Studies of Immobilized and Cross-linked α–Chymotrypsin to Explore Solvent Stabilization

By

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Youngstown State University
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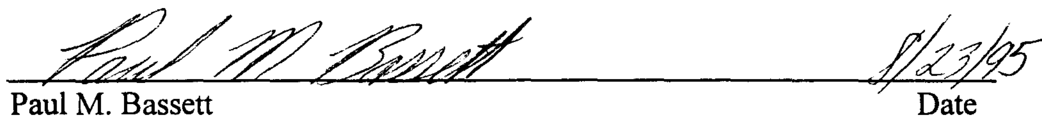

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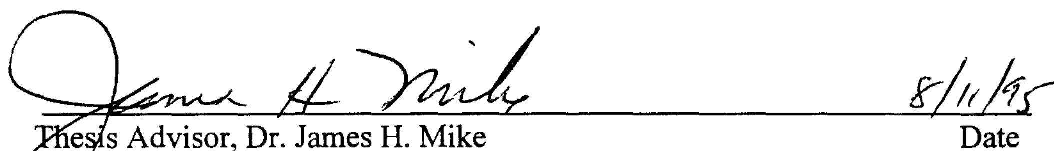

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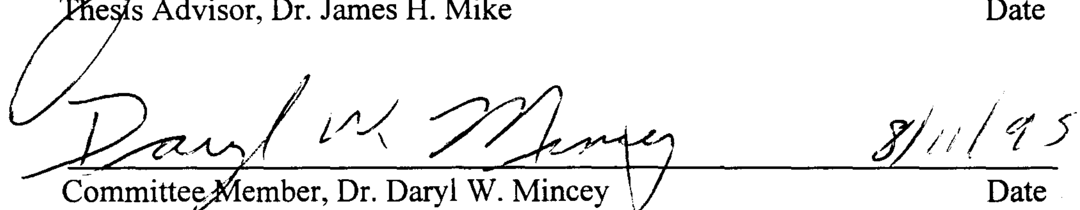

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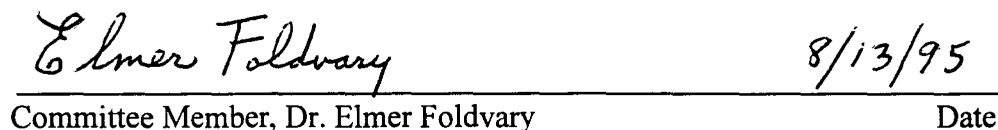

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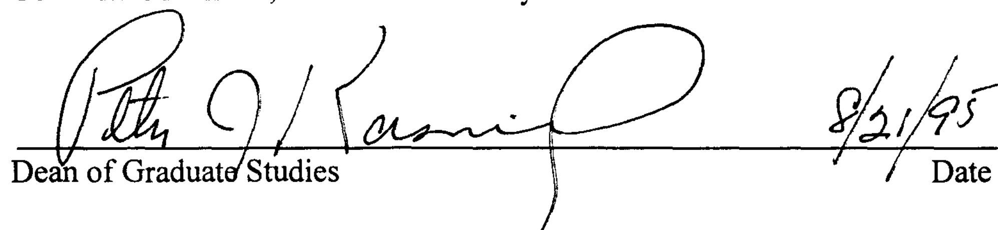

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Abstract

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Immobilized enzyme post-column reactors in HPLC systems yield good sensitivity and selectivity in biochemical analyses. The problem, however, is that the mobile phase, usually methanol based, readily denatures the enzyme column unless a dilution system is employed prior to this component. The dilution element not only makes the entire system cumbersome, but also introduces various conditions that complicate the procedure. This paper describes an immobilization study that used intramolecular and intermolecular cross-linking techniques to examine whether enzymes retain their catalytic activity when exposed to high methanol concentrations as in HPLC systems. An investigation of the activity of several immobilized enzyme columns with respect to methanol concentration were performed. The activities of free, non-immobilized, immobilized, and intra- and intermolecularly cross-linked α-chymotrypsin were studied in increasing concentrations of methanol by monitoring the hydrolysis rate of N-benzoyl-L-tyrosine ethyl ester (BTEE) at 256nm. Plots of activity vs. methanol concentration were done to determine the stability of the free, immobilized, and immobilized/cross-linked enzymes in methanol. The resultant activity plots revealed that the free enzyme yielded very little activity in a 50% methanol environment, while the immobilized enzyme column demonstrated maximum activity in 60%-70% methanol matrices. Intramolecular cross-linking techniques that were applied to the immobilized enzyme lead to inactive columns. The intermolecular techniques proved to be more successful since activity was observed in these columns up to and including 100% methanol.

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List of Symbols and Abbreviations

HPLC	High Performance Liquid Chromatography
BTEE	N-Benzoyl-L-Tyrosine Ethyl Ester
λ	Wavelength
nm	Nanometer
Km	Michaelis-Menton constant
Vmax	Maximum velocity
S	Substrate concentration
NoXL	No Cross-linking
s_e	Emergent substrate concentration
Q	Flow rate through the column
C	Reaction capacity
ln	Natural log
K'm	Apparent Michaelis-Menton constant
k_3	Breakdown rate of enzyme-substrate complex
k'_s	Substrate inhibitor constant
PCR	Post Column Reactor
Na_2HPO_4	Dibasic Sodium Phosphate
EDAC	1-ethyl-3(3-dimethylaminopropyl)carbodiimide
cc	Cubic Centimeter (mL)
mg	Milligram

Δ	Change
CG	Chymotrypsin-Glutaraldehyde complex
t_V	Residence time
η	Viscosity
t	Time
IE	Immobilized Enzyme
NaOH	Sodium Hydroxide
IMER	Immobilized Enzyme Reactor
g	Gram
mM	Millimolar
°C	Degrees Celcius
MeOH	Methanol
V_L	Void volume
[E]	Enzyme Concentration
E	Molar concentration of the enzyme in the PCR
V_t	Total volume of the packed bed
P	Amount of reacted substrate in the column
β	Column void space
CHY	Chymotrypsin
C'	Column reaction capacity
APG	Aminopropyl Glass
GLUT	Glutaraldehyde

ENZ	Enzyme
AG	Aminopropyl Glass - Glutaraldehyde Complex
AGE	Aminopropyl Glass - GLUT - Enzyme Complex
CPG	Controlled Pore Glass
TiO_2	Titanium Oxide
ZrO_2	Zirconium Oxide
Al_2O_3	Alumina
SiO_2	Silicon Oxide
h	Decreased plate height
L	Length of the reactor column
K_0	Permeability constant
mL	Milliliter
NADH	Reduced NAD^+
AMG	Amyloglucosidase
NAD^+	Nicotinamide Adenine Dinucleotide
GDH	Glucose Dehydrogenase
CME	Phenoxazine derivative modified graphite electrode
e^-	Electron
AP	Alkaline Phosphatase reactor
IDH	Inositol dehydrogenase
LDH	Lactate dehydrogenase
LOD	Lactate oxidase

HRP	Horseradish peroxidase
H_2O_2	Hydrogen Peroxide
3-HSD	3-α-Hydroxysteroid dehydrogenase
THAM	Tris-(Hydroxyaminomethane)
ED	Ethylene Diamine
TMD	Tetramethylene Diamine
M	Molar
μL	Microliter
AGEG	APG - GLUT - ENZ - GLUT Complex
σ_t	Band Broadening
dp	Diameter of packed particles
Δp	Pressure drop across the reactor bed

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Chapter 1

Introduction

The term immobilized enzyme has several functional definitions. For example, as applied to these experiments, it means the covalent attachment of an enzyme through a bifunctional agent to a water insoluble support. Another application of the term immobilized enzyme can be found in microencapsulation entrapment immobilization methods, where an enzyme is trapped inside a semipermeable membrane. The entrapped enzyme, although confined to a restricted area by the membrane, still exists in the same fashion as an enzyme in an aqueous solution.

The number of methods used to covalently bind enzymes is great, however the concepts involved in describing the enzyme are the same. These concepts include: equilibrium, covalent bonding, kinetics, polymerization, and physically changing the micro-environment of the enzyme.

Equilibrium is important because during the immobilization process the solid support is not in solution as is the enzyme. A solid-liquid phase boundary is created in which the enzyme is in one phase and the support material is in another. This poses a challenge because the phase transition energy (the interaction between the two phases) must be low enough to allow a maximum number of enzyme molecules from solution to attach to the solid support without becoming deactivated. One way to do this is to use bifunctional or multifunctional organic molecules to create a bridge between the solid support and the enzyme molecules. Figure 1 illustrates the use of a bifunctional agent to attach an enzyme to a solid support.

Once immobilized, the enzyme's micro-environment changes in several ways. First, since the enzyme is immobilized, its general structure becomes more rigid than if the enzyme were free in solution. This increased rigidity may cause the active site of the enzyme to become more defined, which would allow the substrate to fit into the active

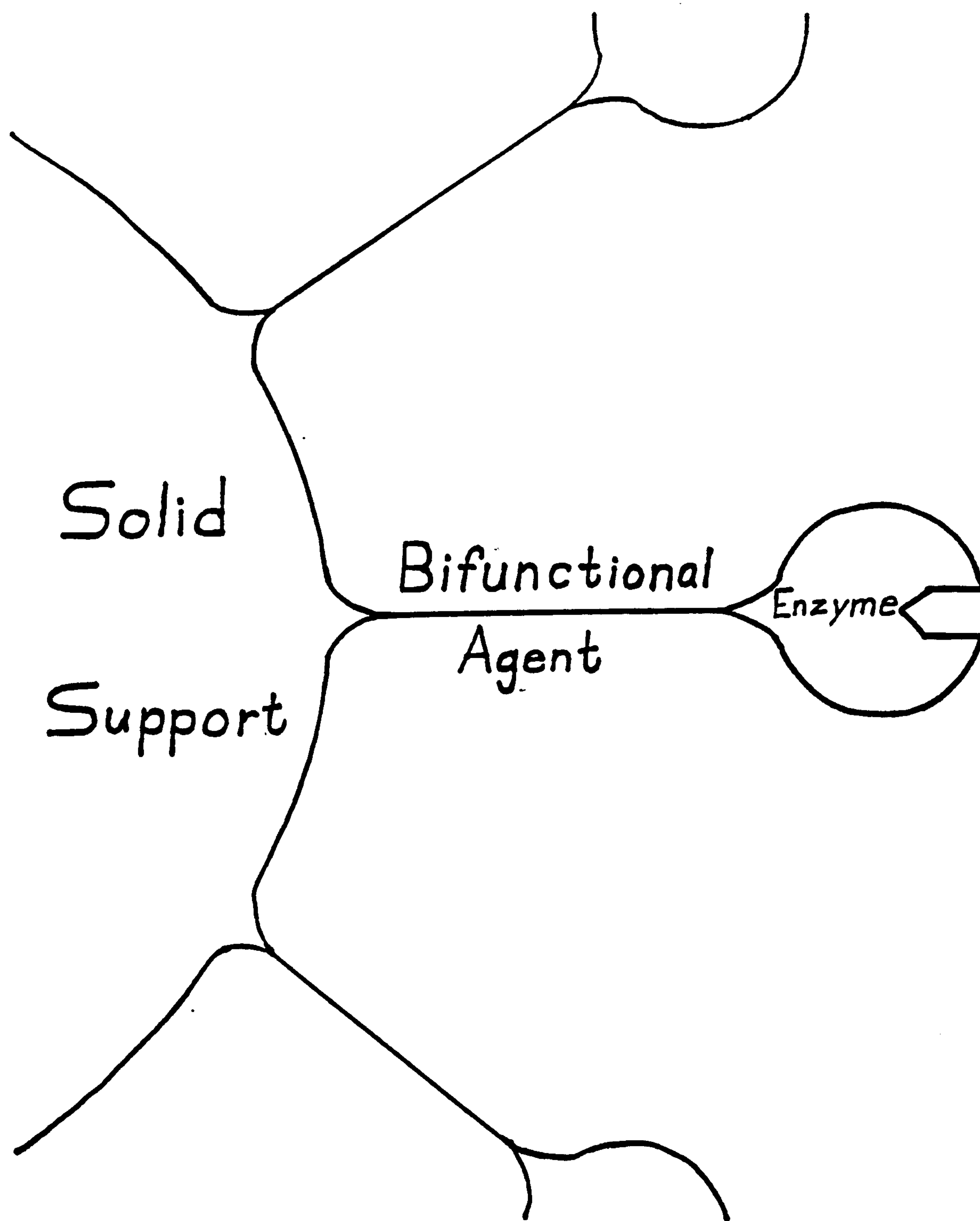


Figure 1: The linkage of a solid support to an enzyme through a bifunctional reagent.

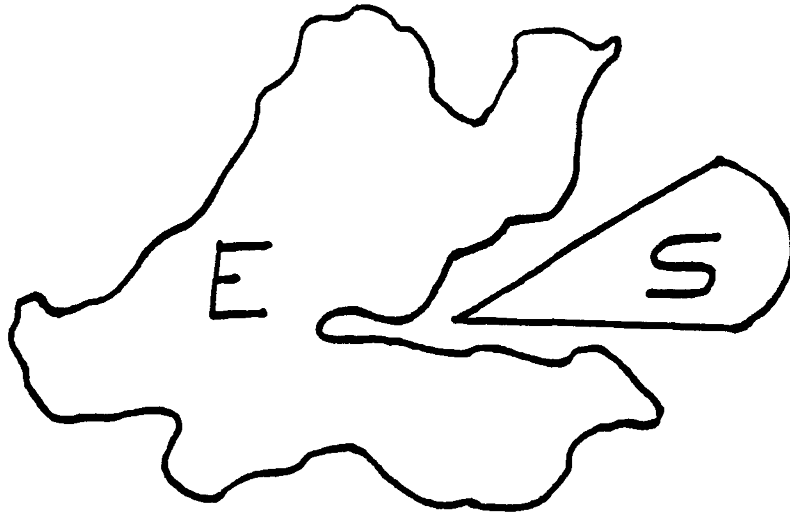
site easier, thus providing an increase in activity over a non-immobilized or free enzyme. This, however, is not the case because, for example, if the enzyme requires conformational changes for the reaction to proceed, then the activity may actually be lower than that of the soluble enzyme. Figure 2 shows the general rigidity of an enzyme that exists in an aqueous environment compared to that of an immobilized enzyme.

Another factor that affects the enzyme microenvironment is the quantity of enzyme immobilized. If not enough enzyme is used, then the immobilized enzyme may not exhibit enough activity to be practically useful. On the other hand if the quantity of enzyme is exceedingly large, then the surface of the support may be so heavily populated that activity levels are almost non-existent since crowding causes the enzyme to denature. It is also possible that the use of large concentrations of enzymes could cause denaturing to occur during even mild immobilization procedures.

The next source of enzyme microenvironment change is described below by Trevan, M.D.[1]

> "There are two distinct ways in which a polymer support might affect the micro environment surrounding an immobilized enzyme. The first may be considered a partitioning effect. By virtue of its own physical chemistry, the polymer may attract (or repel) substrate, product, inhibitor, or other molecules to its surface thus concentrating (or depleting) them in the immediate vicinity of the enzyme. The second way in which the polymer may effect the enzyme's micro environment may be by presenting itself as a barrier to the free diffusion of molecules both to and from the enzyme. Either partitioning or diffusion limitation effects may be present on their own in a given immobilized enzyme system or they may both be present, acting either synergistically or antagonistically."

The final micro-environment consideration is discussed by Bender, M.L.[2]. His work described a method that eliminated the two effects mentioned above by Trevan. It



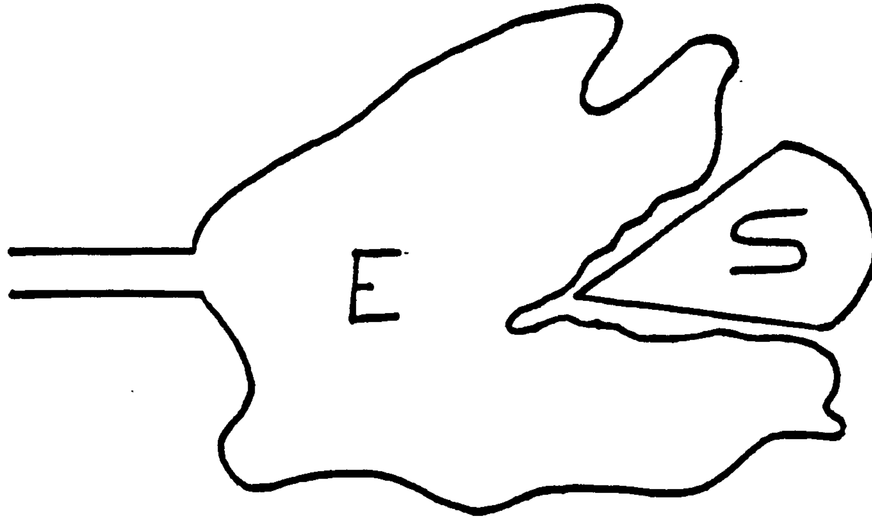


Figure 2: a) A more flexible enzyme in an aqueous solution. b) A more structured active site can be seen in the immobilized enzyme.

involved the immobilization of an enzyme on a solid support that yielded the same level of activity as observed for the soluble enzyme. To accomplish this he covalently immobilized the enzyme far enough away from the solid support so as to eliminate any interactions (constructive or destructive) between the enzyme and support. He begins the attachment process first by diazotization of the amine as reported by Wheetall[8], followed by condensation of the diazonium salt to either a phenolic tyrosine group or imidazole group of a histidine of the enzyme via an azolink[2,3]. The result of the four step process produced an immobilized enzyme that had identical catalytic properties as the soluble counterpart. By achieving the same level of catalytic activity, the kinetic treatment of this system could be executed by using either the soluble or the immobilized form.

