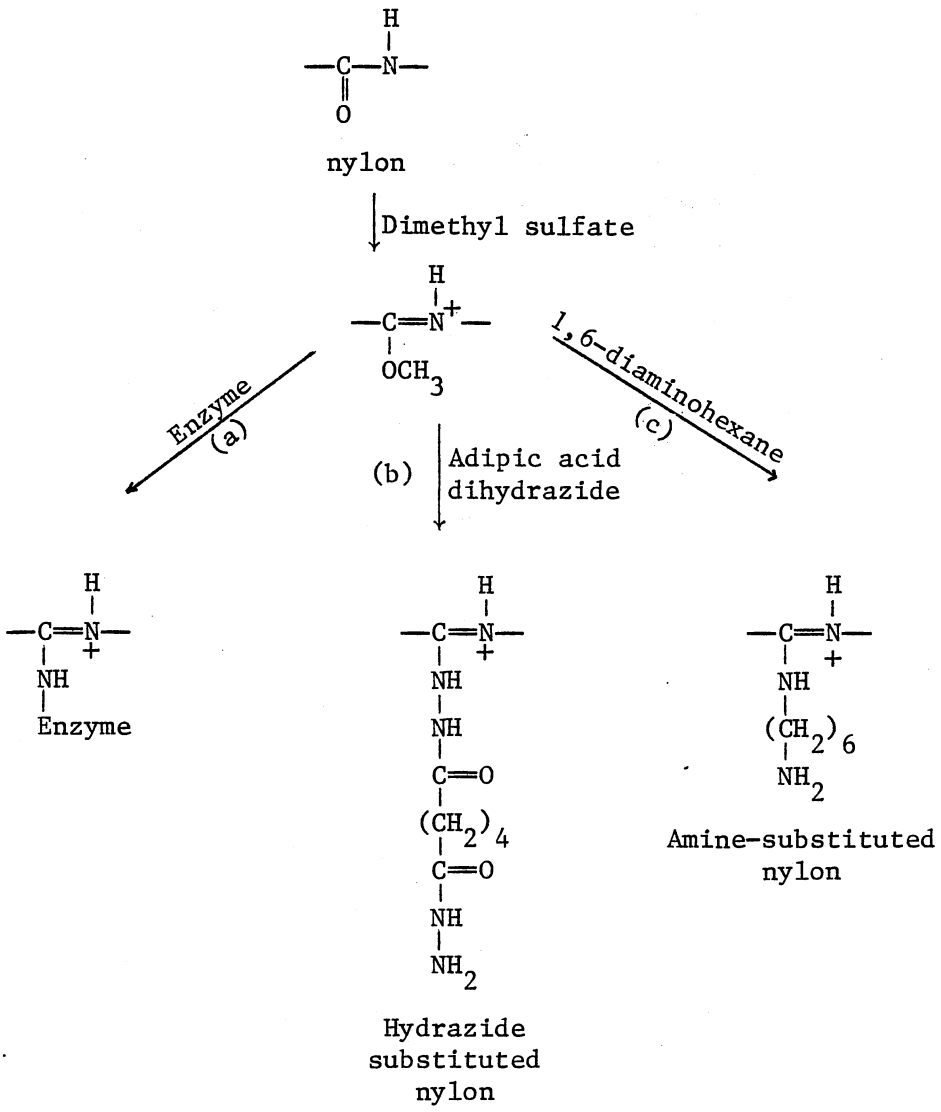


Figure 11. O-alkylation of Nylon and Reaction of the Imidate with Enzyme, Hydrazide and Amine.

Both the hydrazide-substituted nylon and the amide-substituted nylon can be activated for enzyme coupling in a variety of ways. Urease has been immobilized on nylon supports using almost all of these procedures (18, 36, 37, 39, 40).

CHAPTER IV

EXPERIMENTAL METHODS AND PROCEDURES

Apparatus

pH Meter

A Beckman 35507 flat-bottom pH electrode with a Corning model 7 pH meter was used initially. Later it was replaced by an Orion Research model 601A pH meter.

Peristaltic Pump

A variable speed Master flex model 7020C with speed controller and 7014 pump head was used in the flow system.

Filter and Amplifier

A Spectrum 1021A combination filter and amplifier was used.

Injector

A sliding valve injector with tygon sample loop was used to intercalate the sample into the flow system.

Recorder

A Hewlett-Packard 5880A GC terminal, level four, was used in some parts of this work when recording was necessary.

Reactor

The new enzyme reactor consists of a flat-bottom pH electrode and a nylon chamber, 4.0 cm in length, 3.0 cm outer diameter, and 1.2 cm internal diameter (Figure 12). It contains a small magnetic stirring bar 1.0 cm in length and 0.30 cm in diameter at the bottom. Nylon shavings (0.08 g) containing immobilized urease were placed in the lower part of the chamber. A perforated circular nylon disc kept the nylon shavings and the magnetic stirring bar separated from each other. The electrode cannot be inserted into the chamber beyond a certain distance because of the presence of a rim on the inside wall. The chamber became sealed when the electrode was inserted into it. The void volume of the reactor (with shavings and electrode) is 2.8 ml. It has a sample inlet in one side and a small outlet on the other side, for the removal of air bubbles.

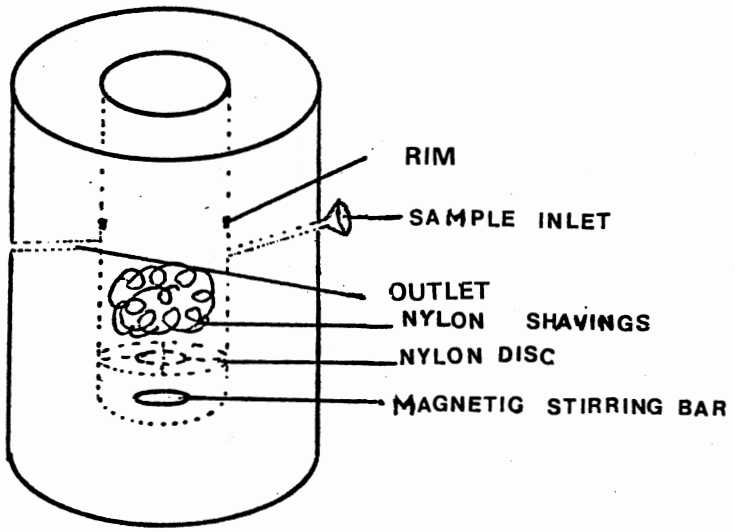


Figure 12. Nylon Shavings Enzyme Reactor

When this reactor was used with a continuous flow system, threads were placed on the upper part of the chamber using a die. An O-ring and a nylon cap were used to seal the reactor thus preventing any leakage when used under pressure. The original sample inlet was sealed and a new one was drilled at the lower part of the chamber.

Reagents and Solutions

All reagents were AR grade. The water used for solution preparation was deionized and distilled.

Phosphate Buffer (0.100M)

0.609 g of $NaH_2PO_4 \cdot H_2O$ (Fisher Scientific Co.) and 0.018 g of disodium ethylene-diamine tetraacetate (Fisher Scientific Co.) were dissolved in 45 ml of distilled water. The pH of this solution was adjusted to 7.0 with 5.00M NaOH (Fisher Scientific Co.). It was then diluted to 50 ml.

Phosphate Buffer (0.013M)

0.086 g of $NaH_2PO_4 \cdot H_2O$ (Fisher Scientific Co.) and 0.292 g of NaCl (Fisher Scientific Co.) were dissolved in 45 ml of distilled water. The pH was adjusted to 7.00 and then diluted to 50 ml. Phosphate buffers (1.30×10^{-2}M) of pH 4.00, 5.00, 6.00 and 8.00 were also prepared by a similar procedure.

Acetate Buffer (0.013M)

0.013M acetate buffers of pH 4.00, 5.00, 6.00, 7.00 and 8.00 were prepared by dissolving 0.085 g of $CH_3COONa \cdot 3H_2O$ (Fisher Scientific Co.)

and 0.290 g of NaCl (Fisher Scientific Co.) in 45 ml of water. The pH was then adjusted with 5M NaOH or 5% HCl followed by dilution to 50 ml.

Tris Buffer (0.013M)

0.075 g of Tris (hydroxymethyl aminomethane) $C_4H_{11}NO_3$ (Fisher Scientific Co.) and 0.292 g of NaCl were dissolved in 45 ml of water. The pH was adjusted to 7.00, and the solution was diluted to 50 ml. Tris buffers (0.013M) of pH 4.00, 5.00, 6.00 and 8.00 were prepared by a similar procedure. Tris buffers of concentrations 5.0×10^{-4}M, 1.0×10^{-3}M, 5.0×10^{-3}M and 1.0×10^{-2}M (pH 7.00) were also prepared by a similar procedure.

Physiological Salt Solution (Sodium Chloride 97 mM, Sodium Acetate 58 mM, Calcium Chloride 3.5 mM, Potassium Chloride 1.5 mM, Magnesium Chloride 0.50 mM)

2.83 g of NaCl, 3.94 g of $CH_3COONa \cdot 3H_2O$, 0.130 g of KCl (Mallinckrodt), 0.050 g of $MgCl_2 \cdot 6H_2O$ (Baker analyzed reagent) and 0.194 g of $CaCl_2$ (Fisher Scientific Co.) were dissolved in 450 ml of distilled water. The pH was adjusted to 7.40 and the solution was then diluted to 500 ml.

Urea (Mallinckrodt)

Methanol (Fisher)

Dimethyl Sulfate (Aldrich)

Nitric Acid (Mallinckrodt)

Enzyme

The enzyme used for the entire work was urease (EC 3.5.1.5) type III, (Sigma Chemical Co.) from Jack beans. The specific activity of urease was 3900 units/g of solid. One Sigma unit ≡ 11.4 International units.

Support

The material used as the support for the enzyme immobilization was Nylon 6 shavings (Hyde Plastic Co., Greenloch, New Jersey).

Immobilization Procedure

The enzyme was immobilized by direct O-alkylation of nylon by following the procedure reported by Sundaram (46). The nylon shavings were cleaned by boiling (1-2 minutes) in 5% nitric acid and then washed thoroughly with distilled/deionized water. The shavings were then treated with dimethyl sulfate in a small glass vial and heated in a boiling water bath for 4-5 minutes. The vial was immediately removed from the water and dipped in an ice bath, after which the shavings were washed sequentially with ice-cold methanol and ice-cold water. The rinsed shavings were then treated with a 5% urease solution in 0.100M phosphate buffer (pH 7.00) containing 1.00 mM EDTA and left overnight at $4^{o}C$. Washing with 1.00M NaCl solution and water was then performed to remove any adsorbed enzyme. Urease concentrations of 0.5%, 1% and 10% were also used for immobilization in some cases. The enzyme reactor was stored in water at $4^{o}C$. The reactions involved

in this procedure have been illustrated in Chapter III, Figure 11a.

Apparatus Set-Up and Procedure

Batch Method

The electrode was connected to the pH meter and inserted into the reactor. The substrate (urea) solution was injected into the reactor through the sample inlet using a syringe. The substrate came into contact with the immobilized enzyme and at the same time, rotation of the magnetic stirring bar aided mixing. In most cases, changes in pH were recorded when the reactions were complete. However, in some parts of this work, changes in pH were monitored at fixed time intervals prior to completion of the reaction. After each measurement, the electrode was removed from the reactor and both the electrode surface and the shavings were rinsed with distilled water. In the later experiments, a recorder was used to monitor the pH change.

Experiments with Flow System

All measurements were performed using a simple experimental set-up (Figure 13). The carrier steam was pumped by a peristaltic pump (P). The injection valve (S) (Internal volume = 25.2 µl) was furnished with a bypass, so that while the sample loop was being filled with sample, the carrier solution passed through the bypass and, after the valve was turned, the sample was intercalated into the stream and carried through the reactor (R). The changes in pH were measured (in terms of peak height) during the passage of the sample through the reactor and were recorded with a recorder (RE). The use of the filter and

amplifier (AF) resulted in amplification of all signals by twofold and reduced the noise level by about 50%.

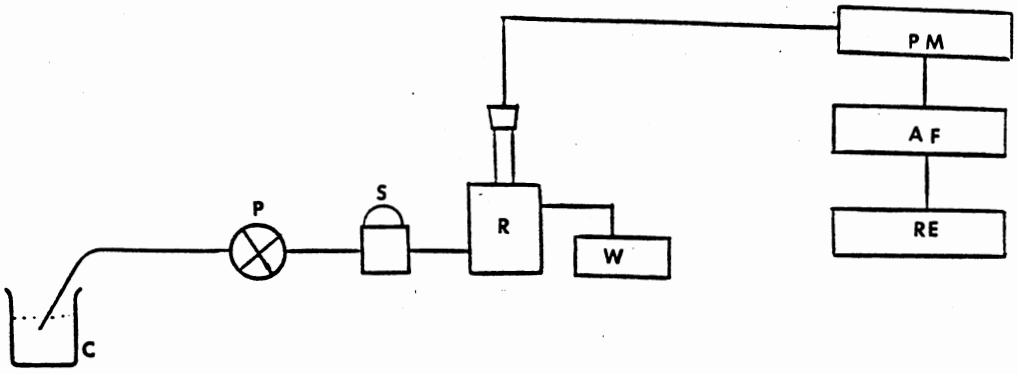


Figure 13. "Nylon Shavings" Enzyme Reactor Incorporated into a Continuous Flow System. C-carrier, P-pump, S-sample injector, R-recorder, PM-pH meter, AF-amplifier and filter, RE-recorder, W-waste.

CHAPTER V

RESULTS AND DISCUSSION

Batch Method

Effect of pH

The pH of the buffer used can affect various characteristics of an urease reactor, e.g. the percentage of transformation of $\overset{+}{NH}_4$ to NH_3 (Equation 4) and the enzyme activity. In general, enzymes are active only over a limited range of pH, and in most cases a definite optimum pH is observed. The effect of pH on enzymes, like all pH effects, is due to the changes of the states of ionization of the components of the system. As the catalytic activity of most enzymes is usually confined to a relatively small range of pH, it seems likely that only one of the ionic forms of the enzyme is catalytically active. Upon immobilization, the pH of optimum activity may be displaced to higher or lower values or may remain unchanged (40). Although it has been reported that the optimum pH for a particular enzyme can vary with the buffer system, no explanation has been presented for this phenomenon (28). The reported optimum pH for both soluble and immobilized urease in various buffer systems are shown in Table I.

It must be kept in mind that the optimum pH for a particular enzyme reactor can also depend on the type of the sensor used. For example, when an air-gap or gas sensing electrode is used as part of

an urease reactor a pH higher than the pH optimum for immobilized urease is commonly used. Alkaline conditions favor the formation of gaseous products (Equation 4), although loss in enzyme activity has been reported (9). These urease reactors can exhibit higher pH optima for the same buffer system. Table I shows the values of optimum pH's for urease reactors, where the sensor (electrode) came in direct contact with the substrate solution.

TABLE I

REPORTED VALUES OF OPTIMUM pH FOR SOLUBLE AND IMMOBILIZED UREASE IN VARIOUS BUFFER SYSTEMS*

Buffer	Soluble Urease	Immobilized Urease	This Work	Reference
Phosphate	6.9	7.0	7.0	(28, 41)
Tris	8.0	N.A	7.0	(29)
Acetate	6.3	N.A	6.0	(28)

N.A = Not available.

*Ionic strength = 0.11; Temperature = 25°C.

The pH at which the new reactor exhibits maximum response was determined by monitoring the changes in pH in different buffer systems at a constant urea concentration of 0.050M. Total buffer concentration in all cases was 0.013M in 0.100M NaCl solution, and experiments were

performed at room temperature. A pH range from 4.00 to 8.00 was used in all cases. The changes in pH after completion of the reaction were recorded. The results are shown in Figure 14. The peak of each curve denotes the pH for optimum response of the reactor. The pH optimum of the new reactor is about the same as observed with the soluble enzyme for one buffer, i.e., phosphate, and lower in other two buffers. The mechanism of this response is not well understood.

Long-term Stability Studies

Long term stability of the new urease reactor was studied by using four different enzyme concentrations, i.e., 0.5%, 1%, 5% and 10% urease. A 0.050M urea solution (in 0.013M Tris buffer in 0.100M NaCl, pH 7.00) was used in each case and changes in pH were noted when the reactions were complete. All experiments were performed at room temperature and the reactor, when not in use, was stored at $4^{o}C$ under water. Before use, the reactor was taken from the refrigerator and sufficient time was allowed to reach room temperature. The results are shown in Figure 15. All curves were normalized to 100% at the time of preparation. As shown in Figure 15, after 70 days about 71%, 70%, 26% and 24% of the initial activity was retained by the enzyme preparations immobilized with 10%, 5%, 1% and 0.5% urease solutions, respectively.

There is no significant difference in long-term stability between urease reactors immobilized with 5% and 10% urease. This indicates that a 5% urease solution has saturated almost all available binding sites of the nylon support. In the rest of this work urease was immobilized by using a 5% enzyme solution. It is also clear from Figure 15 that urease