The third concept involved is the kinetic parameters of K_m (The Michaelis-Menton constant of the enzyme for a particular substrate), V_{max} (The maximum velocity toward which the rate approaches at infinitely high substrate concentrations), S (Substrate concentration), partitioning, diffusional limitations, pH, and enzyme inhibition. These parameters are typically explained in a biochemistry text book for enzymes that are free in solution. When the enzyme is immobilized and packed into a column the explanation in terms of these kinetic entities becomes much more difficult because the enzyme is no longer free in solution. Since the amount of enzyme immobilized to the solid support is dependent upon the method and the type of enzyme employed, a direct comparison between free and immobilized enzyme would not provide an accurate picture. Studies must be performed to determine the amount of enzyme that was attached to the support before any kind of comparison may be applied. This must also be done in order to study the kinetic parameters mentioned above. Once this is done, a concentration of enzyme present in the entire system can be determined and then this can be used to express the kinetic performance of the immobilized enzyme.

Lilly and coworkers[3] described the Michaelis-Menton treatment for an immobilized enzyme column in detail. They presented equation (1) for a single substrate reaction that is the integrated form of the Michaelis-Menton equation. Where s_o= concentration of substrate, s_e= the emergent substrate concentration after flowing through a column of constant diameter at a rate Q ($^{volume}/_{unit\ time}$). C ($^{mass}/_{unit\ time}$) is the reaction capacity of the column, and K'_m= the apparent Michaelis constant. By plotting (s_o - s_e) vs. $\ln(s_e / s_o)$ with a constant flow the slope will yield the value of K'_m. The ratio $^C/_Q$ can be determined by extrapolation of the plot to $\ln(s_e / s_o) = 0$.

$$s_o - s_e = K'_m \ln(s_e / s_o) + {}^C/_Q \qquad (1)$$

They also went on to modify a substrate-inhibited single substrate enzyme equation that began with the following relationship.

$$v = \frac{k_3[E]}{1 + \left(\frac{K_m}{s}\right) + \left(\frac{s}{K_s}\right)} \qquad (2)$$

Where k_3 and [E] are the rate of breakdown of the enzyme-substrate complex and the enzyme concentration. From here a relationship is derived to reflect the behavior of the column.

$$s_o - s_e = K'_m \ln(s_e/s_o) + {}^C/_Q - (0.5K'_s)(s_o^2 - s_e^2) \qquad (3)$$

Where K'_s is the apparent substrate-inhibitor constant.

They describe the residence time, t, in relation to the void volume, V_L, and the flow rate through the column, Q, as the ratio:

$$t = V_L/Q \qquad (4)$$

and the amount of enzyme in the packed column bed as

$$[E] = E / V_t \qquad (5)$$

with E being the molar concentration of enzyme in the packed bed and V_t being the total volume of the bed.

When they combined equations 1, 4, 5, the resulting equation was:

$$(s_o - s_t) - K'_m \ln (s_t / s_o) = k_3 (E/Q)(V_L / V_t) \quad (6)$$

By designating P as the amount of reacted substrate in the column, and β as the column void space and substituting into equation (6), the following three equations result:

$$P = (s_o - s_t)/s_o \quad (7)$$

$$\beta = V_L / V_t \quad (8)$$

$$Ps_o - K'_m \ln (1-P) = k_3 (E\beta/Q) = C/Q \quad (9)$$

with the column reaction capacity $C' = k_3 E\beta$.

Lilly and his coworkers found that the column capacity and the flow rate were independent of each other, that K'_m increased with ionic strength, and that equation (9) described the catalytic action of a packed bed of immobilized enzyme.

The concepts of polymerization come into play since enzymes are proteins which are biopolymers of amino acids. Also, enzymes can be immobilized by both intermolecular and intramolecular cross-linking methods. These will be discussed later in the introduction. Another immobilization method employed is that of cross-linking enzyme molecules directly to one another thus forming active, stable, and insoluble cross-linked enzyme crystals(5). What cross-linking does for immobilized enzymes is provide another degree of rigidity above that of immobilized enzymes. Figure 3 compares the increased rigidity of a free, immobilized, and immobilized/cross-linked enzyme. Again multifunctional agents can be used, only this time as cross-linking agents.

The activity of an enzyme is representative of its catalyzing abilities. An enzyme will cause a specific reaction to occur at a certain rate depending on the concentration of

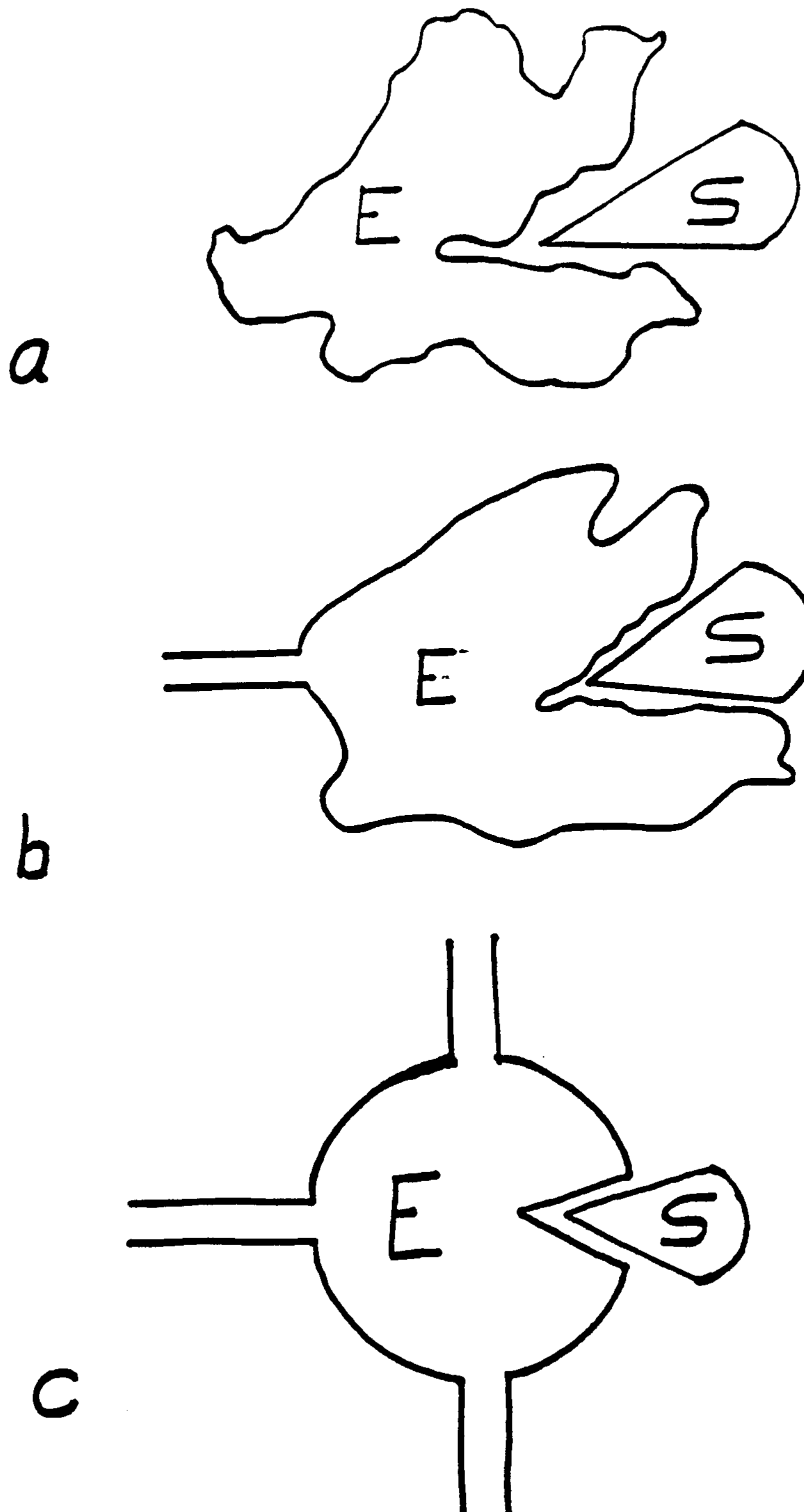


Figure 3: a) Free enzyme with the least amount of rigidity. b) Immobilized enzyme with increased rigidity. c) Immobilized/cross-linked enzyme with the most rigidity.

enzyme and substrate, as well as the kinetic parameters mentioned previously. An immobilized enzyme, since it is held in a fixed position, possesses more rigidity than its free enzyme counterpart. This instilled rigidity will cause the active site of the enzyme to also take on a more fixed position. The substrate would then ideally fit into the active site much easier and therefore react much faster than the free enzyme. This ideal case, however, does not occur. What is actually observed is a decrease in activity. This may be due to several factors.

First, if the enzyme undergoes conformational changes in order to catalyze a reaction, then immobilizing this type of enzyme would inhibit this movement thus decreasing the activity. Furthermore, by immobilizing and cross-linking the enzyme, the apparent activity would be expected to exhibit even smaller levels of activity since the conformational changes are even more restricted.

Second, during the immobilization process it is possible that some of the enzyme molecules will attach in a non-ideal fashion. Ideally the enzyme would be tied down so that the active site would be facing in a direction that would yield easy access for the substrate. What actually happens is a nonuniform attachment of the enzyme. Figures 4, 5, and 6 provide an excellent description of this process[4].

The third factor that may cause the decreased activity of immobilized enzymes is conformational. During the attachment process the enzyme may bind in a way that the active site is distorted to the point where a proper fit of the substrate is no longer possible. Also, if the enzyme must undergo a conformational change in order for catalysis to occur then its observed activity would decrease because its movement is now restricted.

The fourth and final consideration in the decreased activity of immobilized enzymes is that of cross-linking. When an enzyme is first immobilized the attachment is not completely uniform (as can be seen in Figures 4, 5, and 6). But when cross-linking techniques are employed, three additional things can happen. First, the cross-linking agent used may bond to the active site thus blocking substrate penetration. This can be

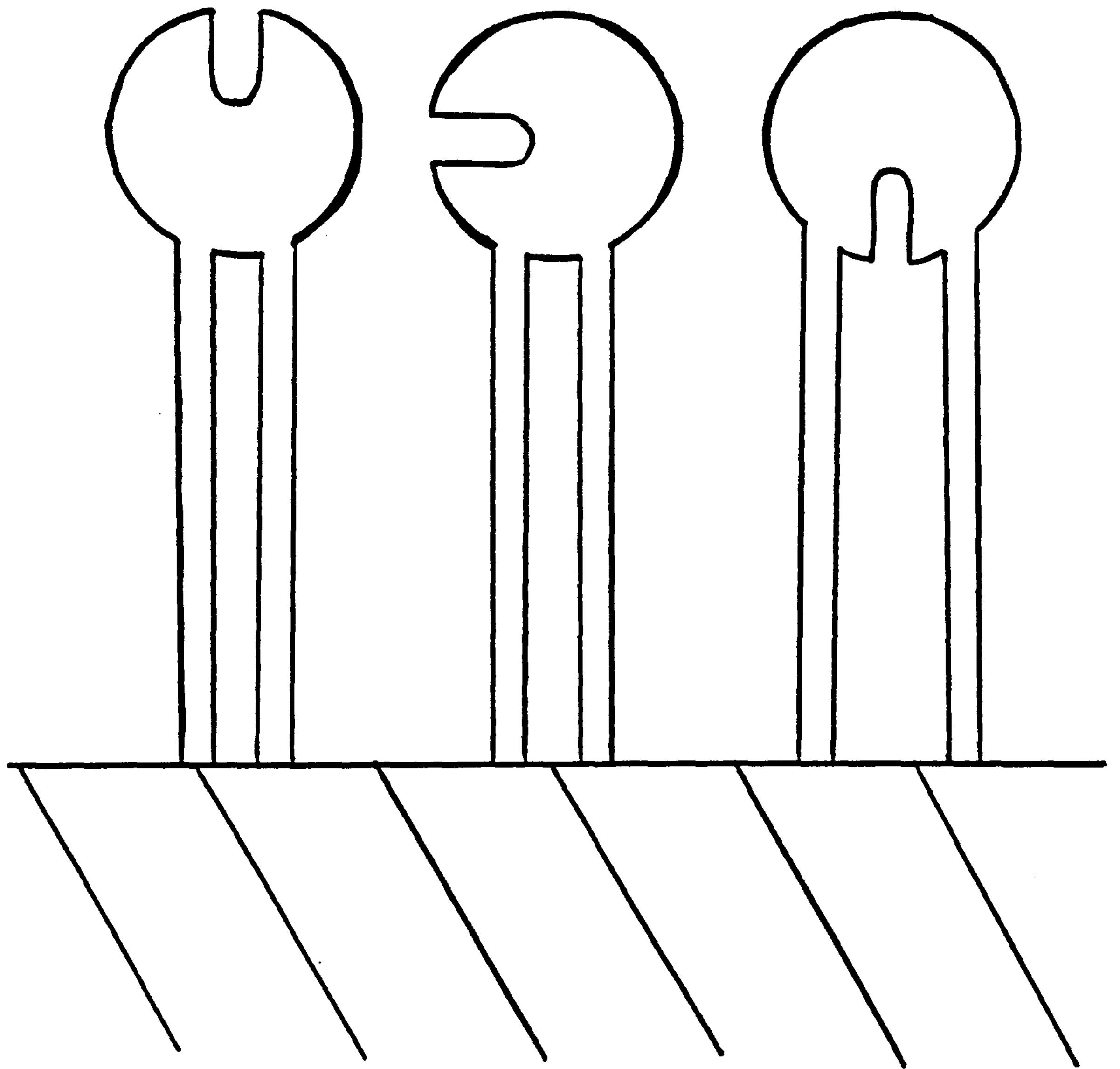


Figure 4) The non-ideal attachment of the enzyme is shown above with the active site facing directly into the mobile phase, partially blocked, and completely blocked. Random covalent binding causes a heterogeneous distribution of the enzyme on the solid support surface.

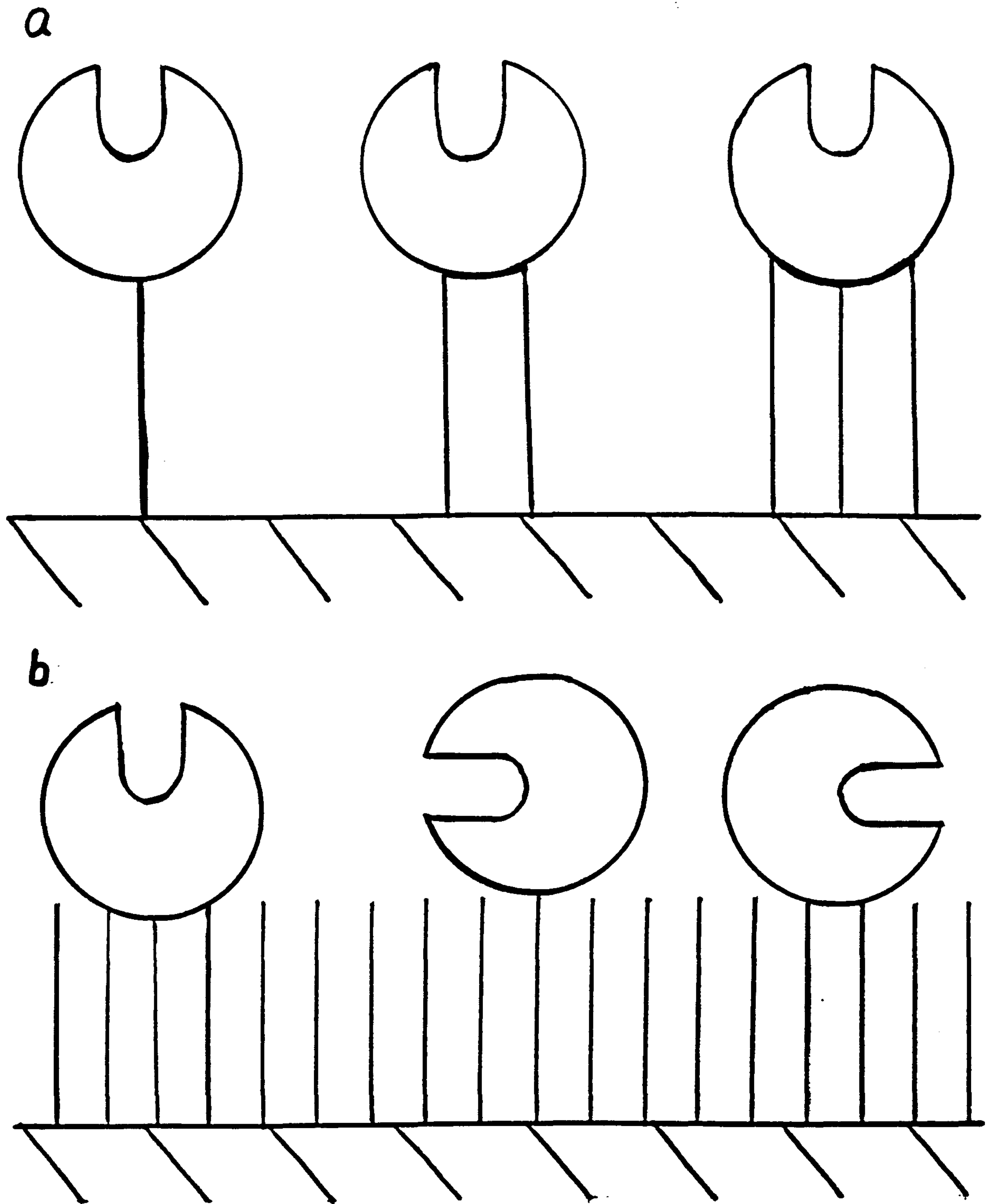


Figure 5) a) If the bridge between the solid support and the enzyme is not uniformly distributed then when immobilized , some enzyme molecules will be bound by one link, two links, four links, etc. (b) Groups on the enzyme's surface that are reactive with the bridge may not be evenly distributed thus causing a heterogeneous bed of immobilized enzymes.