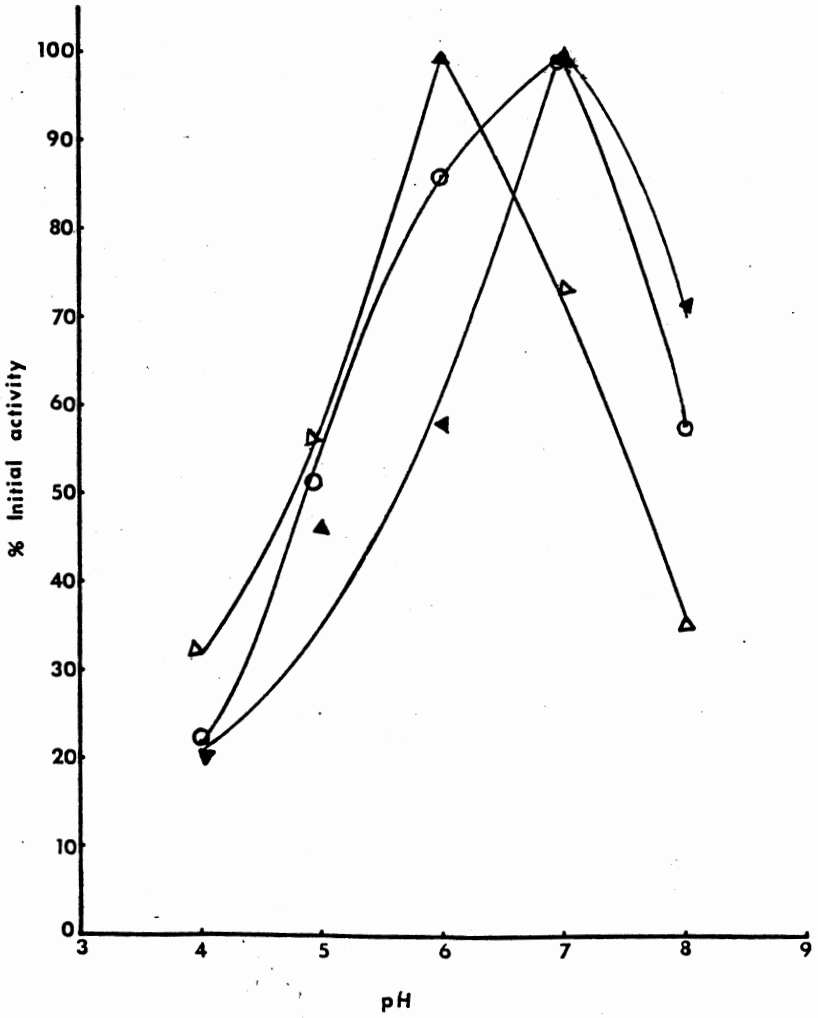


Figure 14. Effect of pH on the Activity of Immobilized Urease. △ acetate buffer; ○ phosphate buffer; ▲ Tris buffer.

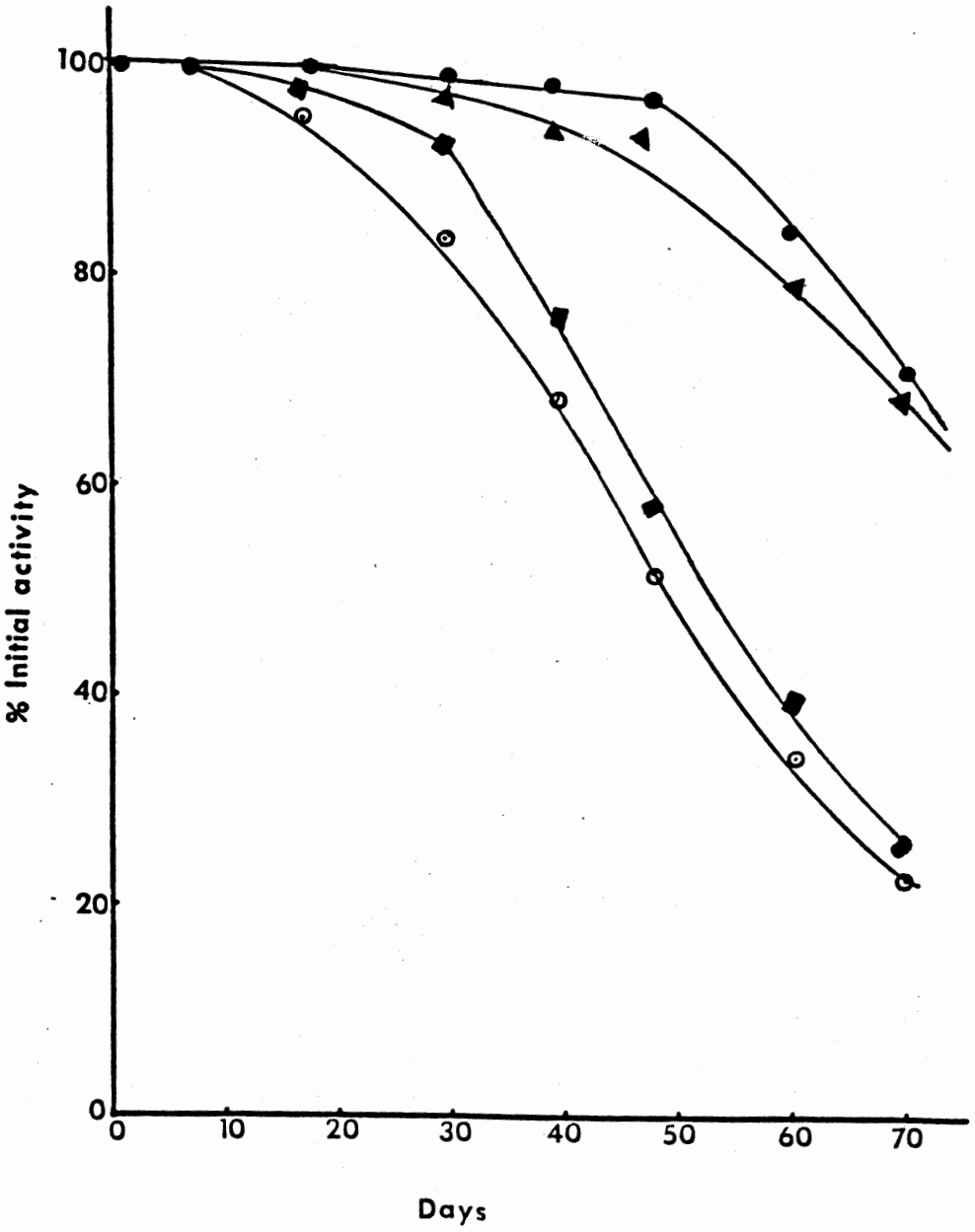


Figure 15. Long Term Stability of the Nylon Shavings Enzyme Reactor. ● 10% urease; ▲ 5% urease; ■ 1% urease; ⊙ 0.5% urease.

immobilized with lower concentrations of the enzyme showed a more rapid and relatively larger loss in activity compared to that of urease preparations with higher enzyme concentrations. This is due to the inactivation of some of the active sites as a result of partial denaturation of the enzyme with time. This may be explained in the following way. For example, let the particular amount of nylon shavings (0.080 g) have a total of ten binding sites for enzyme coupling, and the enzyme activity present in the enzyme bound to the seven of these sites is sufficient to catalyze reaction of all of the particular amount of substrate used. As mentioned previously, urease immobilized with higher enzyme concentrations will have saturated all of the available binding sites of the support (in this case ten). The enzyme immobilized with lower urease concentration will occupy fewer binding sites of the support. For example, eight binding sites may be occupied. Let, after a certain period of time, all the immobilized enzyme preparations lose the activity due to three of the binding sites. The concentrated enzyme preparations will have activity due to seven binding sites remaining and, as a result, no loss of enzyme activity will be observed since enzyme activity present in seven binding sites is sufficient for the amount of substrate present. On the other hand, the enzyme preparations immobilized with lower urease concentrations will only have activity due to five binding sites remaining and, as a result, loss of initial activity will be observed.

Thermal Stability of the Reactor

The rate of the enzyme catalyzed reaction increases with temperature up to a certain temperature. Beyond that temperature, the reaction

rate decreases as the temperature is increased. This decrease of reaction rate at higher temperatures is probably due to the denaturation of the enzyme (31). Very little information about the thermal-stability of urease reactors has been reported. No optimum temperature has been recommended for urease reactor either.

The thermal stability of the new urease reactor was determined by injecting a 5.00×10^{-2}M urea solution in 0.013M Tris buffer (pH 7.00 in 0.100M NaCl) and measuring the resulting pH change. Six different temperatures were used (25°C, 35°C, 48°C, 60°C, 75°C and 90°C). The nylon chamber containing the immobilized urease and distilled water were incubated in an oven at the particular temperature of interest. The residual enzyme activity was determined by measuring the change in pH at fixed time intervals. After each measurement, the shavings and the electrode were washed with distilled water at the same temperature. The result is shown in Figure 16. The change in pH prior to incubation was taken as 100% activity in each case. Injection of substrate solution at room temperature following completion of the experiment (at a higher temperature) resulted in a smaller pH change than that observed prior to incubation. This indicates partial denaturation of the enzyme.

As shown in Figure 16, the reactor retained about 80% of its initial activity after one hour at temperatures of 75°C or less. Increased thermal stability is important from an analytical viewpoint because it allows higher analytical throughput due to lower response time and in some cases, a higher sensitivity.

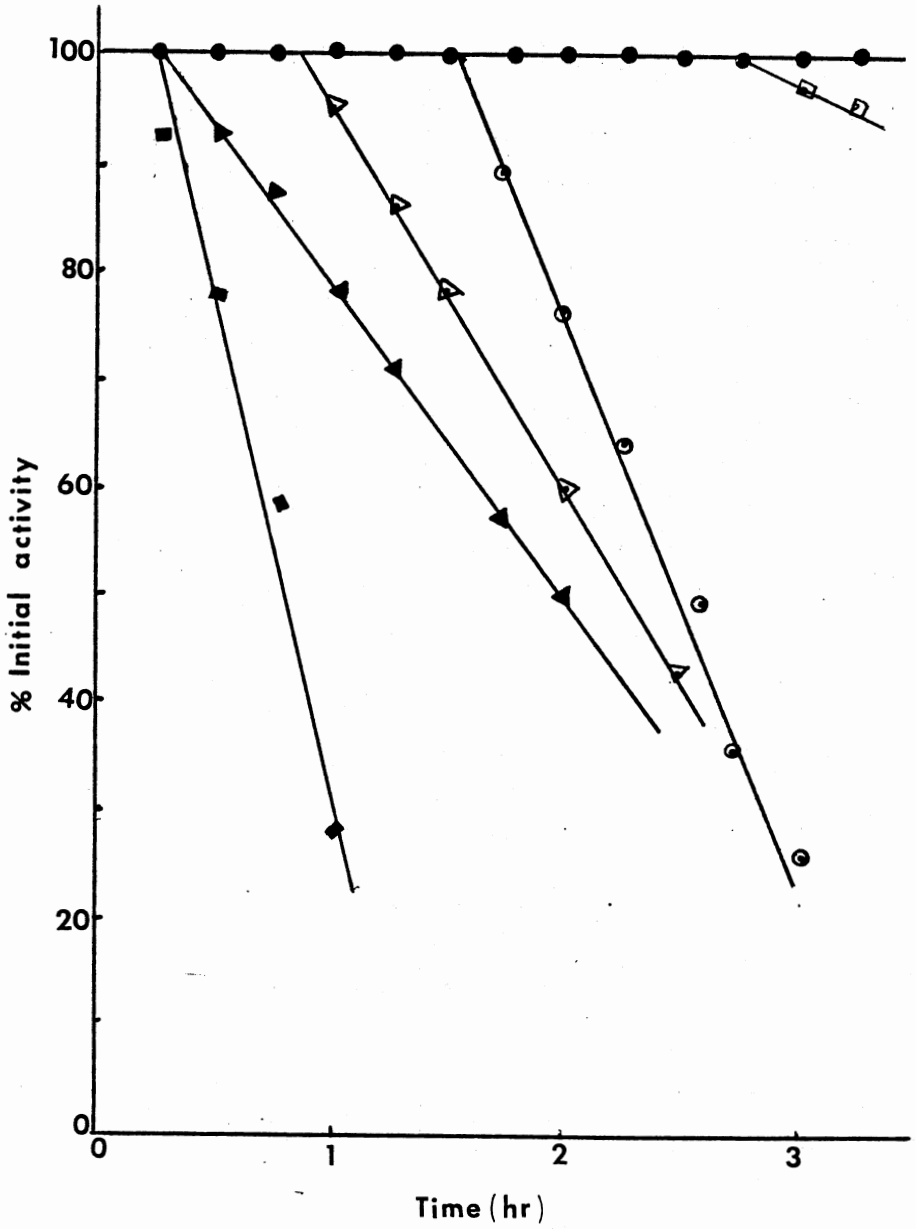


Figure 16. Stability of the New Reactor at Elevated Temperatures. ● 25°C; ⊡ 35°C; ⊙ 48°C; △ 60°C; ▲ 75°C and ■ 90°C.

Effect of Substrate Concentration

In order to determine substrate concentration in a sample it is necessary to ascertain the range of substrate concentrations where the change in response is directly proportional to the substrate concentration. Calibration curves were made by using two approaches:

(i) Kinetic approach - urea solutions of concentrations 1.0 x 10^{-1}M, 1.0 x 10^{-2}M, 5.0 x 10^{-3}M, 1.0 x 10^{-3}M, 1.0 x 10^{-4}M, 1.0 x 10^{-5}M and 1.0 x 10^{-6}M were used. All solutions were made in 0.013M Tris buffer, pH 7.00 (in 0.100M NaCl). Initial reaction rates were calculated from first 10% of the total pH range. The results are shown in Figure 17. The curve was linear within the range of 1.0 x 10^{-6}M to 1.0 x 10^{-1}M urea with a slope of -9.9 x 10^{-8} mole/l/min/decade of urea concentration.

In this method, completion of reaction is not necessary. As a result, a larger number of samples, relative to the equilibrium approach, could be analyzed in a given period of time. Although temperature control has been recommended for this procedure, a satisfactory calibration curve was obtained by performing the experiment at room temperature. The standard deviations (of 3 determinations) at different urea concentrations were 1.5 x 10^{-8} mole/l/min, 1.4 x 10^{-8} mole/l/min, 1.2 x 10^{-8} mole/l/min, 1.0 x 10^{-8} mole/l/min, 1.3 x 10^{-8} mole/l/min, 8.0 x 10^{-9} mole/l/min and 3.0 x 10^{-9} mole/l/min for 1.0 x 10^{-1}M, 1.0 x 10^{-2}M, 5.0 x 10^{-3}M, 1.0 x 10^{-3}M, 1.0 x 10^{-4}M, 1.0 x 10^{-5}M and 1.0 x 10^{-6}M urea, respectively. Nonlinearity in the calibration curve (which has been observed in the equilibrium method; Figure 18) was not observed in this method. As a result, a larger linear dynamic

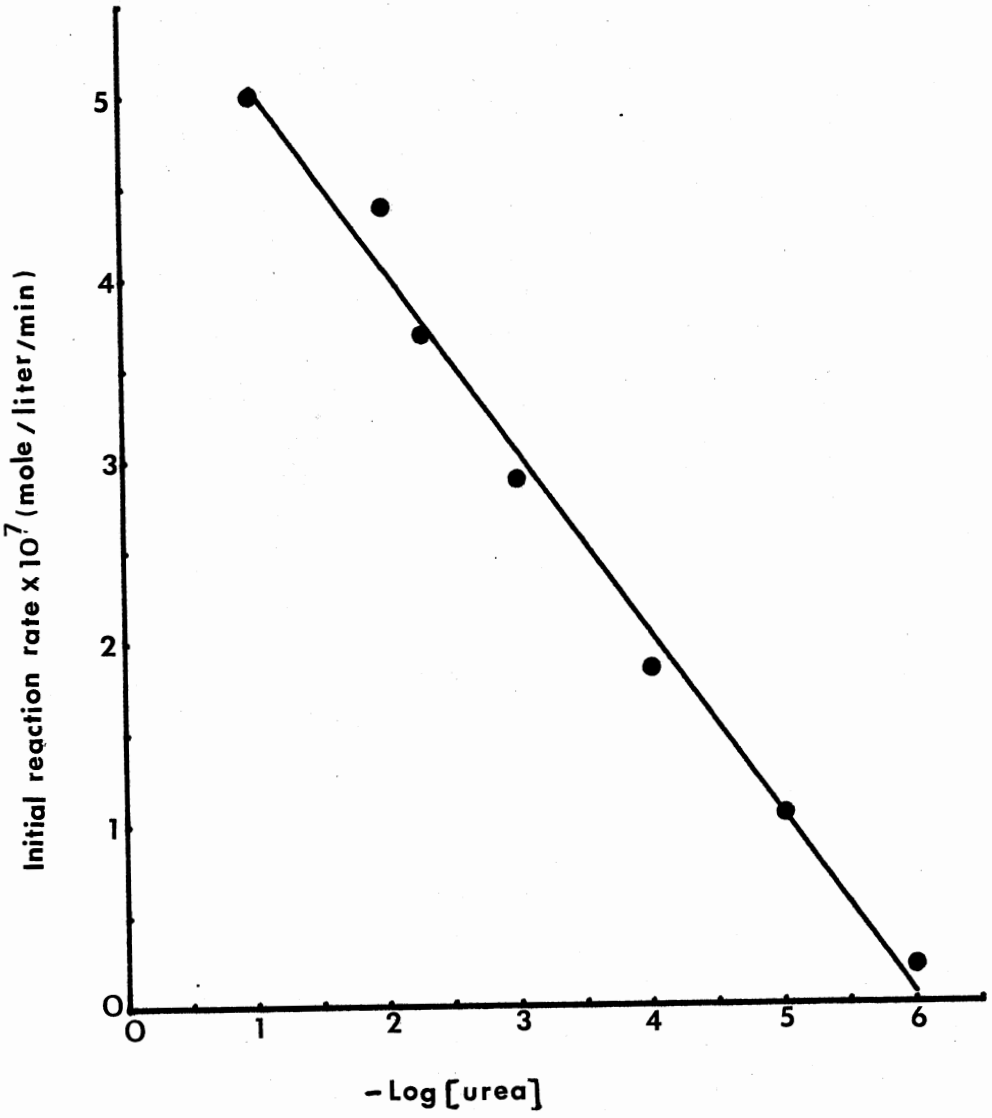


Figure 17. Calibration Curve in Tris Buffer (Kinetic Method).