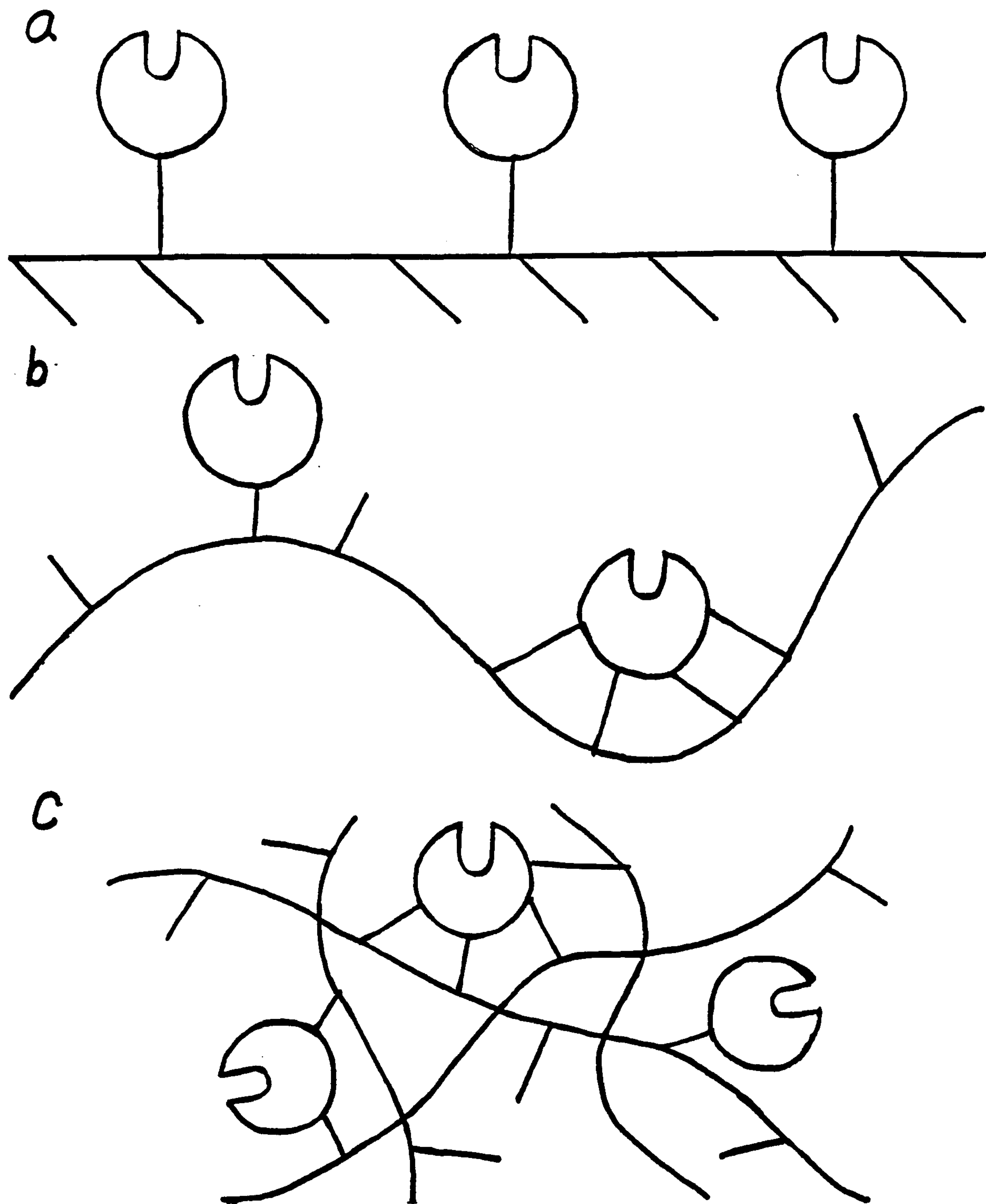


Figure 6) Here, a variety of support materials are shown to induce certain degrees of heterogeneous attachment. A flat, solid surface should be better than an irregular surface. Porous and fibrous supports often exhibit the least amount of binding uniformity.

avoided by choosing a cross-linking agent that does not favorably react with those amino acid residues in or near the active site. Second, the active sites of the non-ideally attached enzymes may be blocked by the intermolecular cross-links that form thus denying substrate the opportunity to react with those enzyme molecules. Third, a blanket effect may occur in which the cross-linking agents form a coating on top of the immobilized enzyme layer. This covering then inhibits enzyme-substrate interaction.

The immobilized enzyme will also be more stable (i.e. more resistant to changes in pH, solvent concentrations, and other denaturing considerations). An immobilized/cross-linked enzyme would then be even more stable since it is not only tied down to a solid support, but is also part of a cross-linked polymer matrix.

The micro-environment of the enzyme in all three scenarios is quite different. In the free enzyme system, the micro-environment is nothing more than the amino acid residues of the enzyme interacting with the solvent directly, which leads to an increase in enzyme stability. With an immobilized enzyme the solvent effects are not as prevalent because a number of the enzyme's amino acid residues are not able to interact directly with the solvent because they are bonded to or in close proximity to the support material. An immobilized/cross-linked enzyme has a significantly less number of interactions with the solvent since it is not only bound to the support material but also may possess intermolecular cross-links, intramolecular cross-links, or a combination of both. This restricts the degree of interaction the amino acid residues may have with the solvent. These cross-links also help maintain the primary, secondary, tertiary, and quaternary structure that the enzyme possesses, which makes the enzyme much more resistant to situations that would otherwise denature the enzyme.

Types of Immobilized Enzymes

Covalent Attachment: In order to immobilize an enzyme it must be attached to a support material of some type. Types of support materials will be discussed later in the introduction. Since covalent attachment techniques of water soluble enzyme molecules

via nonessential amino acid residues to water insoluble, functionalized supports is the most prevalent method for immobilizing enzymes[6], and also the method used in this project, it will be discussed first.

Covalent bonding fundamentally involves the sharing of electrons. When a solid support such as controlled-pore glass (CPG) is used it must first be prepared by attaching a small molecule like an aminopropyl group to it. This provides a viable link that can be used to attach a multifunctional bridge between the CPG and the enzyme (in this case α-Chymotrypsin). Glutaraldehyde (GLUT), a bifunctional organic molecule with two aldehyde groups can be used as the bridge. Robinson, Dunnill, and Lilly[7] studied and proved that α-chymotrypsin(CHY) immobilized to aminopropyl controlled-pore glass(APG) via the carbonyl group of GLUT[8] is in fact a covalent attachment. Figure 7 shows the covalent reactions involved with the immobilization of CHY to APG via GLUT.

The above illustrates a typical covalent immobilization via a bifunctional agent. The protein will employ amino acid residues that contain an amine group to form the covalent bonds with the GLUT. It is for this reason that GLUT works nicely with CHY. CHY does not have any amino acid residues with amine groups near the active site. Therefore the attachment process will not bind up the active site and thus render the enzyme inactive.

***Adsorption**:* The adsorption of an enzyme onto a water insoluble support involves several key parameters such as pH, solvent characteristics, ionic strength, protein and adsorbent concentration, and temperature[9]. It is also the simplest way to immobilize an enzyme because the primary necessity is that an aqueous solution of enzyme comes in contact with a surface active adsorbent such as alumina, anion exchange resins, cation exchange resins, cellulose, glass plates, as well as many others[10].

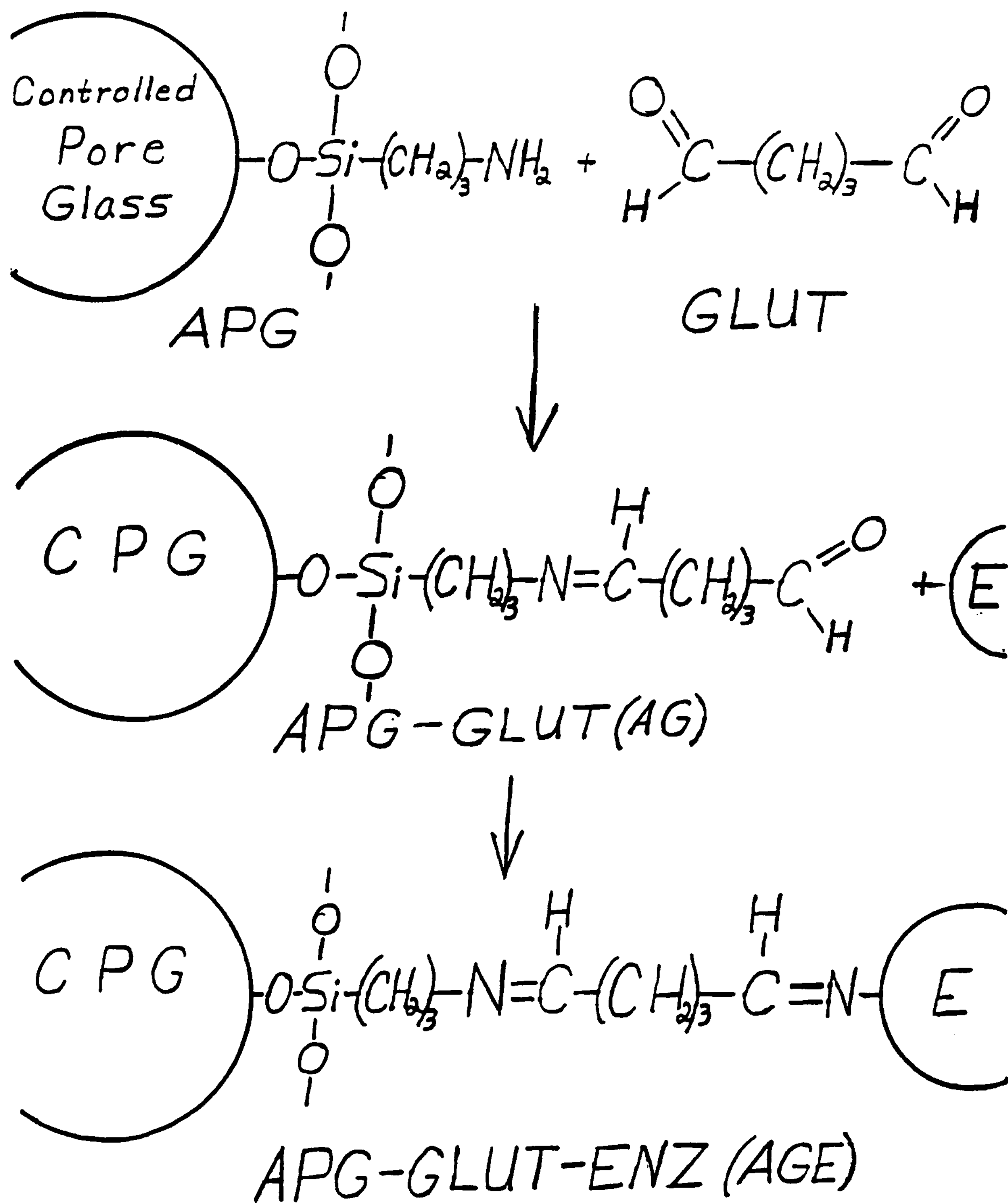


Figure 7) Reactions involved in the covalent attachment of CHY to APG via GLUT.[8]

Some of the advantages of adsorption immobilization include simple immobilization procedures that usually do not deactivate the catalytic activity of the enzyme, reversible adsorption processes, and finally, it is possible to separate and purify enzymes during immobilization[9]. On the other hand, there are some disadvantages that have limited the usefulness of this technique. Covalent attachment is a chemical method where as adsorption is a physical method that tends to bind enzymes weakly. Also, because the interaction between the enzyme and the support is weak, the amount of enzyme that may be bound is small. Since the enzyme is entrapped in a matrix, the support material may affect the interaction of the substrate with the enzyme. Finally, the parameters of pH, ionic strength, the matrix the immobilized enzyme is adsorbed to, and temperature[9] are very important because the adsorbed enzyme will only be slightly more resistant to changes in these parameters than the free enzyme. Zittle[11], James and Augustine[12], McLauren and Coworkers[13-15], and Hummel and Anderson[16] have studied the effects of these parameters extensively.

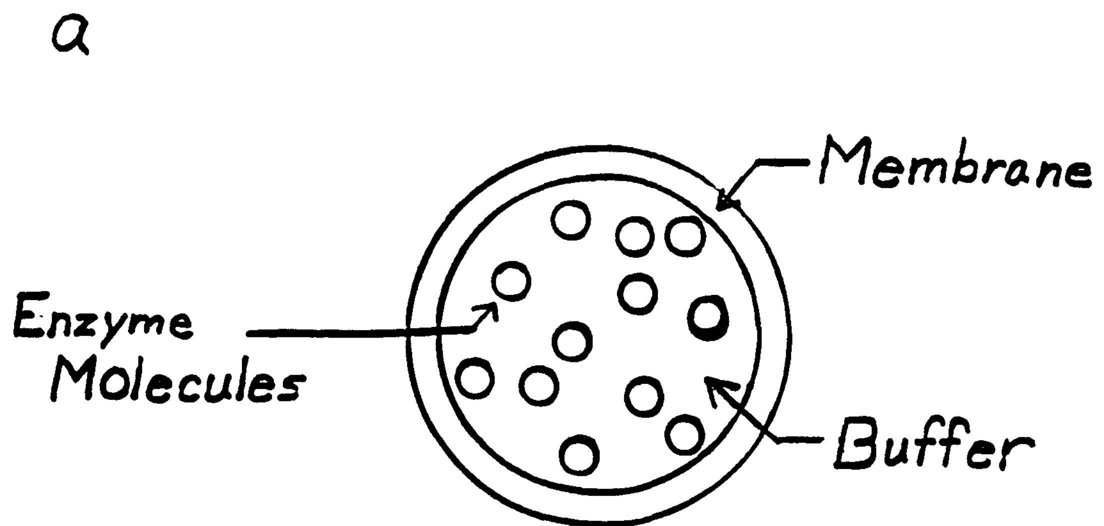
CHY has been adsorption immobilized to several supports such as: carboxymethyl ether cellulose[17], cellulose nitrate[17], cellulose phosphate[17], glass(no activity observed)[18], kaolinite[19-22], and metal coated glass plates with barium stearate and/or uranyl acetate[23, 24]. Each support exhibited different adsorbing capabilities as well as different activity levels. Which one was best depended upon the application.

Microencapsule Entrapment: Microencapsulation entrapment is an immobilization process that is quite different in that the enzyme molecules are not bound to a solid support like the covalent and adsorption methods. The enzyme molecules, along with buffer, are entrapped within a semipermeable membrane that is prepared by using emulsion polymerization techniques. An aqueous solution of enzyme molecules is first emulsified in an organic solvent matrix that may contain one or more organic solvents[25].

This matrix must contain a surfactant that is soluble in organic solvents[25]. The organic-solvent-surfactant-enzyme mixture is then mixed vigorously which results in a polymer membrane that contains the enzyme molecules. The reason that the organic matrix may contain one or more solvents is due to specific gravity considerations. If the density of the organic matrix is greater than one, then the microcapsules tend to float at the top of the solution[25]. On the other hand, if the solvent density is less than one, then the microcapsules will sink to the bottom of the vessel thus causing an extreme, nonreversible aggregate[25]. Figure 8 on the next page illustrates a typical microencapsule immobilized enzyme system.

Other considerations in the microencapsulation entrapment immobilization of enzymes are the thickness, permeability, mechanical strength of the membrane, and the entrapped enzyme characteristics. Membrane thickness and permeability are dependent upon the organic matrix composition, rate of formation of the microencapsules, and the concentration of chemicals used to synthesize the membrane[25]. The entrapped enzymes exhibit characteristics similar to those of aqueous enzyme solutions. This similar behavior is due to the fact that the entrapped enzymes are not chemically modified and exist within the membrane as free in solution[25]. They do however, display differences in activity levels which is due to rate of mass transfer of substrate and products through the membrane.

Support Materials: Support materials for immobilizing enzymes are just as important as the enzyme being immobilized. There are both organic and inorganic as well as natural and synthetic support materials available. An example of an organic support was mentioned in the microencapsulation section above. The membranes that comprise the microcapsules are made of organic polymers such as nylon 6,10[25]. Enzymes may also be immobilized via entrapment in organic cross-linked polymer matrices like polyacrylamide gels. Both the microencapsule and the cross-linked polymer gel supports tend to display a lower order of crystal structure since they are more flexible



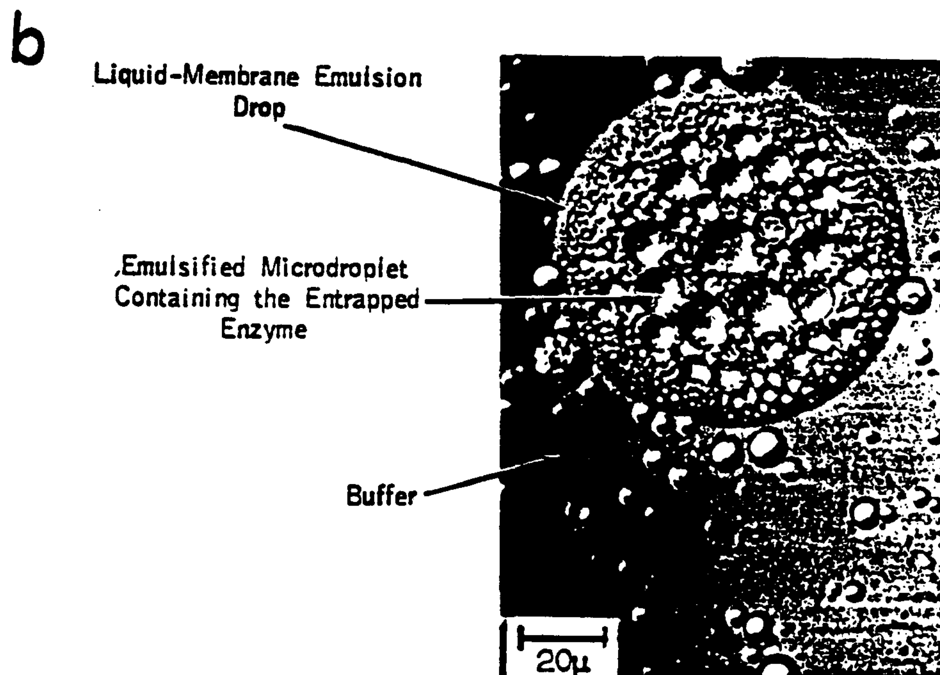


Figure 8: a) A typical microencapsule entrapped immobilized enzyme. b) An interface contrast photomicrograph of liquid surfactant membrane microcapsules containing phenolase. Prepared by T. Hucal and S.W. May[25].

and compressible than their inorganic counterparts. Most inorganic solid supports such as CPG and alumina are more rigid than the polymer supports, therefore creating "specialty-like" applications for each support. D.L. Eaton[8, 26] discusses a decision tree that can be of great help in choosing a support that will fit properly in an immobilized enzyme system. This tree is presented below.

Immobilized Enzyme Solid Support Decision Tree

1) Does the pore morphology permit the entry of the enzyme?
2) Can the enzyme be immobilized on the support?
3) Is the immobilized enzyme durable in a) acid b) base c) high salt?
4) Can the support material be conveniently handled?
5) Does the carrier have compression strength?
6) Is the maximum enzyme loading adequate for the system?
7) What is the maximum tolerable pressure drop?
8) How is the above affected by particle size, flow rate, and particle shape?
9) What is the operational half life of the system?
10) How is the half life affected by temperature, pH, and other conditions?
11) Under what conditions can the derivative be stored?

Polymers: Polymers can be used for covalent and adsorption immobilization, microencapsulation entrapment, gel entrapment, and the coimmobilization of enzymes. For the enzyme CHY there are forty-six parent polymers to which the enzyme may be covalently bound. These supports range from polyacrylamide[27] to cellulose[28] to sephadex[29]. A few polymers that may be used for adsorption immobilization are carboxymethyl cellulose[17], CPG[18], anion, and cation exchange resins. In microencapsulation immobilization the membrane that holds the enzyme inside is a semipermeable polymer. The most popular is a nylon prepared by a condensation reaction of hexamethylene diamine and sebacoyl chloride[30-34]. Others are cellulose nitrate[31-37, 40-42], polystyrene[31, 40], and ethyl cellulose[43]. Immobilizing enzymes by entrapment uses polymers exclusively to trap enzyme molecules within the cross-links of the polymer matrix. Some of the materials used to entrap CHY are poly(N,N'-methylene

bisacrylamide)[44], and silicone rubber[45]. A primary advantage to using polymers comes from the nature of a polymer. Polymers are made of many repeating units. Those repeating units in the polymer will allow for a more uniform distribution of enzyme throughout the matrix.

Gels: Gels are primarily used in enzyme entrapment applications with the most widely known system being that of polyacrylamide gels. These gels are made by the polymerization of acrylamide and N,N'-methylene bisacrylamide. The fashion in which an enzyme is incorporated into the gel is relatively simple. The polymerization reaction is performed in the same manner as for synthesizing a polyacrylamide gel, only now the enzyme is introduced to the reaction mixture. When the polymerization occurs the enzyme becomes entrapped in the polymer matrix.

Ceramics: Ceramics, including CPG and alumina, are the primary inorganic support used for the immobilization of enzymes. Ceramic supports are an excellent choice for immobilized enzymes in post column reactors in High Performance Liquid Chromatography (HPLC) because they are the only supports rigid enough to withstand the pressure in an HPLC system. The ceramic supports are resistant to microbial degradation and also they can be prepared with strict control over pore diameter, and mesh size. They can be made of TiO_2, ZrO_2, Al_2O_3(alumina), and SiO_2(CPG). While these are all categorized as ceramics, they can have quite different applications. For example, if an immobilized enzyme system is going to be run in the alkaline pH range, TiO_2 may be a better choice than CPG because of durability factors[8].

Cross-Linking: Cross-linking is the best way to decrease the freedom of motion of a molecule. Enzymes already have a small amount of cross-linking in them. They are usually disulfide linkages between amino acid residues that help the enzyme retain its three dimensional structure. These links are not very strong and when an enzyme denatures, it is usually due to their breakage and the subsequent relaxing or unfolding of the enzyme into a randomly oriented chain of amino acid residues.

There are two ways cross-linking can occur. First is intermolecularly, or between molecules. When applied to enzymes, these cross-links would occur between nonessential amino acid residues on adjacent enzyme molecules. These cross-links provide added stability to the structure of each enzyme molecule involved. Figure 9 on the next page shows how the intermolecular cross-links give an extra level of structural strength to enzymes immobilized to a solid support.

Quiocho and Richards(46) have done cross-linking studies of an enzyme system and have shown that there is in fact a significant increase in the mechanical stability after the enzyme is cross linked. They included diffraction pattern studies that prove that the intermolecular cross-links cause only a small change in the general molecular structure of the enzyme. The primary concern is that if the cross-linking agent is too small, it could possibly denature the enzyme by stretching it out, or if the cross-linking agent is too long then the hydrocarbon chain may induce partitioning effects on the substrate.

The intramolecular cross-linking of enzymes involves the use of cross-linking reagents to add additional structural order to the enzyme. The reason for this is because this type of cross-link is internal to the molecule. Both ends of a usually small organic molecule become attached to certain amino acid residues (depending on which cross-linking agent is used) within the same enzyme molecules. Cross-links that form add a great amount of strength to the tertiary structure of the enzyme because they are much stronger than the already present disulfide bonds that aids in the prevention of the unfolding of the amino acid chain. Figure 10(47) schematically illustrates the fundamentals of intramolecularly cross-linking, which is based on the principal thermodynamic quantity for this system - entropy. Entropy is mainly in effect when the enzyme denatures. By strategically placing cross-links within the enzyme the potential for disorder decreases.

There are many more considerations involved with intramolecular cross-linking methods as opposed to intermolecular cross-linking techniques. For instance, the size of

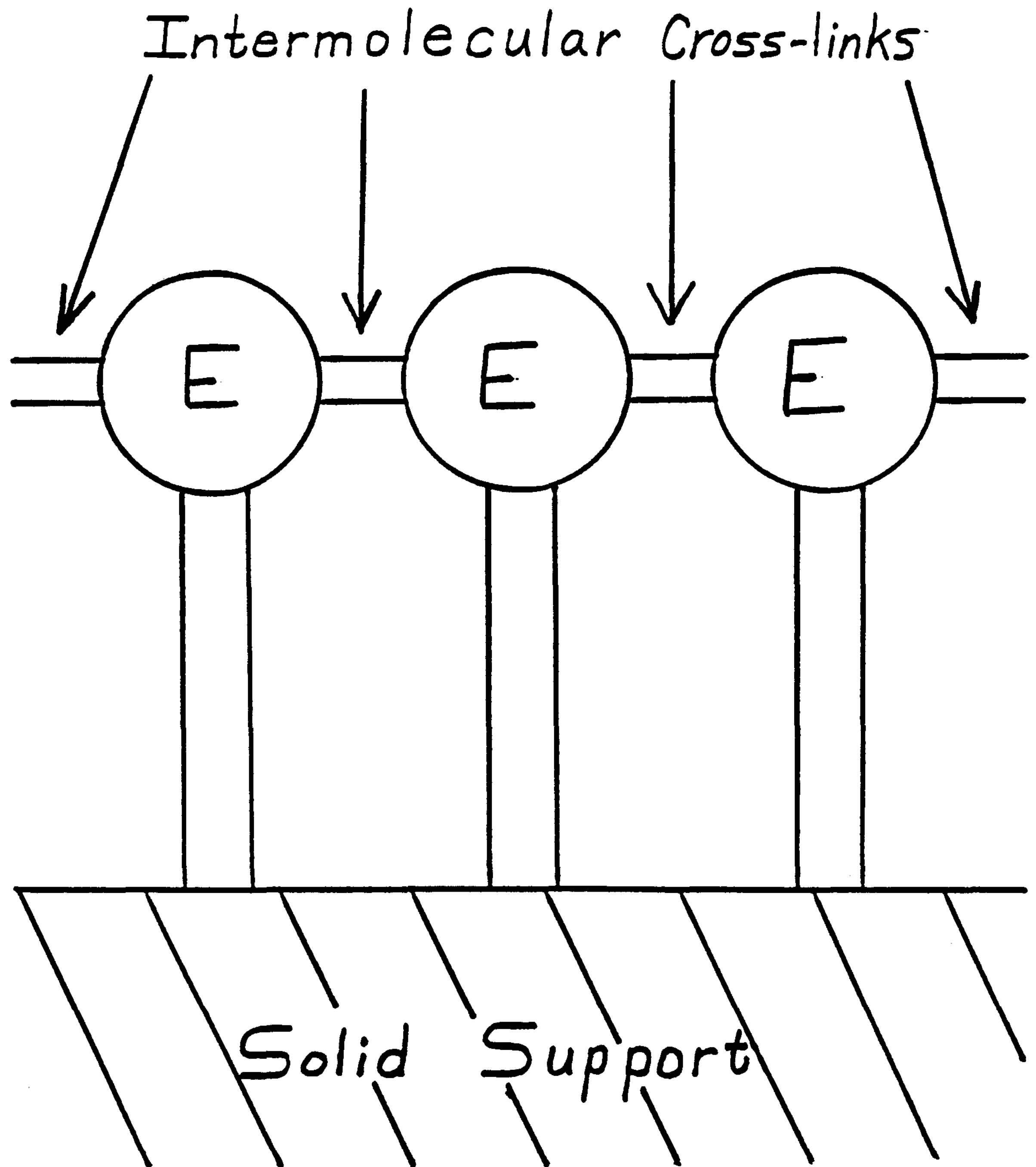


Figure 9) Intermolecular cross-links between covalently immobilized enzymes add stability to the enzyme structure. The cross-links are shown above with a double line.

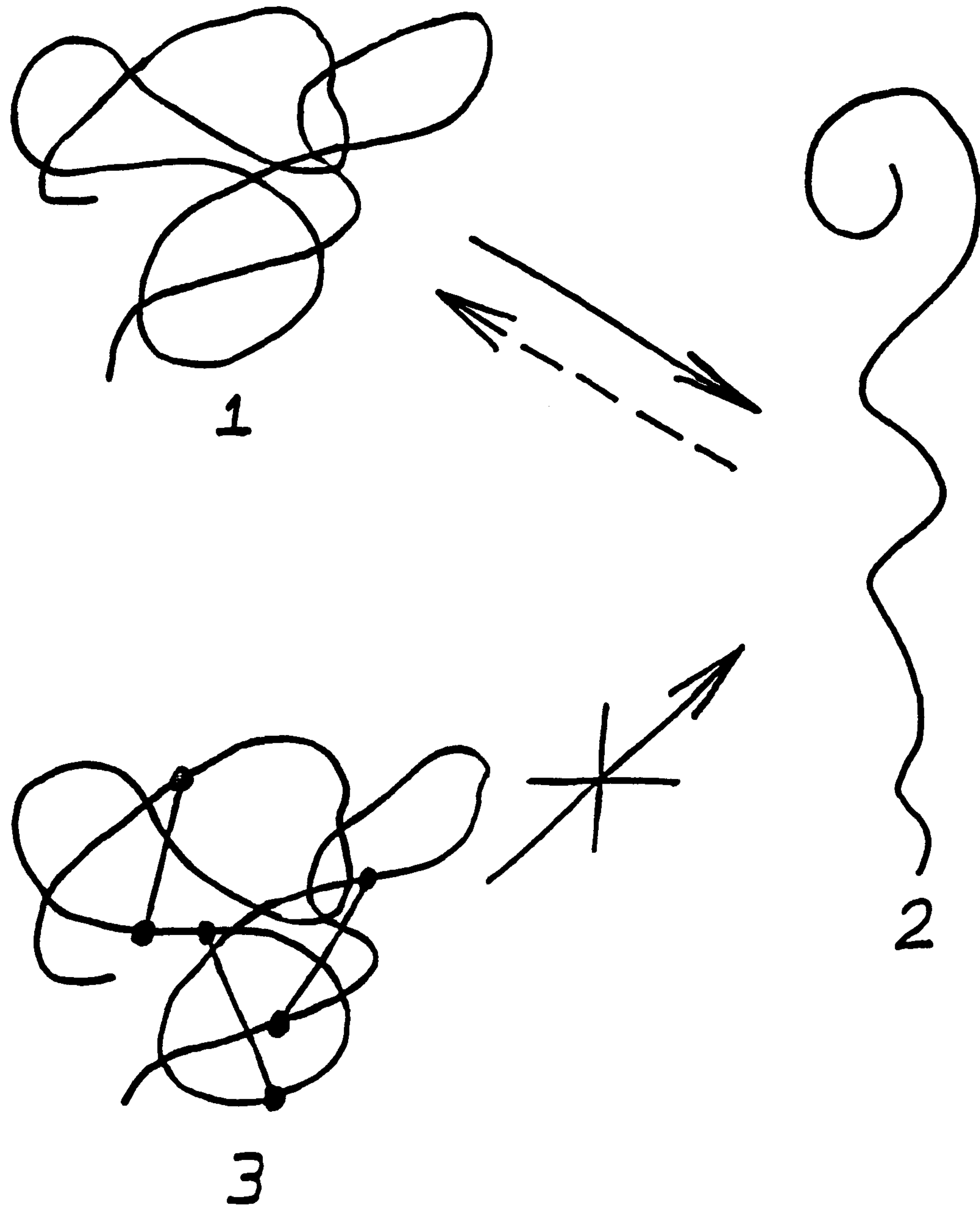


Figure 10) 1, An enzyme in its original form possibly containing a certain degree of hydrogen and disulfide bonding can denature into 2. 3, An intramolecularly cross-linked enzyme cannot denature.

the cross-linking agent is important since it will be within the enzyme molecule. If it is too long or too short it may deactivate the enzyme or cause some other form of cross-linking to occur, however choosing a cross-linking agent of optimal length can pose a challenging and/or frustrating situation.