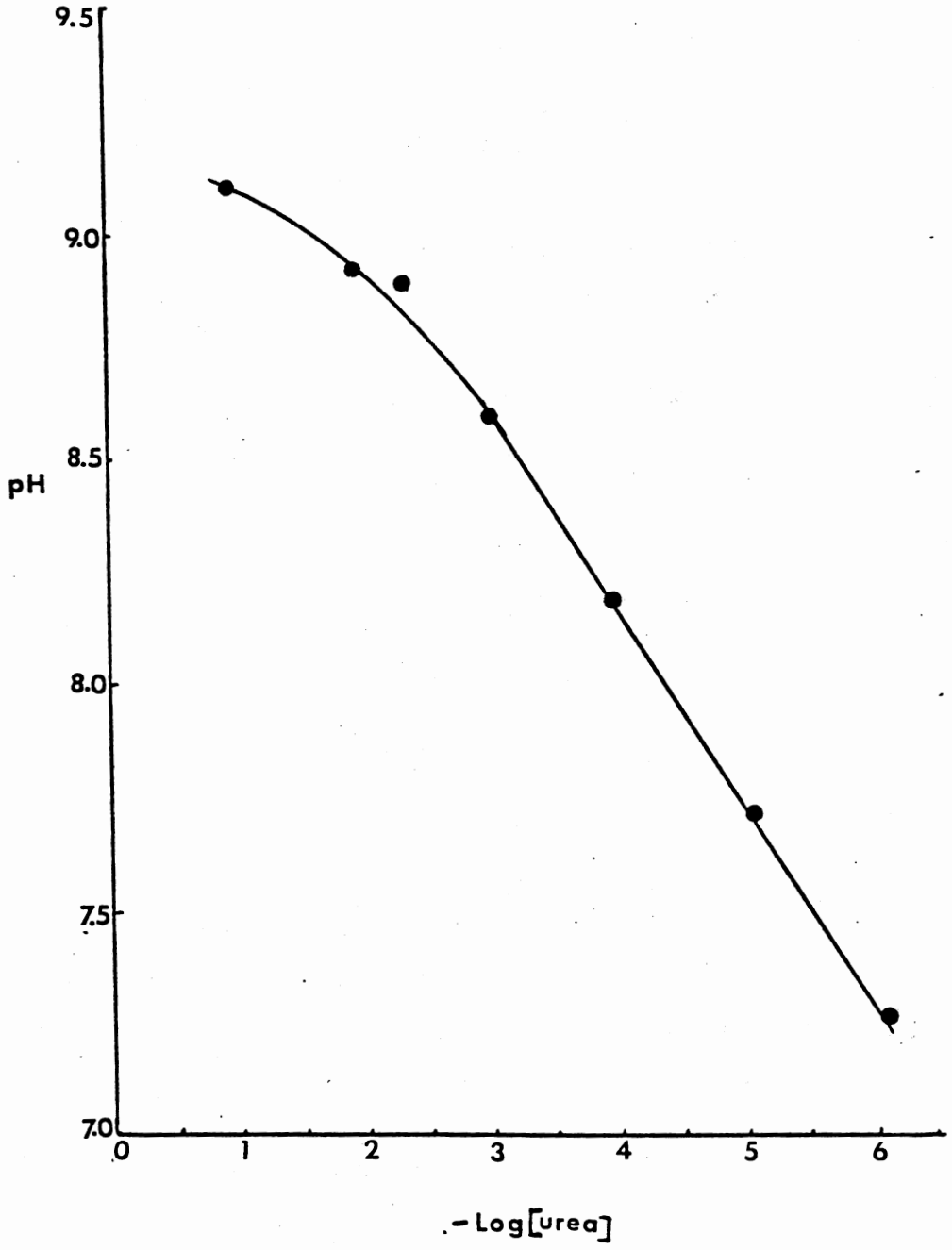


Figure 18. Calibration Curve in Tris Buffer (Equilibrium Method).

range can be realized.

(ii) Equilibrium approach - urea solutions of concentrations 1.0×10^{-1}M, 1.0×10^{-2}M, 5.0×10^{-3}M, 1.0×10^{-3}M, 1.0×10^{-4}M, 1.0×10^{-5}M and 1.0×10^{-6}M were used for the construction of the calibration plot. All urea solutions were made in 0.013M Tris buffer, pH 7.00 (9n 0.100M NaCl) and changes in pH were recorded after completion of the reactions. The results are shown in Figure 18. The curve was linear within the approximate range of 1.0×10^{-6}M to 5.0×10^{-3}M urea. The slope of the linear portion of the calibration curve is -0.433 pH unit/decade urea concentration. The standard deviations of 5 determinations were 0.010 pH unit, 0.015 pH unit, 0.017 pH unit, 0.022 pH unit, 0.031 pH unit, 0.040 pH unit, 0.050 pH unit for 1.0×10^{-1}M, 1.0×10^{-2}M, 5.0×10^{-3}M, 1.0×10^{-4}M, 1.0×10^{-5}M and 1.0×10^{-6}M urea, respectively.

Deviations from linearity at higher substrate concentrations have been observed in many enzyme reactors (3, 6). The common cause to which this effect has been attributed is diffusional problems. The deviation from linearity at high substrate concentrations in this case, however, is probably due to the decrease in enzyme activity as a result of the relatively large increase in pH of the reaction mixture. However, irreversible loss of enzyme activity was not observed. This was confirmed by injecting another urea solution after washing the reactor with distilled water.

The normal urea concentration in human serum is in the range of 8.0×10^{-4}M to 4.0×10^{-3}M (42). Thus, the range of normal serum urea is within the linear range of the calibration curve in both kinetic and equilibrium cases. This indicates the possibility of

using an urease reactor such as the one studied for the determination of urea in serum.

A calibration curve similar to one described above was obtained by using physiological salt solution (pH 7.40) in place of Tris buffer. Urea solutions were prepared in the following concentrations, 5.0×10^{-2}M, 1.0×10^{-2}M, 1.0×10^{-3}M, 5.0×10^{-4}M, 1.0×10^{-5}M and 1.0×10^{-6}M and the experiments were performed at room temperature. The reactor response was found to be linear within the range of 1.0×10^{-6}M to 1.0×10^{-3}M of urea (Figure 19), and the slope of the calibration curve was -0.375 pH unit/decade of urea concentration. The smaller slope of this calibration plot compared to that in the Tris buffer may be due to the higher ionic strength of the salt solution. This effect has also been observed in another urease reactor (43). It should be mentioned that the slope of the calibration curve dictates the sensitivity of the particular method. However, the calibration plot in physiological salt solution indicates that it should be possible to use the reactor in the presence of ions normally present in biological fluids.

Determination of the Michaelis-Menten Constant (Km)

From an analytical point of view, determination of Km for a particular enzyme and substrate is important because it implies that the linearity of a particular enzymatic method extends to a substrate concentration equal to the value of Km. Recently, however, it has been reported that it is possible to measure substrate concentrations up to about 3.5 Km by fitting rate vs. time data to the Michaelis-

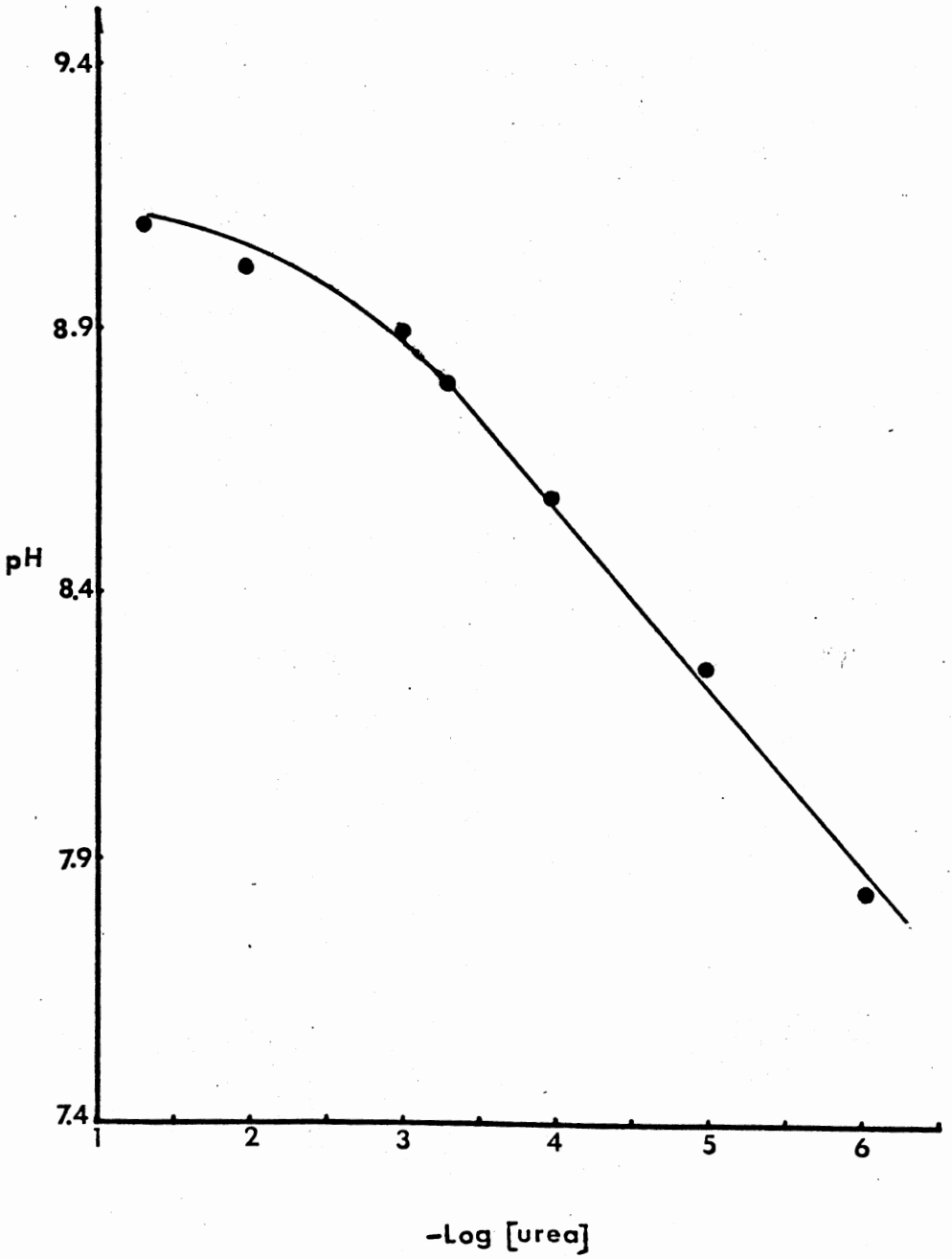


Figure 19. Calibration Curve in Physiological Salt Solution.