Martinek, K., and Torchilin, V. P., [47] propose three ways to optimize the number of intramolecular cross-links: first, the optimal length of the cross-linking molecule may actually be chosen[47, 49, 50], or the enzyme may be premodified by substituting additional reactive groups for the enzyme's surface[49, 51]. Finally, a mixture of multifunctional reagents (preferably bifunctionals) with different lengths could be used which would allow the enzyme to choose the best cross-linking agents automatically[51].

Multifunctional Agents: Multifunctional agents are compounds that have more than one functional group that can be used in a reaction. The simplest of this category are the bifunctionals. They consist of two reactive groups that are usually located at opposite ends of the molecule. The most popular bifunctional agent, which was mentioned earlier, is GLUT. It contains two aldehyde groups separated by three methylene groups. Aldehydes in general react well with amine groups. GLUT also follows this principle, so it will react with the amine groups on the amino acid residues of the enzyme under mild conditions as can be seen in Figure 7 and as demonstrated by Wheetall[8]. GLUT may be used not only as a bridge between the solid support and the enzyme, but as both an intra- and intermolecular cross linking agent[46]. In serving as an intermolecular cross-linking agent, GLUT reacts nicely with amine groups on the surface of the enzyme molecule. This reaction, as illustrated in Figure 11a, causes one of the aldehyde groups of the GLUT to bond with the amine group of an amino acid residue on the surface of the enzyme, while leaving the second aldehyde group of the GLUT molecule to react in a variety of ways. One possible way is that if another enzyme molecule is in close proximity to the open aldehyde moiety and has an amine group on its surface, the second enzyme molecule may become linked to the first one through the

GLUT bridge. Another possibility can occur if the enzyme molecules are too widely spread out on the surface of the solid support. When the enzyme molecules are dispersed in this fashion the GLUT bridge is too short to do the job. A solution to this problem can be reached by using another bifunctional molecule with an amine group on each end to fit between the two GLUT aldehyde groups. This bifunctional's amine groups would react with the open end of the GLUT molecules thereby forming a longer intermolecular cross-link (Figure 11b). The length of the bifunctional amine must be considered since the enzyme molecules on the support's surface could be greatly distorted otherwise (see Figures 35 and 36). Other bifunctionals that can be used in the same fashion include carbodiimides(47), diisocyanates(8), and diimidoesters(8).

Another class of multifunctionals that are widely used are the trifunctional compounds. Trifunctionals, as their name implies, have three reactive functional groups. The most popular ones in use are the triazine compounds such as cyanuric chloride(52, 53, 54). Trifunctionals are used primarily in immobilization procedures as a bridge between the support and the enzyme. They are rarely used as cross-linking agents, if at all.

POST COLUMN REACTORS

Post Column Reactors (PCR's) are used to enhance the detection of analytes in HPLC systems. This is accomplished by inserting a column after the analytical column and before the detector. These columns contain a derivatization source such as a catalyst that can be used over and over to convert the analyte from a difficult to detect compound to an entity that can be seen by the detector with relative ease.

On-line PCR's have several advantages(55) including the actual reaction that takes place in the column. This reaction does not need to go to completion or be stable. The on-line reaction just needs to be reproducible. Next, the sample can be separated without special treatment thus allowing the use of already established HPLC procedures. Multidetection methods can be employed to enhance selectivity and sensitivity. For example, one detector can be placed between the analytical column and the PCR and a

different detector can be placed after the PCR, and finally, typical HPLC detection limits can be decreased by at least two orders of magnitude by employing a PCR. The most significant disadvantage of adding a PCR is the loss of chromatographic resolution through an increase in band broadening.

In a packed bed reactor a column is packed with small glass beads (or other solid support material) containing a coating of the reactive material. Since the column contains a packed bed of material it can be considered, under nonretention conditions, an HPLC column in and of itself. Band broadening is then described by the following equation which illustrates that a decrease in particle size or an increase in residence time can reduce band broadening.[55]

$$\sigma_t = \left(t_v h dp / L \right)^{1/2} \qquad (10)$$

Where h is the decreased plate height, dp is the diameter of the packed particles and L is the reactor column length.[55]

Another consideration is the drop in pressure across the bed of the reactor which is related to the same considerations as in equation (10).

$$\Delta p = \eta L^2 / K_o t_v dp^2 \qquad (11)$$

Where K_o is the permeability constant with a value typically between 0.001-0.002.[55]

In order to practically employ these two equations for a packed bed reactor, reactions that usually take longer than 20 seconds yields a good medium between band broadening and pressure drop effects.[55]

For this project an enzyme immobilized to glass beads and packed into a column served as a reactor similar to that used for HPLC. The enzyme was immobilized and then

cross-linked in several different ways and assayed for activity. Also studied was the stability of the immobilized and cross linked enzyme in different concentrations of methanol.

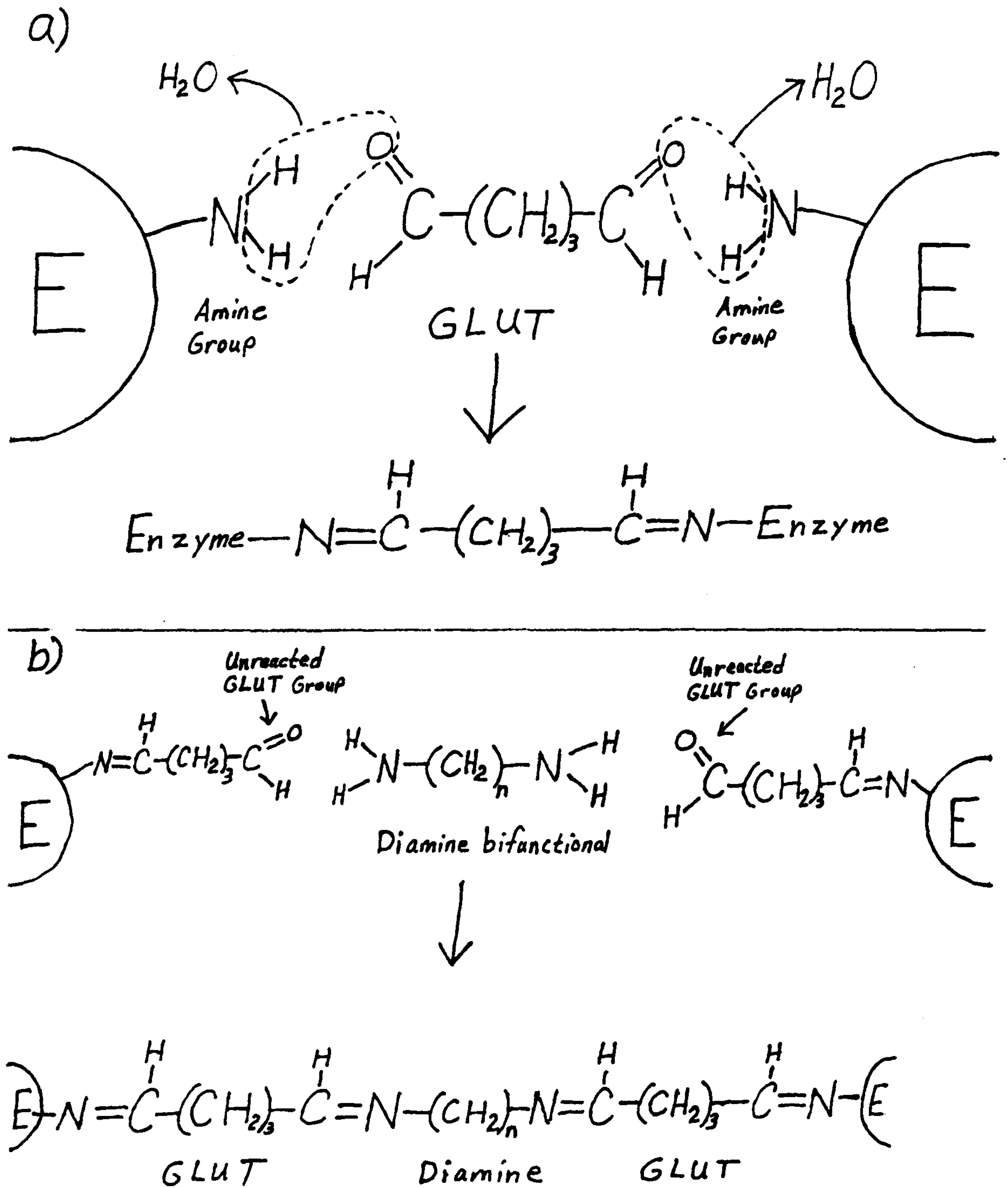


Figure 11) a) Bifunctional reaction of GLUT between two enzyme molecules to form an intermolecular cross-link. b) Reaction of a diamine bifunctional between two open ended GLUT molecules to form an intermolecular cross-link.

Chapter 2

Historical

The development of immobilized enzymes (IE's) came about in the late 1940's. Since then, both the immobilization techniques and the applicability of IE's have been extensively studied. One application, though relatively new, introduces IE's to the world of post-column reactors for enhanced detection in HPLC. The way this is accomplished involves using a packed-bed reactor in which a support is coated with a layer of enzyme, rendering it immobilized, and then packing the active material into a column. This column can be inserted after the analytical column in an HPLC system and used to convert the separated components into easily detectable products, thus allowing for more sensitive and selective analyses.

In 1966, Hicks and Updike(57) were one of the first teams to use IE's in a column application for chemical analysis. They immobilized the enzyme to a polyacrylamide matrix that led to a block polymerized enzyme-gel compound. This compound was then mechanically dispersed into very small particles, washed, and lyophilized. The lyophilized particles had a final size of 20-40 units. The gel could then be rehydrated and slurry packed into a 1 mL syringe for use. The column was used to react with the substrate in an on-line analysis scheme. Sample would flow through the column, react, mix with a coloring agent and be detected photometrically.

Some early work in the use of a catalyst in a packed-bed reactor was done in the late 1970's by J.F. Studebaker(56). His work led to a method of detection for thiols and disulfides. This was accomplished by causing the compounds to release a chromophore from the packed bed in a column placed after the analytical column in an HPLC system. The chromophore could then be easily detected.

A group led by Potter(58) was the first to use immobilized enzyme reactors with electrochemical detection for the analysis of acetylcholine and choline from rat brain tissue in 1983. They accomplished this by immobilizing acetylcholinase and cholinase to

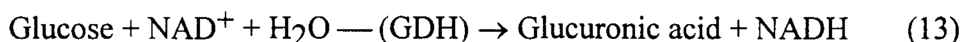
solid supports and also to anion-exchange resins via adsorption. Acetylcholinase and cholinase would produce hydrogen peroxide that could be amperometrically detected.

Carbohydrates, both large and small, can be difficult to chromatograph due to poor selective detection and separation. Immobilized enzyme reactors have helped solve these problems when coupled with various LC methods. Huang and Kissinger[59] designed a system using glucose oxidase as a PCR that produced hydrogen peroxide. This system enabled the detection of glucose in serum, foods, and in vivo microdialysis.

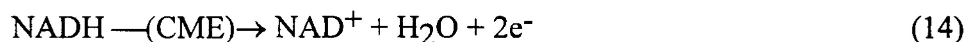
Immobilized glucose dehydrogenase was used to determine glucose and lactose in penicillin fermentation broths[60]. With the setup employed here, six other mono- and disaccharide entities could be analyzed[61].

Amyloglucosidase was used as a PCR for the analysis of maltooligosaccharides by hydrolyzing the α–(1,4), α-(1,6), and α-(1,3) glucosidic bonds from the non-reducing end of di-, oligo-, and polysaccharides[62, 63, 64]. The immobilized enzyme reactor produced NADH that was oxidized and detected[65, 66, 67] by the following reactions.

$$\text{Oligosaccharide} \text{ — (AMG)} \rightarrow (\beta\text{-D-glucose})_n \qquad (12)$$



$$\text{Glucose} + NAD^+ + H_2O \text{ — (GDH)} \rightarrow \text{Glucuronic acid} + NADH \qquad (13)$$



$$NADH \text{ —(CME)}\rightarrow NAD^+ + H_2O + 2e^- \qquad (14)$$

Marko-Varga et.al.[68, 69] designed a chromatographic system for the analysis of myo-inositol and inositol phosphates by using three immobilized enzyme reactors in series and an electrochemical detector. The reaction scheme is shown in Figure 12.

The analytical column used anion exchange with the first PCR being alkaline phosphatase (PCR1), the second being a co-immobilized inositol dehydrogenase, lactate dehydrogenase, and lactate oxidase (PCR2), and the third containing horseradish

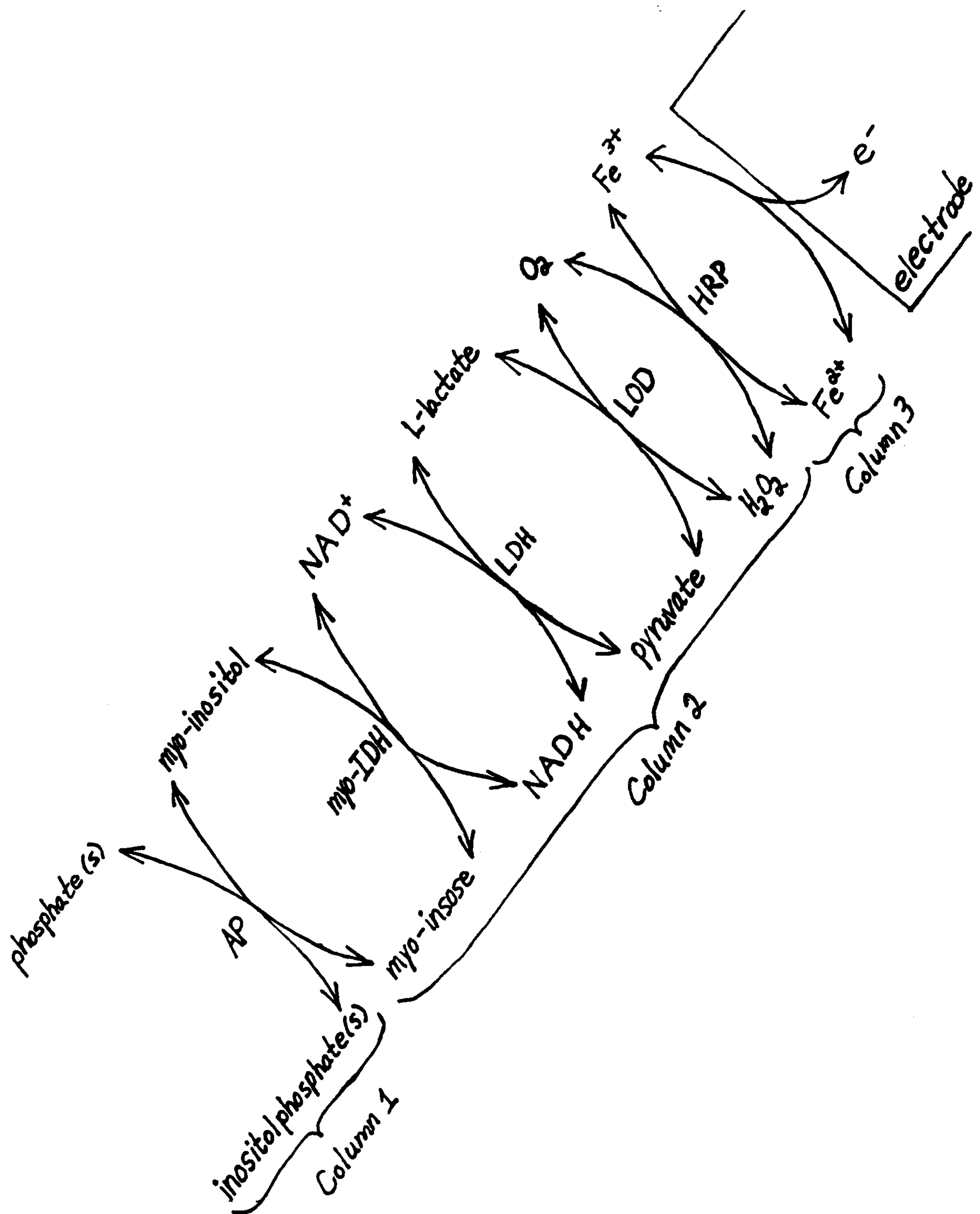


Figure 12) Three immobilized enzyme post-column reactor reaction mechanism for the electrochemical determination of inositol phosphate.

peroxidase. This complex system resulted in a linear quantification from 30μM to 10mM with 20μL sample injection volumes.

For the analysis of bile acids an expensive enzyme, 3-α hydroxysteroid dehydrogenase (3-HSD), was immobilized and used to oxidize the 3-hydroxy group in the bile acid to form a keto group. At the same time NAD^+ was reduced to NADH, which could be detected electrochemically [70] or fluorometrically[71, 72, 73, 74].

Other examples of immobilized enzyme post column reactors can be found in the analysis of xanthine and hypoxanthine[75], purine bases and their nucleosides[76, 77, 78], nicotine amide bases[79], glucuronides and cyanogenic glucoronides[80], alcohols[81, 82], amino acids[83, 84, 85, 86], adenosine phosphates [87], creatinine[88], and urea[89].