Menten rate equation (44). This is done in order to increase the linear dynamic range of the method. An enzyme catalyzed reaction may be represented in the following manner:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E + P \qquad (5)$$

where E = enzyme, S = substrate, P = product, ES = enzyme-substrate complex, $Km = \frac{k_{-1} + k_2}{k_1}$.

The Km value is generally expressed in units of mM or M since Km is that substrate concentration which results in an initial reaction rate that is equal to half of the maximum initial reaction rate (Vm) obtainable.

Urea solutions of different concentrations, i.e., 1.0×10^{-3}M, 2.0×10^{-3}M, 3.00×10^{-3}M, 4.00×10^{-3}M, 5.00×10^{-3}M, 6.00×10^{-3}M, 8.00×10^{-3}M, 1.00×10^{-2}M were prepared in 0.013M Tris buffer pH 7.00 (in 0.100M NaCl). Changes in pH were recorded with a strip chart recorder. Initial reaction rates were calculated from the first 10% of the total pH change.

The Michaelis-Menten rate equation (45) for an enzyme catalyzed system is

$$V_o = \frac{Vm[S]_o}{Km + [S]_o} \qquad (6)$$

where, Vo = initial reaction rate at a given substrate concentration, Vm = maximum initial reaction rate, $[S]_o$ = initial substrate concentration.

From equation (6) it can be shown that

$$\frac{1}{Vo} = \frac{Km}{Vm[S]_o} + \frac{1}{Vm} \qquad (7)$$

A straight line was obtained by plotting $\frac{1}{V_o}$ vs. $\frac{1}{[S]_o}$. This plot is known as the "Lineweaver-Burke plot". The Km value was obtained from the X-intercept of the straight line at which $\frac{1}{[S]_o} = -\frac{1}{Km}$. This can be shown by setting $\frac{1}{V_o} = 0$ in Equation (7).

The Km value for the immobilized urease was found to be 1.65 ± 0.35M. Some reported Km values for immobilized urease are presented in Table II.

TABLE II

REPORTED Km VALUES FOR IMMOBILIZED UREASE

Type of reactor	Km (M)	Reference
Tubular reactor	3.50×10^{-3}	(18)
	17.0×10^{-3}	(46)
Packed bed reactor	4.30×10^{-3}	(47)
'Nylon shavings' reactor	1.65×10^{-3}	This work

The lower Km value obtained in this work compared to the other urease reactors (Table II) may be an indication of higher initial rates of reaction. This may be explained by using an initial reaction

rate (IRR) vs. substrate concentration plot (Figure 20). In Figure 20 region AB is first order and region CD is zero order with respect to substrate concentration.

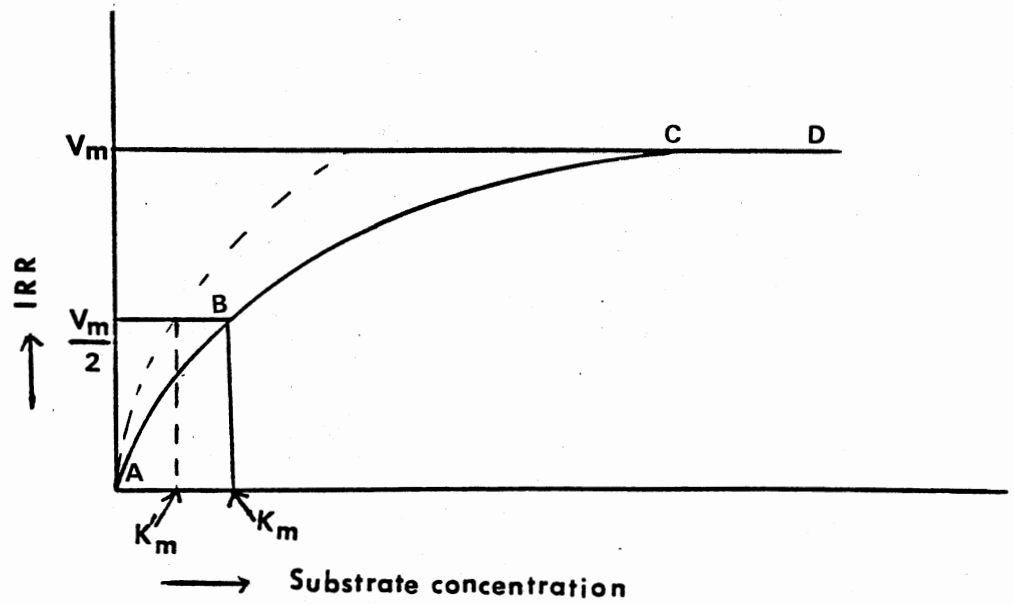


Figure 20. Initial Reaction Rate vs. Substrate Concentration Plot.

It has been mentioned earlier that the Km is the substrate concentration which results in an initial rate equal to half of the maximum initial reaction rate (Vm). Therefore, when a higher initial reaction rate is obtained, the IRR vs. substrate concentrations curve becomes steeper at low substrate concentrations which results in a smaller Km value.

Response Time of the Reactor

It is important to determine the response time of an enzyme reactor because it indicates the analytical throughput. In other words, it indicates the number of samples that can be processed per unit time. The response time of the new urease reactor was determined by using urea solutions in Tris buffer (pH 7.0). Urea solutions of the following concentrations were used, e.g., 1.0×10^{-2}M, 1.0×10^{-3}M, 1.0×10^{-4}M, 1.0×10^{-5}M and 1.0×10^{-6}M. Changes in pH were recorded at fixed time intervals until a stable pH was obtained. All experiments were performed at room temperature. The results are shown in Figure 21. As shown in Figure 21, the response time of the urease reactor varies with the concentration of the substrate, ranging from about 5 minutes for 1.0×10^{-6}M to 1.0×10^{-3}M urea and about 2 minutes at higher urea concentrations. This indicates that the reactor should be able to analyze about 11-24 samples per hour (including the wash time of the reactor) depending on the substrate concentration, when used under equilibrium conditions. Use of the same reactor under kinetic conditions, however, could increase this analytical throughput. The variation in response time was due to the fact that, at a fixed enzyme concentration, the rate of the enzyme catalyzed reaction is proportional to the substrate concentration. The response times of various types of enzyme reactors are shown in Table III.

The response time of this reactor is comparable to the response time of the air-gap and gas sensing enzyme reactors (Table III). This indicates little or no diffusion problems present in this reactor.