On a more fundamental level, chymotrypsin's action as a hydrolase is well known. It hydrolyzes peptide bonds where the carbonyl group is contributed by amino acids containing an aromatic ring. Chymotrypsin can cleave ester linkages and also simple amides[90].

The aromatic ring of the substrates of chymotrypsin seems to be necessary for position purposes as illustrated in Figure 13[90]. Proof of this stems from the fact that chymotrypsin will exhibit activity on synthetic substrates that contain large hydrophobic alkyl groups instead of the aromatic groups of the amino acids.

Structurally, chymotrypsin is comprised of three polypeptide chains that are covalently attached through two disulfide cross links as can be seen in Figure 14. Chymotrypsin is synthesized through trypsin's hydrolysis actions on chymotrypsinogen. As a result of trypsin's reaction with chymotrypsinogen, two dipeptide groups are removed from positions 14-15 and 147-148 (Figure 14).

The amino acid residues that give life to chymotrypsin are histidine 57 and aspartic acid 102 from the B chain and serine 195 in chain C. While these residues are far apart on the linear chain, they have been determined to be in close proximity of each other in the actual enzyme structure[90].

Substrate

Hydrophobic Positioning Group

R

HC – C – X

O

Susceptible Acyl Bond

Positioning Site

Catalytic Site

Active Site

Chymotrypsin molecule

$C_6H_5-CH_2-CH(NH_3^+)-C(=O)-NHCH_2COO^-$ Peptide

$C_6H_5-CH_2-CH(NH_3^+)-C(=O)-NH_2$ Amide

$C_6H_5-CH_2-CH(NH_3^+)-C(=O)-OCH_3$ Ester

Figure 13) Description of how substrate molecules fit into the active site of chymotrypsin with an illustration of the susceptible acyl bond. Also shown are the general forms of peptide, amide, and ester substrate molecules.

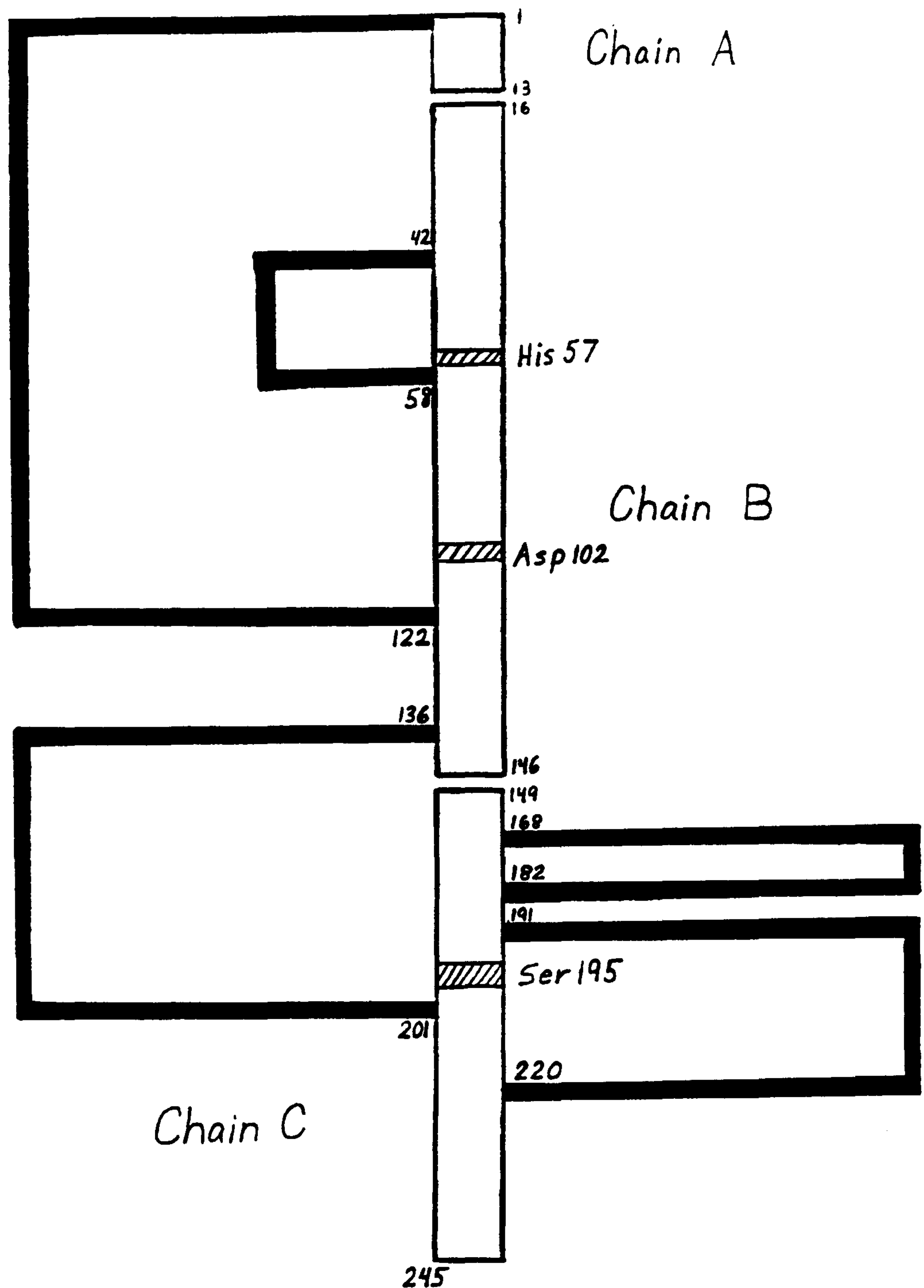


Figure 14) Linear model of chymotrypsin showing the three peptide chains A, B, and C, disulfide bonds between residues 1 and 122, 42 and 58, 136 and 201, 168 and 182, 191 and 220, and also the three amino acid residues responsible for the catalytic activity (His 57, Asp 102, and Ser195). While these three residues are far apart in the chain, they are actually in close proximity to each other in the three dimensional structure.

Chapter 3

Statement of Problem

Immobilized enzyme reactor systems have allowed for the development of more selective and sensitive analysis methods. One of the primary concerns is that of enzyme stability and reactivity in a vast array of matrices.

In one application, an immobilized enzyme is packed into a column and used as a post-column reactor in HPLC analyses. To perform analyses by HPLC methods, a mobile phase usually containing a certain mixture of aqueous and organic components is required. One of the most common organic solvents used in mobile phases is methanol. Methanol, being less polar than water, readily denatures enzymes. All of the current HPLC/immobilized enzyme reactor (IMER) systems combat the denaturing effects of the mobile phase with the use of a mixing tee between the analytical column and the IMEC. This mixing tee is used to introduce specific reagents that allow for sample detection, like fluorescing agents, and also to dilute the mobile phase to an aqueous/organic ratio that will not destroy the enzyme column (usually 20-30% organic).

The need for diluting the mobile phase through the mixing tee provides useable analysis methods, but does suffer some significant drawbacks like dilution effects. In order to eliminate the dilution effects present, the immobilized enzyme in the column must be stabilized. By immobilizing the enzyme, it automatically becomes more resiliant, but not enough to exhibit significant activity in mostly organic mobile phases. The enzyme needs to be modified. Enzyme modification must be done carefully so as to retain its catalytic properties. Immobilization alone can decrease the activity of the enzyme molecules, so further changes can also decrease the enzyme's ability to function.

Therefore, the concept of enzyme immobilization coupled with specific intra- and intermolecular cross-linking techniques should provide a stable and active column when assayed in high concentrations of methanol. Several bifunctional agents were used in conjunction with various cross-linking procedures to provide a column or columns with appreciable activity levels in high methanol concentrations.

Chapter 4

Materials and Apparatus

Table 1: This table contains a list of all chemicals and equipment used including lot numbers, model/catalog numbers, and supplier where applicable. All chemicals used were of analytical reagent quality. All equipment was run through periodic calibrations to ensure accurate and reliable data.

Materials/Equipment	Lot Number	Model/Catalog #	Supplier
Tris(Hydroxyaminomethane) (THAM)	4833	T 1503	Sigma, St. Louis , MO
N-Benzoyl-L-Tyrosine Ethyl Ester (BTEE)	69F5400	B 6125	Sigma, St. Louis, MO
Calcium Chloride Dihydrate ($CaCl_2 \cdot 2H_2O$)	KHJY	4160	Mallinckrodt, Paris, KY
α-Chymotrypsin (EC 3.4.21.1) (CHY)	81H7155	C 4129	Sigma, St. Louis, MO
Hydrochloric Acid (HCl)	7647-01-0	VW 3110-3	VWR Scientific, Pittsburgh, PA
Methanol, Anhydrous, HPLC Grade	H09259	9093-03	Baker, Phillipsburgh, NJ
Sodium Phosphate, Dibasic (Na_2HPO_4)	702409	S-374	Fisher Scientific, Pittsburgh, PA
Controlled Pore Glass, Aminopropyl (APG)	34H8250	G-5019	Sigma, St Louis, MO
Sodium Hydroxide Pellets (NaOH)	34145030	SX 0590-1	EM Science, Gibbstown, NJ
Glutaraldehyde (GLUT)	34H0318	G-6257	Sigma, St Louis, MO

Materials/Equipment	Lot #	Model/Catalog #	Supplier
Siliconized Glass Wool	364	191022	Regis, Chicago, IL
Deionized Water Reagent	NA	NA	YSU Reagent
Succinic Anhydride	D931	868	Kodak, Rochester, NY
Ethylene Diamine	724202	E 479	Fisher Scientific, Chicago, IL
1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC)	82H0782	E-7750	Sigma, St Louis, MO
Tetramethylene Diamine	24H3400	P 7630	Sigma, St Louis, MO
pH Meter Calibration Buffers 7, 10	S10611	566575	Beckman, Fullerton, CA
pH Meter	NA	720A	Orion, Boston, MA
pH Combination Electrode	NA	91-56	Orion, Boston, MA
pH Electrode Storage Solution	2-0029	05664-00	Cole Parmer, Niles, IL
Magnetic Stirrer	NA	04639	Cole Parmer, Niles, IL
Automatic Pipettes 100µL - 10mL	NA	NA	Ranin, Ridgefield, NJ
Variable Peristaltic Pump	NA	LP-1	Amicon, Danvers, MA
Phar-med Solvent Resistant Sample Lines	1800092641	95701-48	Cole Parmer, Niles, IL

Materials/Equipment	Lot Number	Model/Catalog #	Supplier
Computer	NA	Personal XT	IBM, White Plains, NY
Diode Array Spectrometer	NA	8452A	Hewlett Packard, Newark, DE
Spectrometer Computer Software	NA	89530A	Hewlett Packard, Newark, DE
Quartz Sample Cuvette 1cm	NA	6Q	Beckman, Fullerton, CA
Quartz Sample Flow Cell	NA	NA	Beckman, Fullerton, CA
Analytical Balance	NA	AE 100	Mettler, Toledo, OH
1cc Syringes	NA	309597	Becton Dickinson & Co., NJ
Drierite Dessicant Mesh Size 8	NA	23005	Hammond Drierite Co., Hammond, OH
Analytical Glassware	NA	NA	NA
Parafilm	NA	PM 992	Cole Parmer, Niles, IL
Isocratic Water Bath	NA	910	Fisher Scientific, Chicago, IL
#42 Filter Paper	0566	NA	Whatman, Maidstone, England

Chapter 5

Experimental

Enzyme Assay and Enzyme Methanol Profile

Reagents: The reagent solutions used in the enzyme assay and enzyme methanol profile, as well as their preparation are listed below.

80 mM THAM Buffer pH=7.8	0.96 g THAM dissolved in 100 mL Deionized Water. pH adjusted with 1M HCl.
1.18 mM BTEE Reagent	18.5 mg BTEE dissolved in 31.7 mL Methanol and 18.3 mL Deionized Water
2 M Calcium Chloride	5.5 g $CaCl_2$ in 25 mL Deionized Water
1 mM HCl	16.8 µL of 11.9 M reagent HCl in 200 mL Deionized Water
2-5 units/mL CHY in cold 1 mM HCl	5.5 mg CHY in 50 mL 1 mM HCl

Procedure

Enzyme Assay(54,55): This method of analysis was a continuous monitoring of the reaction between CHY and the substrate BTEE by diode array spectrometry.

In two quartz cuvettes (blank and test) 1.42 mL of THAM buffer, 1.40 mL BTEE, and 0.08 mL $CaCl_2$ were added using Ranin automatic pipettes. These cuvettes were equilibrated to 25°C. In the mean time, a spectrometer blank scan was done with THAM, $CaCl_2$, and HCl. In the blank cuvette, the absorbance at λ=256 nm was monitored until constant. Once constant, 0.10 mL of 1 mM HCl reagent was pipetted into the cuvette and mixed three times by inversion. This cuvette was then placed back in the spectrometer and its absorbance was measured for ten minutes. The blank was removed from the spectrometer and the test solution was inserted. The absorbance was monitored until it was constant, then 0.10 mL of the enzyme solution was pipetted into the cuvette and this solution was mixed three times by inversion. The cuvette was placed back into the

spectrometer and the absorbance was recorded for ten minutes. In a three milliliter reaction mixture the final concentrations of the reagents were 38 mM THAM buffer, 0.55 mM BTEE solution (30% methanol), 53 mM $CaCl_2$, 0.033 mM HCl, and 0.2-0.5 units of CHY[55].

The graphs of the blank and test runs, shown on the next page in Figure 15, were plotted using linear regression analysis. From these plots, the slope for each was determined. The two slope values were then used in the equation shown below, as provided by Sigma Chemical Co., St. Louis, MO[58] for the calculation of units/mg of enzyme. The millimolar extinction coefficient for BTEE at 256nm is 0.964.

$$\frac{\text{Units of Enzyme}}{\text{mg of Enzyme}} = \frac{\Delta_{256}\,\text{Test} - \Delta_{256}\text{Blank}}{(0.964)\,(\text{mg enzyme} / \text{mLreaction mixture})} \tag{4}$$

Soluble Enzyme Methanol Profile: After the enzyme assay was performed, a methanol profile of free enzyme was done. That is, the stability of the enzyme towards increasing methanol concentrations was examined. To accomplish this, the same procedure was followed as performed for the enzyme assay above except the overall concentration of methanol was modified. The assay method was repeated in the same fashion as before except instead of having 30% methanol in the final test solution, 1% methanol was used. This was used as the starting point instead of 0% methanol because BTEE was not soluble in water. After this run was completed and the spectra recorded, the final methanol concentration was increased to 10% methanol in a new sample and run again. This was repeated until no catalytic activity could be detected. All reagents were made in the same fashion as mentioned in the original assay method. The only solution that was changed was the BTEE water-methanol composition. This meant that the concentration of BTEE was not altered in any way, but the methanol/water ratio was

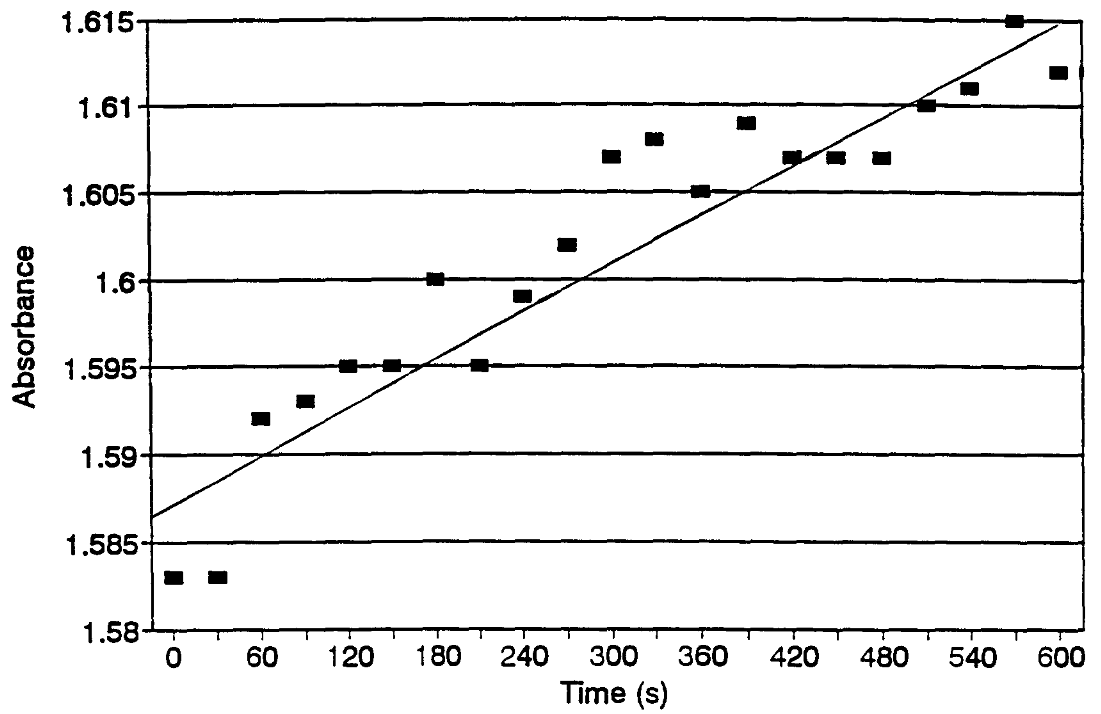


Figure 15) The plot of the data from the enzyme assay of time vs. absorbance for the blank scan.

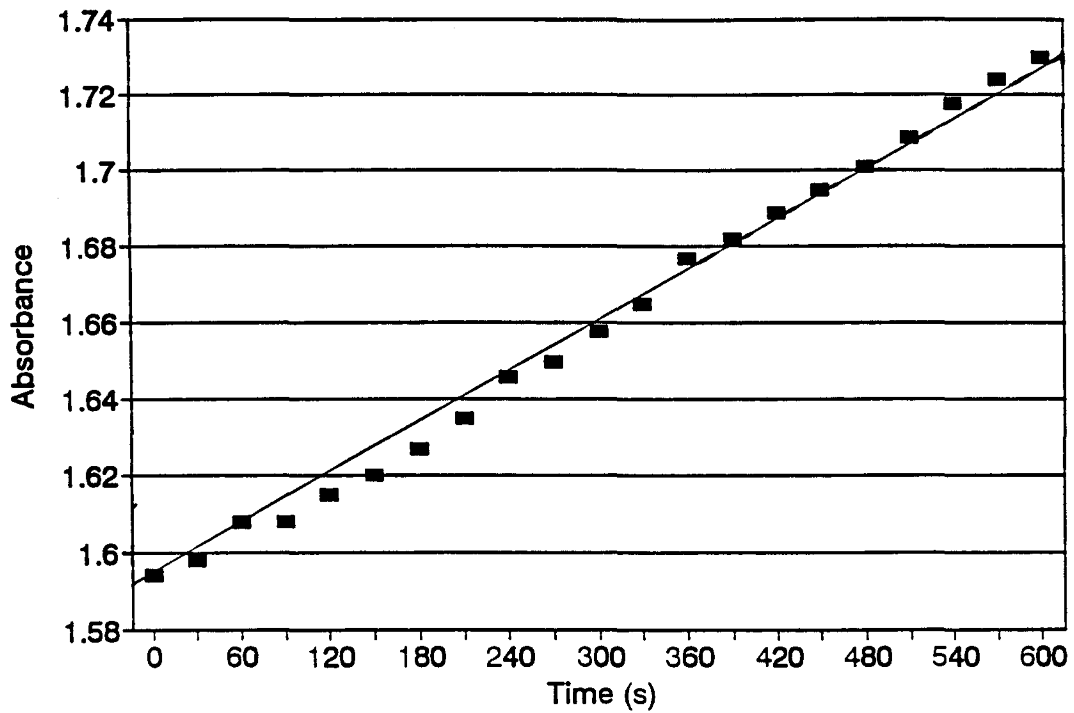


Figure 16) The plot of the data from the enzyme assay of time vs. absorbance for the test scan.

changed to yield final methanol concentrations of 1%, 10%, 20%, 30%, 40%, and 50%. Table 3 lists the composition of the different BTEE solutions. Only 10 mL of each was made in order to reduce the volume of waste generated.

The method as mentioned above was again used. After the absorbance was measured for all the sample runs, the activity was calculated for each run by using formula (1) given above. Once the activity was calculated for each sample, a plot was made comparing the activity of each run vs. % methanol. This plot was converted to % activity vs. % methanol, with the activity at 1% methanol being considered to be 100% activity. The resulting plot in Figure 17 below shows the denaturing effect methanol exhibited on the enzyme. The decrease in activity fell to near zero around 50% methanol.

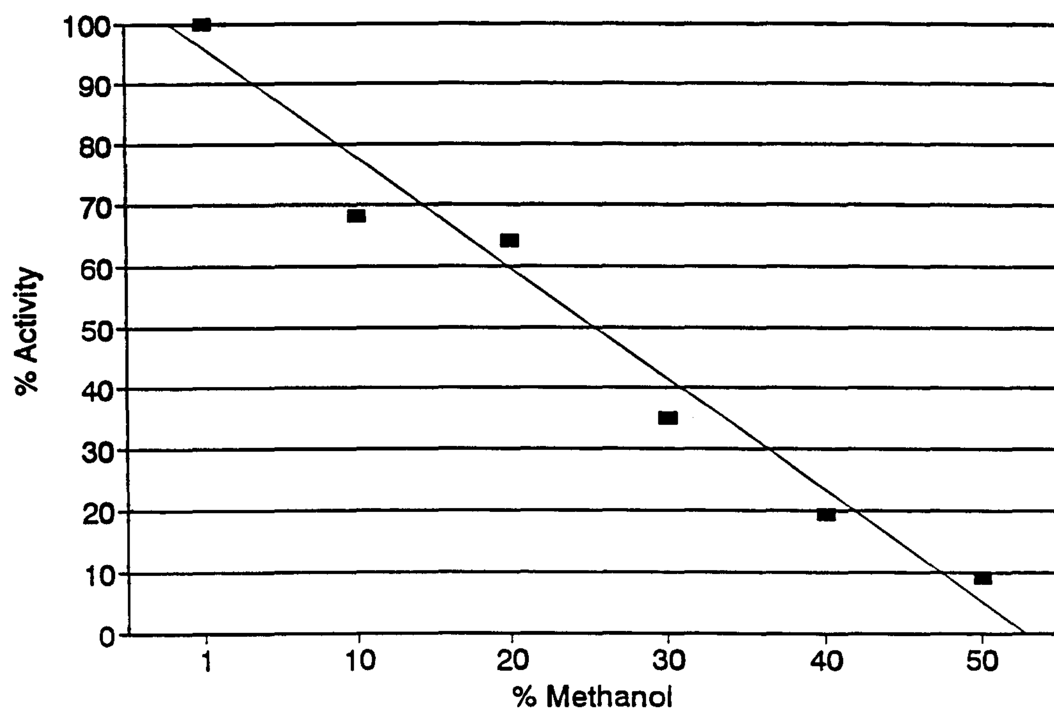


Figure 17) A plot consisting of % activity vs. % methanol shows the effect methanol has on the catalytic activity of CHY.

Immobilized Enzyme Preparation[8] and Methanol Profile

Reagents: The reagent solutions used for the preparation and analysis of the immobilized enzyme are listed below.

Aminopropyl controlled-pore glass	Purchased from Sigma
6 M HCl	50.0 mL reagent HCl was diluted to 100 mL with deionized water
0.05 M Na_2HPO_4 buffer pH=7.8	3.5 g Na_2HPO_4 was dissolved in 500 mL deionized water and adjusted to pH=7.8 with 6 M HCl
Enzyme Solution	0.2 g CHY was dissolved in 2.0 mL of 0.05 M Na_2HPO_4 buffer pH=7.8
2 M $CaCl_2$	55.368 g $CaCl_2$ was dissolved in 200 mL deionized water.
2.5% GLUT Solution	2.50 mL of 25% GLUT reagent was dissolved in 22.50 mL 0.05 M Na_2HPO_4 buffer pH=7.8
BTEE Solution	0.74 g BTEE was dissolved in 10.0 mL of HPLC grade methanol
THAM Buffers	Made from 0-100% Methanol.

Immobilized Enzyme Column Preparation: The original method[8] begins by adding silane groups to the surface of the CPG in order to allow further attachments to occur. It has now become available and much more convenient to purchase the CPG in this already activated form which is known as amino propyl controlled-pore glass (APG). APG makes the immobilization process much quicker, from two days to a few hours.

500 mL of the Na_2HPO_4 buffer was made up first by placing 3.5 g of Na_2HPO_4 in 500 mL deionized water and then adjusting the pH to 7.8 with a Cole Parmer 4390 series magnetic stirrer, an Orion 720A digital pH meter and 91-56 combination pH meter,

and 1.0 M HCl. Once this buffer was made the APG was weighed on a Mettler AE100 balance and then placed in a 50 mL beaker containing the 2.5% GLUT solution and covered with Para-Film. This initial reaction involves the attachment of one end of the GLUT molecules to the aminopropyl groups of the APG and can be seen in Figure 7 of the introduction. The reaction was allowed to proceed for one hour. When time had expired a Buchner funnel was used to filter the material. The APG-GLUT (AG), which had turned color from white to a purple-wine color, was placed on the filtration apparatus with a Pasteur pipette and washed with 1000 mL of deionized water. The AG was then transferred to the enzyme solution containing 0.2 g enzyme in 2.0 mL Na_2HPO_4 buffer at pH=7.8 by drawing up the enzyme solution into a Pasteur pipette and, with tweezers used to hold the filter paper over the beaker of enzyme solution, washing the AG down into the beaker. The filter paper was discarded and the AG in the enzyme solution was covered with Para-Film and allowed to react for four hours. The AG/enzyme complex (AGE) was placed on another piece of #42 filter paper and washed with 200 mL of Na_2HPO_4 buffer at pH=7.8. Once washed the AGE was placed in a 1cc tuburculin syringe, with a siliconized glass wool plug at the end, by slurry packing. The washed AGE was transferred to a beaker containing 10 mL of Na_2HPO_4 buffer at pH=7.0. The AGE was drawn up with a Pasteur pipette and inserted into the 1cc syringe. The AGE would settle while the buffer would remain on top. This was repeated until all of the AGE was packed into the syringe as Figure 18 illustrates. Figure 19 shows a schematic diagram of the diode array spectrophotometer.

Immobilized Enzyme Column Methanol Profile: Now that the column synthesis was complete, the developed methanol profile procedure was performed. To analyze the activity of the column the following set up was constructed. First, a Fisher Scientific 910 water bath was connected to a water jacket (a spiraled glass tube) for the column, then to the Hewlett Packard 8452A diode array spectrometer cell compartment, then to a sample reservoir, and finally back to the water circulator (Figure 19). Although all the lines of

the water bath were wrapped with bubble wrap foam insulation, the path length the water had to travel was substantial, so a temperature profile was done at each site. To do this the water bath was turned on and set to the factory preset of 37°C. A thermometer was placed in each of the three sites. The water was circulated for fifteen minutes and tempreature readings were taken every five minutes at each site. Table 4 yields the temperature profile of the system. Since the 37°C setting produced temperatures significantly lower, the water bath was switched from the factory preset to the variable mode. The dial on the water bath was set to 40°C because the profile at 37°C gave temperatures 3°C too low at each site. The temperatures at each site were then measured again to ensure the proper thermal requirements of the analysis procedure.

Table 2: Temperature profile of the column analysis system.

Time (s)	Water Bath Temperature Setting $^{\circ}$C	Column Water Jacket	Cuvette Holder	Sample Reservoir
5	37.0	30.8	30.6	25.0
10	37.0	34.1	33.8	31.2
15	37.0	34.7	34.1	33.6
5	40.0	35.9	35.2	34.6
10	40.0	36.6	36.0	35.7
15	40.0	37.3	36.7	36.4

After the temperature profile was established, the column was placed into the water jacket. The first assay sample was prepared. Since much more enzyme was assumed to be immobilized than was used in the assay and free enzyme methanol profiles, the BTEE concentration was increased as well. In the scale up process it was found that the same ratio of BTEE/enzyme could not be achieved so a maximum amount

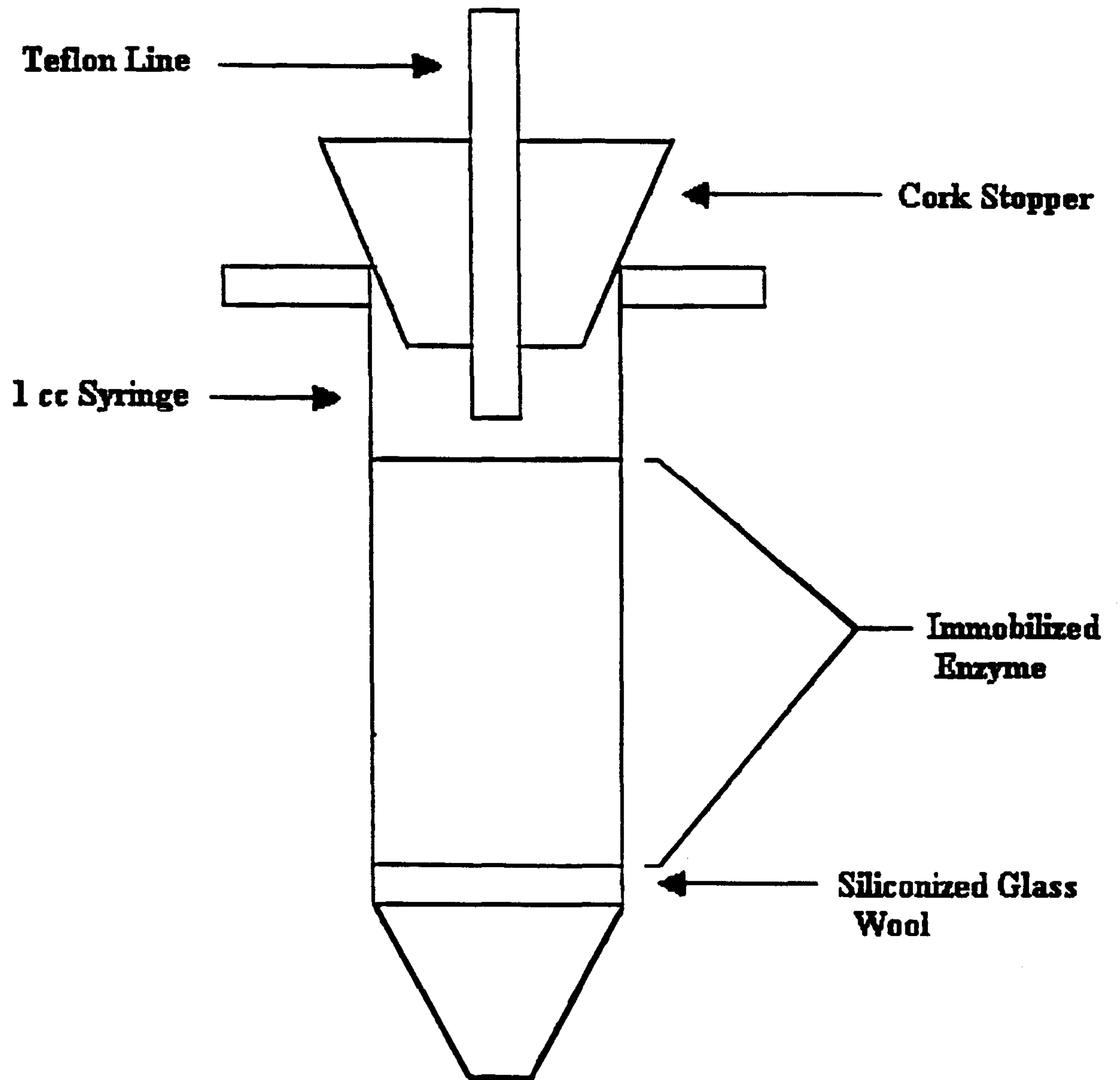


Figure 18): Diagram illustrating the packed column containing the immobilized enzyme CHY.