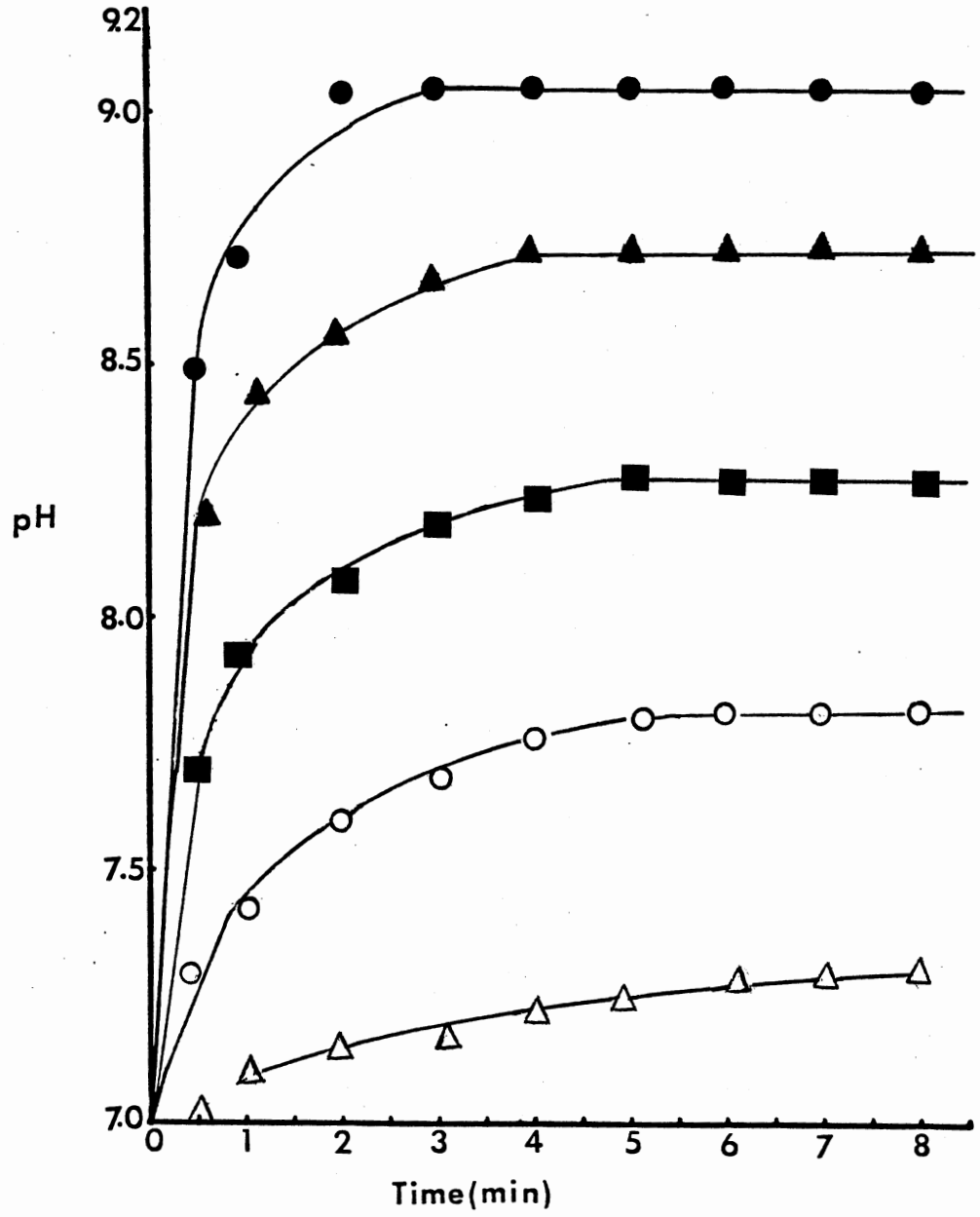


Figure 21. Response Time of the Reactors at Different Concentrations of Urea. ● 1.0×10^{-2}M; ▲ 1.0×10^{-3}M; ■ 1.0×10^{-4}; ○ 1.0×10^{-5}M and △ 1.0×10^{-6}M.

TABLE III

RESPONSE TIMES FOR DIFFERENT ENZYME REACTORS

Type of Reactor	Response Time (min.)	Reference
Layer	5-7	(6)
Disc	1-22	(7)
Gas-sensing	1-5	(48)
Air-gap	2-4	(12)
'Nylon shavings'	2-5	This work

The lack of response which has been observed at lower substrate concentration (Figure 21) during the first minute of reaction is probably due to the presence of the buffer. Some amount of time is required to overcome the buffer capacity.

Continuous Flow System

The urease reactor was implemented into a continuous flow system as part of this work. The apparatus set-up and the experimental procedure used with the flow system have been discussed in Chapter IV.

Several parameters were investigated in order to optimize the performance of the reactor. In almost all cases, a substrate concentration of 1.0×10^{-3}M was used for optimization since it represents the approximate midpoint of the normal human serum urea range. Tris buffer (pH 7.00) was used in the entire work and all readings were

taken at room temperature.

Effect of Buffer Concentration

The effect of buffer concentration on peak height was studied by using Tris buffer (pH 7.00) in the following concentrations: 5.0×10^{-4}M, 1.0×10^{-3}M, 5.0×10^{-3}M and 1.0×10^{-2}M. Sodium chloride was used in all buffers as an ionic strength adjustor. The result is shown in Figure 22. It is difficult to use the reactor in a strongly buffered media since the sensitivity becomes low. A very low buffer concentration will result in a very sensitive reactor which, at first glance, is very attractive from an analytical point of view. However, a large change in pH is undesirable for an enzyme reactor because it can adversely affect the enzyme activity. A buffer concentration of 1.0×10^{-3}M was chosen for this work since it represents the minimum buffer concentration which gives an acceptable pH change in terms of enzyme activity regardless of substrate concentration.

Effect of Sample Size

The effect of sample size on reactor response was studied by using loops of different lengths. 1.0×10^{-3}M urea solution (in pH 7.00 Tris buffer) was used.

Table IV shows the effect of sample size on peak height and time required to return to the baseline (t_{base}).

Peak height increased with sample volume up to a certain point. A further increase in sample volume did not increase the peak height significantly. However, a comparatively large increase in t_{base} was observed. The optimum sample size is that volume of sample which

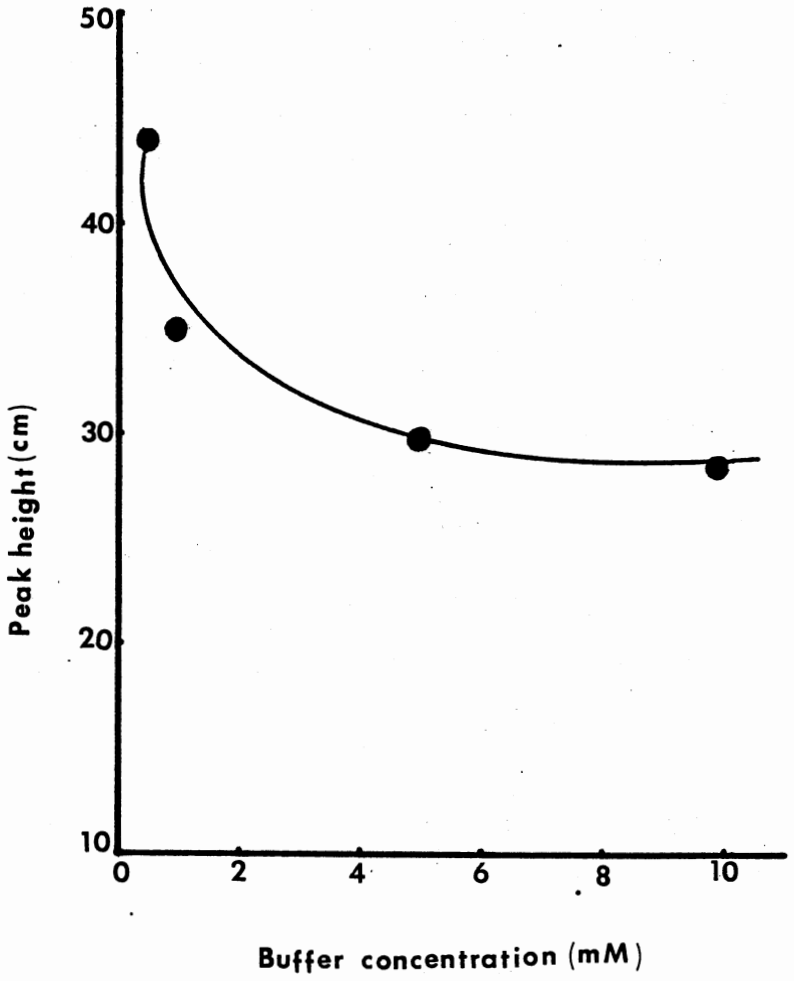


Figure 22. Effect of Buffer Concentration on Peak Height.

produces a large signal, and at the same time a small t_{base}. Considering these two factors, a sample size of 61.6 µl was chosen for this work. Reproducibility of the result was tested by repetitive injection of 61.6 µl samples. The standard deviation for a set of 15 injections was 1.59 cm.

TABLE IV

EFFECT OF SAMPLE SIZE ON PEAK HEIGHT AND t_{BASE}

Sample Size (µl)	Peak Height (cm)	t_{base} (min.)
37.3	24.3	6.20
41.4	25.8	6.30
45.4	28.4	7.20
49.5	29.0	8.00
53.5	31.4	9.60
57.5	33.6	10.8
61.6	35.8	11.2
65.7	36.2	13.8
69.7	36.9	14.2
73.1	37.1	14.9

Effect of Flow Rate

The effect of flow rate on the peak height and the time required to return to the baseline (t_{base}) was studied. Urea solution (1.0×10^{-3}M in pH 7.00 Tris buffer) was used as the sample. The results are shown in Figure 23. As shown in Figure 23, a decrease in flow rate resulted in a larger peak. This is due to the fact that, at a low flow rate, the injected sample spends more time in contact with the enzyme, which results in a larger change in pH. Since change in pH is proportional to the observed peak height, a larger peak is obtained. However, a decrease in flow rate resulted in a larger t_{base}. This increase in t_{base} results in a lower sampling rate which is undesirable due to the greater amount of time required to perform a given number of determinations. A flow rate of 1.10 ml/minute represents a reasonable compromise between sensitivity and analytical throughput and therefore, was chosen as the flow rate for the remainder of this work.

Calibration Curves

Urea solutions of 1.0×10^{-1}M, 1.0×10^{-2}M, 5.0×10^{-3}M, 1.0×10^{-3}M, 5.0×10^{-4}M and 1.0×10^{-4}M were prepared in 1.0×10^{-3}M Tris buffer, pH 7.0 (in 1.00 M NaCl). The result is shown in Figure 24. As shown in Figure 24, the curve was linear in the range of 1.0×10^{-4}M to 1.0×10^{-1}M of urea concentration. The standard deviation at different substrate concentrations were 1.07, 1.13, 1.51, 1.69, 1.85, and 1.85 for 1.0×10^{-1}M, 1.0×10^{-2}M, 5.0×10^{-3}M, 1.0×10^{-3}M, 5.0×10^{-4}M, and 1.0×10^{-4}M urea concentration, respectively. The shift of the linear dynamic range to higher substrate concentrations relative to

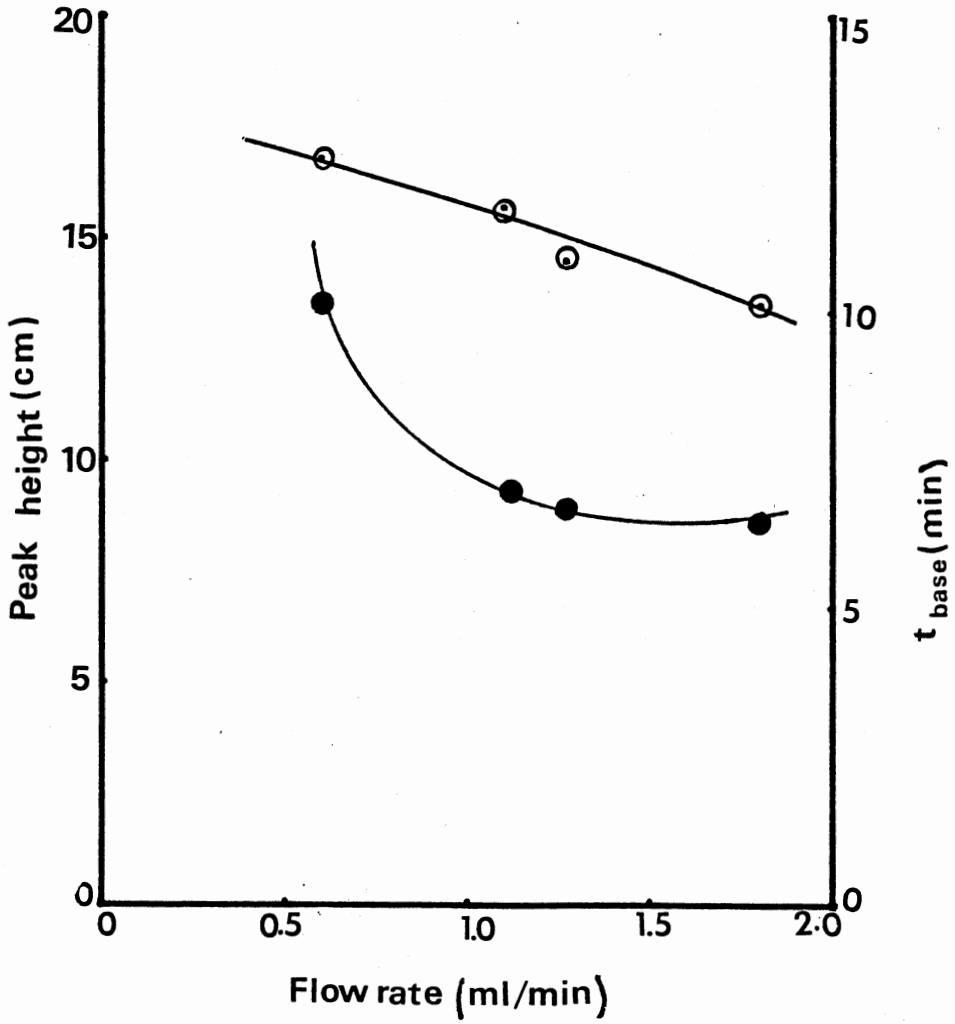


Figure 23. Effect of Flow Rate on Peak Height and t_{base}.
⊙ peak height; ● t_{base}.

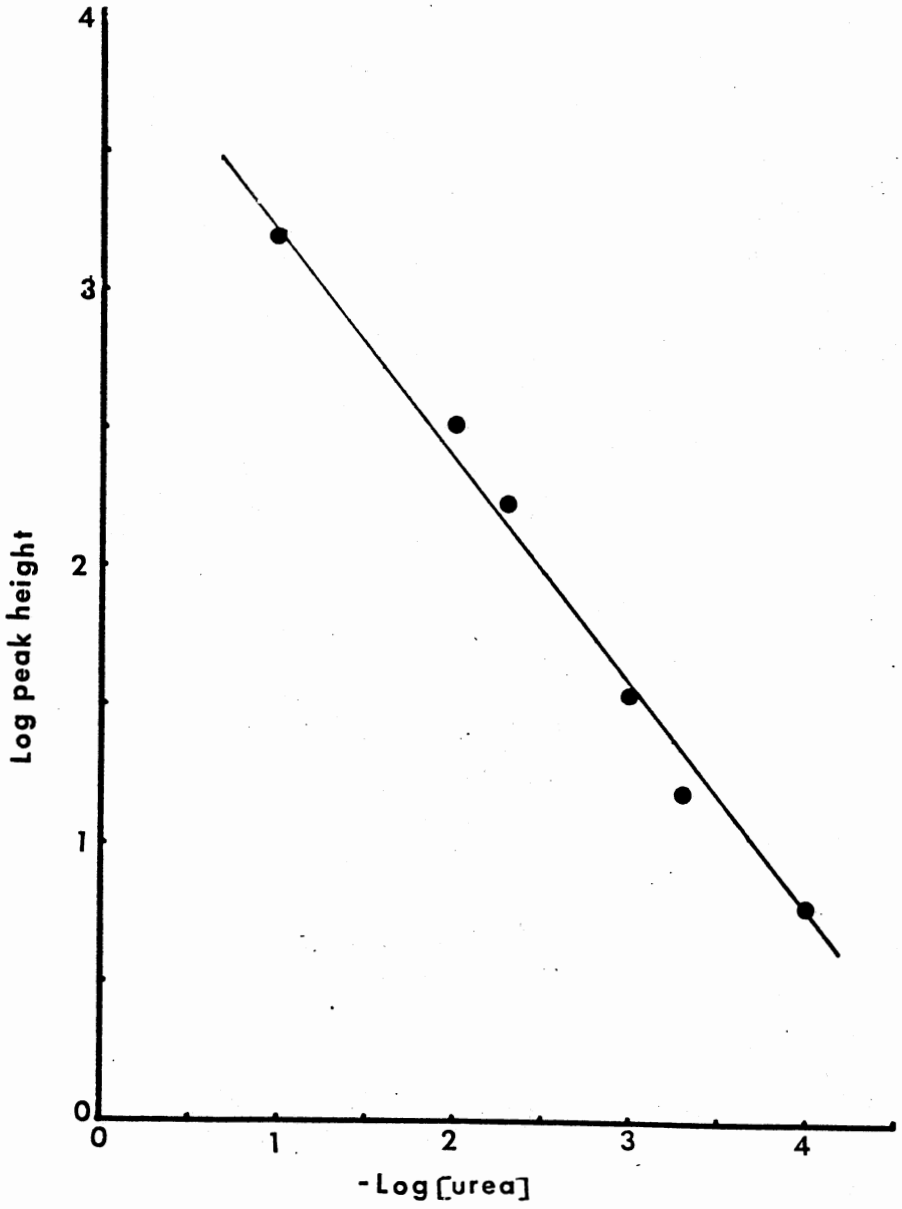


Figure 24. Calibration Plot for Urea by Use of a Continuous Flow System Incorporating the 'Nylon Shavings' Enzyme Reactor.

the linear dynamic range in the batch system is due to dilution of the injected sample in the reactor. Lack of response with urea concentrations lower than about 1.0×10^{-4} M urea is also a result of dilution of the sample.

CHAPTER VI

CONCLUSION

The principal objective of this project was to develop and test a new configuration of enzyme reactor which would minimize the problem of slow diffusion common to many previously developed enzyme reactors. Urease (in immobilized form) was used as a model enzyme system. The new enzyme reactor which was described in detail in Chapter IV consists of a small nylon chamber, nylon shavings containing immobilized urease, a small magnetic stirring bar, and a flat bottom pH electrode. This reactor is very easy to handle. Special precautions, such as complete sealing of the reaction chamber for the air-gap type of enzyme reactor and careful control of the enzyme layer thickness for layer-type or disc-type enzyme reactors, are not necessary for this reactor. The newly developed enzyme reactor has proven to be useful for batch analysis. As discussed in Chapter V, the linear dynamic range obtained by the use of this reactor indicates the possibility of using it for the determination of serum urea. It is free from interferences due to the presence of various ions normally present in biological fluids. This reactor exhibits sufficient stability and its relatively low response time indicates little or no diffusional limitations. The temperature stability (Figure 16) of this reactor suggests the possibility of using it at elevated temperatures, which could result in higher analytical

throughput and in some cases sensitivity. The ease of handling, good stability, and relatively low response time makes this newly developed enzyme reactor attractive for the determination of urea by a batch method.

Although the linear dynamic range obtained when using this reactor in a continuous flow system indicated the possibility of using it for the determination of serum urea, the very large t_{base} (time required to return to the baseline) defeated the main purpose of using continuous flow system. The large internal volume of the reactor compared to the sample volume results in dispersion and a concominant increase in t_{base}. A decrease of the reactor volume could reduce dispersion and t_{base}. As discussed in Chapter V, the lack of response of the reactor at substrate concentration lower than 1.0×10^{-4}M urea results from the dilution of injected sample in the reactor. The decrease of reactor volume could also lower the limit of detection.

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VITA 2

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