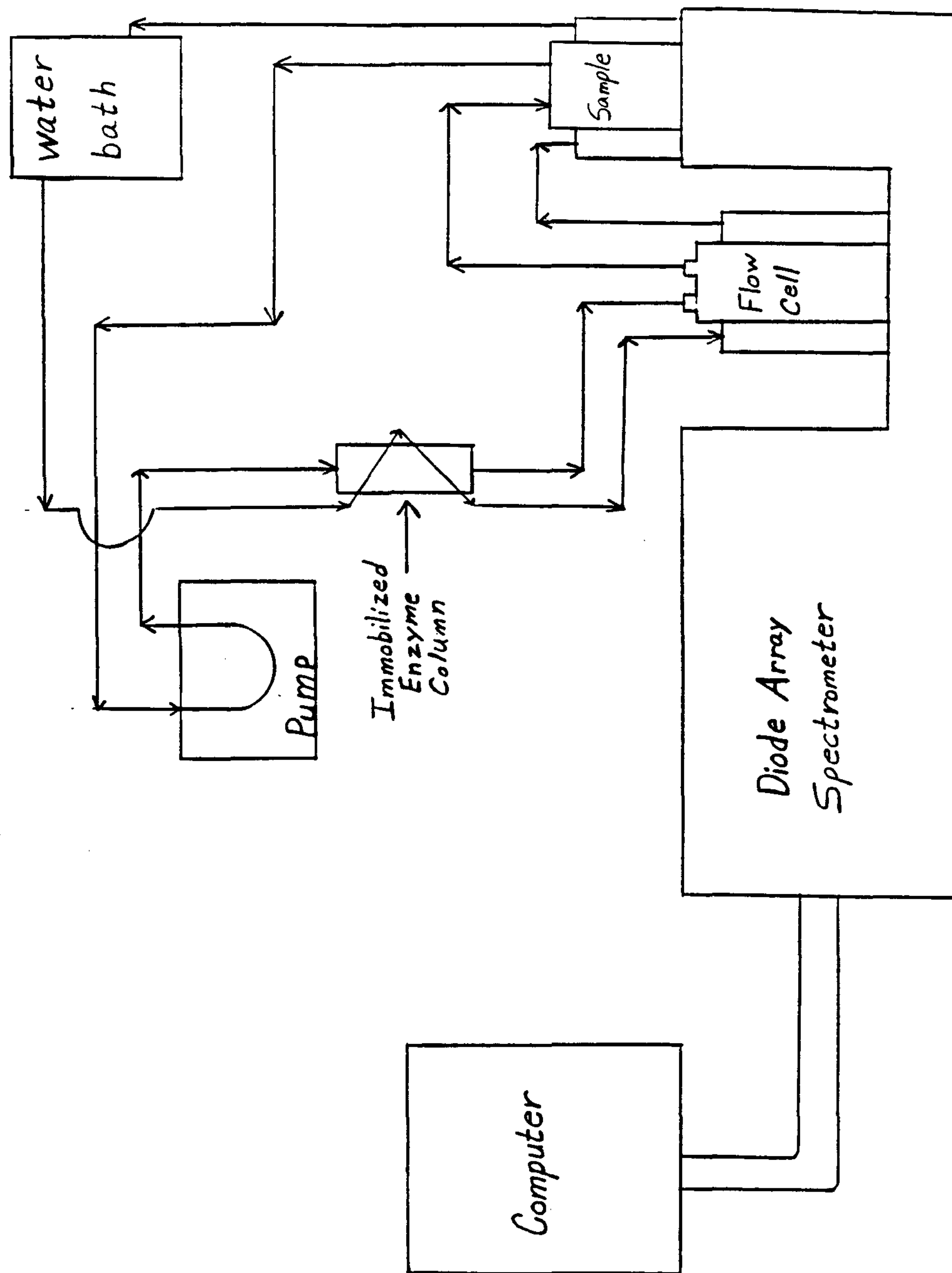


Figure 19) Schematic diagram for the diode array column analysis scheme.

was used that would not hinder the analysis procedure. About 6000 times more enzyme was used here as opposed to the free enzyme study. The BTEE concentration had to be increased but 6000 times was not possible since 3.1 g would be required for each sample run. A factor of 200 times was used instead in order to keep the sample volume reasonably small, which required only 0.31 g of BTEE for each sample run.

A sample was prepared containing 2.84 mL of THAM buffer pH=7.8, 4.20 mL of BTEE in 100% methanol, and 0.32 mL of 2 M $CaCl_2$, and was thermally equilibrated. In the mean time, the lines were filled with 30% methanol/THAM buffer pH=7.8 and connected to the enzyme column. Once equilibrated the pump and scan were started simultaneously. The scan parameters were as follows.

The run time was 0-600 seconds, absorbance was set from 2.2-3.2 A.U., with a cycle time of 5.0 s and an integration time of 2.5 s. After 60 s it was noted that the lines had become packed with precipitated BTEE and the flow was extremely slow. The run was stopped, the column discarded (it was packed with BTEE also), and the lines including the flow cell were cleaned out with water, methanol, and then refilled with 30% methanol THAM buffer pH=7.8.

A new column was inserted into the system and a new sample was prepared. It consisted of 2.84 mL 30% methanol/THAM buffer pH=7.8, 1.40 mL BTEE in 100% methanol, and 1.40 mL of methanol. The sample was thermally equilibrated and then the scan and pump were started simultaneously with the run parameters the same as above. After 300 s no precipitation had occurred and no change in absorbance was observed. At this time 1.40 mL of BTEE in 100% methanol was added. At 360 s no precipitation was observed and the scan was still flat, so 1.40mL of BTEE was added. At 420 s a flat scan was still occurring so 0.32 mL of 2 M $CaCl_2$ was added and, since nothing was happening at 450 s, 1.50 mL of BTEE in 100% methanol was added. No precipitation was seen at this time and the absorbance was beginning to increase. At 600 s the run was complete so the numbers were recorded and the scan resumed on the same sample that

had not stopped circulating for the three minute delay needed to record the numbers. The absorbance was still rising.

After this scan was complete a blank column was made up of an equivalent amount of CPG only and inserted into the water jacket. The lines were cleaned out with water, methanol and then refilled with 30% methanol THAM buffer pH=7.8. A sample was made in the exact same fashion as the one made for the previous run and a blank scan was performed via the successive addition method. The absorbance was recorded and observed under the same conditions as the enzyme column. These graphs are shown later on in Figures 20-24 as the linear regression plots. Once the activity had been obtained, another column was made and a methanol profile was performed. This profile had to begin at 60% methanol since that was the methanol concentration where the BTEE would not precipitate out of solution and plug up the lines and ruin the column. A blank run was performed by a successive addition of the substrate which allowed for the substrate to be evenly distributed in the system before reacting with the assay column. Once the blank column was run and the spectra recorded, the assay column was placed in the water jacket and the scan and pump started together. No successive addition of compounds was required here since that had already been done in the blank column run. A slope was observed at both 60% and 70% methanol but for 80% methanol there was no increase in absorbance detected. At this point the runs were stopped. There was activity at 60% and 70% methanol for the immobilized enzyme column and somewhere between 71% and 80% the enzyme was denatured by the methanol.

The final concentrations of THAM, $CaCl_2$, BTEE, and enzyme (assuming 10% attachment) were as follows. For a total system volume of 14.56 mL there was 0.0156 M THAM, 0.4396 M $CaCl_2$, 0.06828 M BTEE, and 5.72×10^{-4} M CHY (the molecular weight of CHY was calculated as 24,000). Figure 22 illustrates the resultant slope of the 60% methanol run. Figure 23 shows the slope of the 70% methanol run, Figure 24 demonstrates the flat spectra of the 80% methanol run, and Figure 25 presents the

resultant graph of activity vs. % methanol. A plot of % activity vs. % methanol was done but is incomplete since it was not possible to study the column at methanol concentrations lower than 60%.

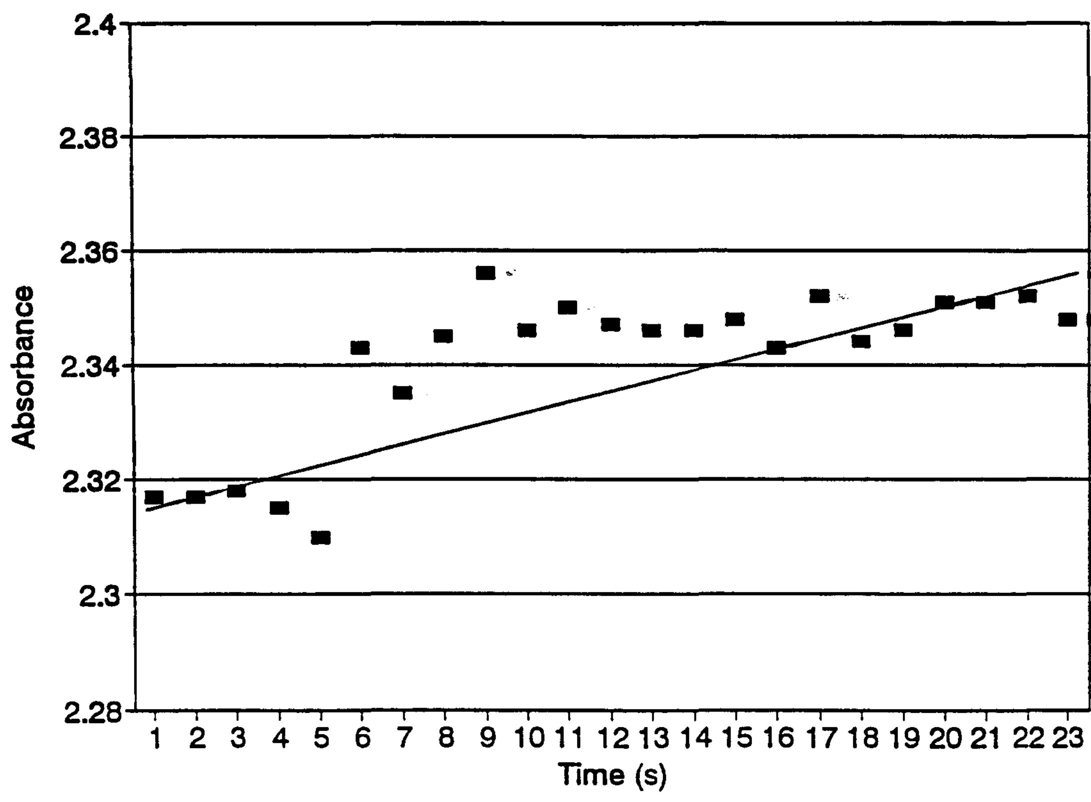


Figure 20) The linear regression plot of the blank run.

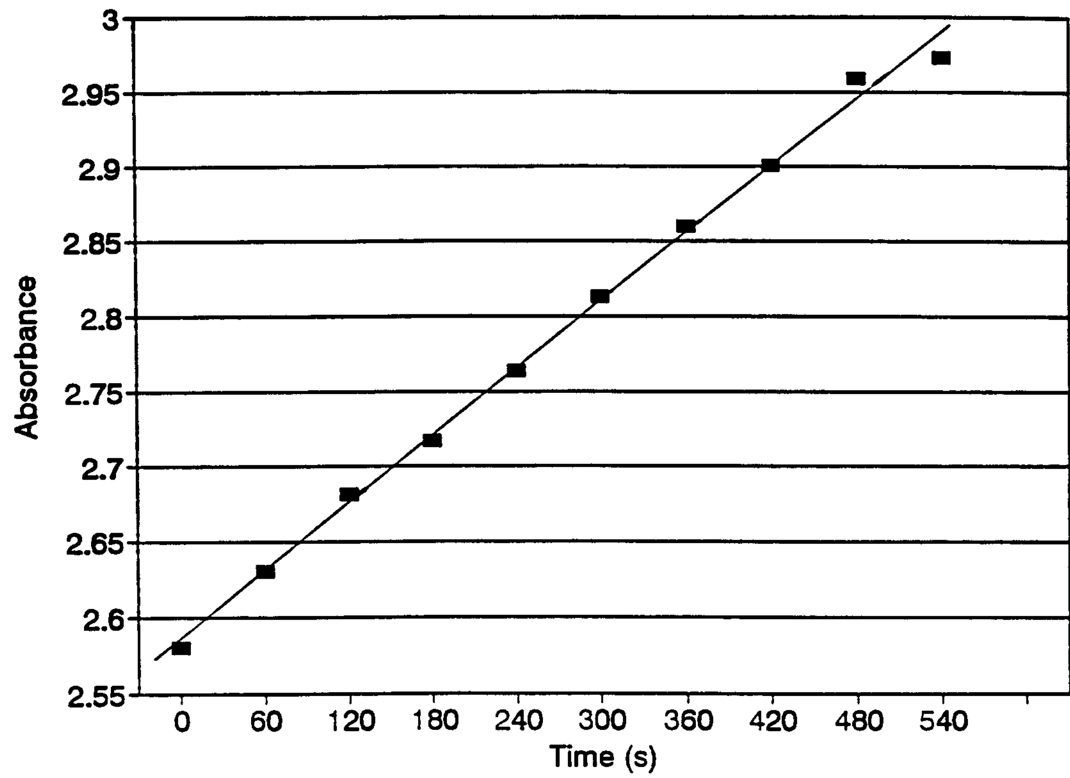


Figure 21) The linear regression plot for the assay run of the immobilized enzyme column.

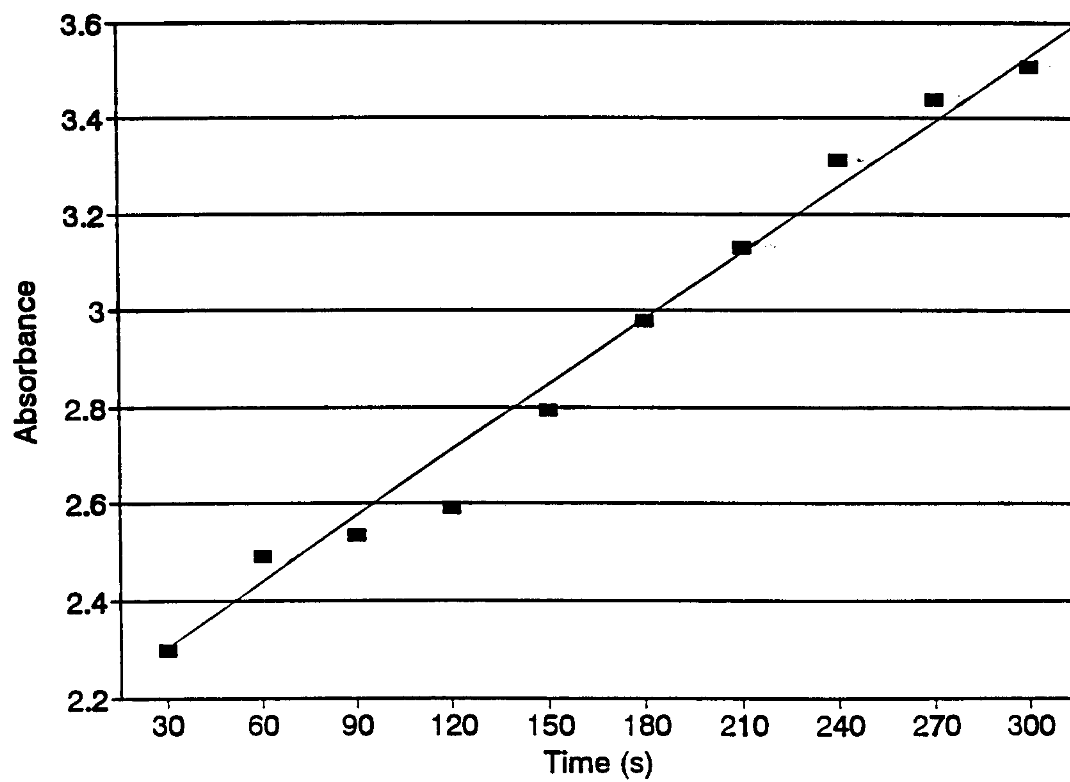


Figure 22) Lineaar regression plot of change in absorbance covalently immobilized CHY at 60% methanol.

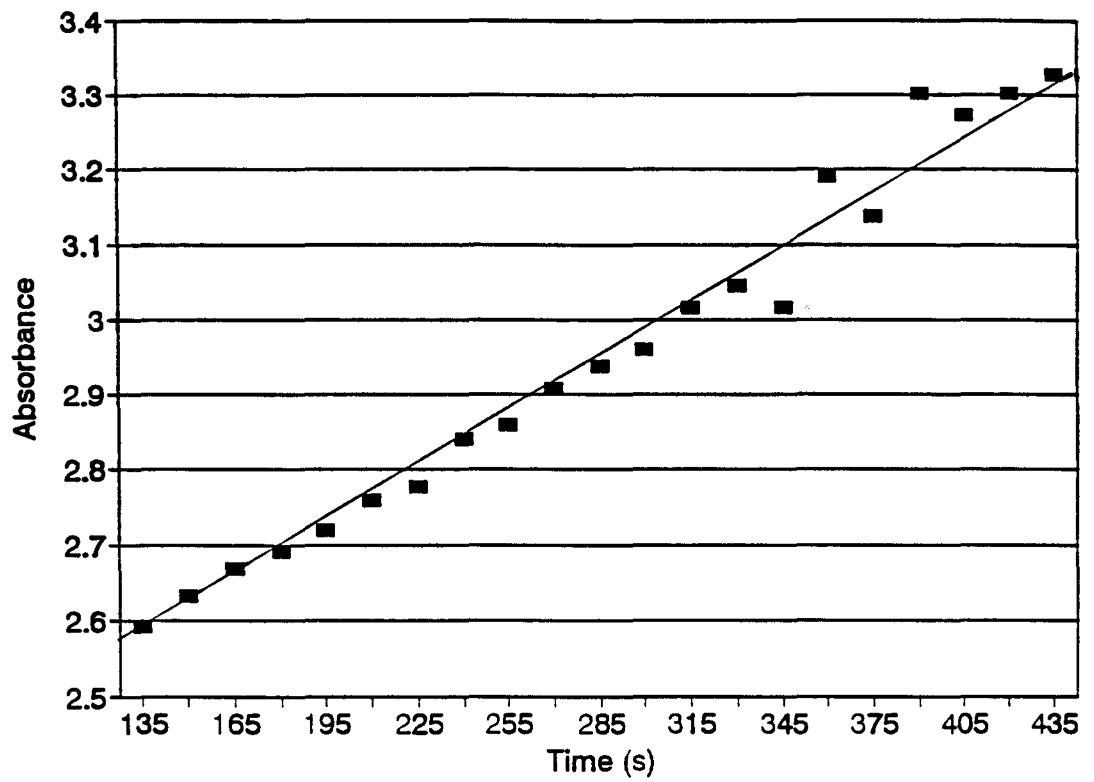


Figure 23) Plot of covalently immobilized CHY at 70% methanol.

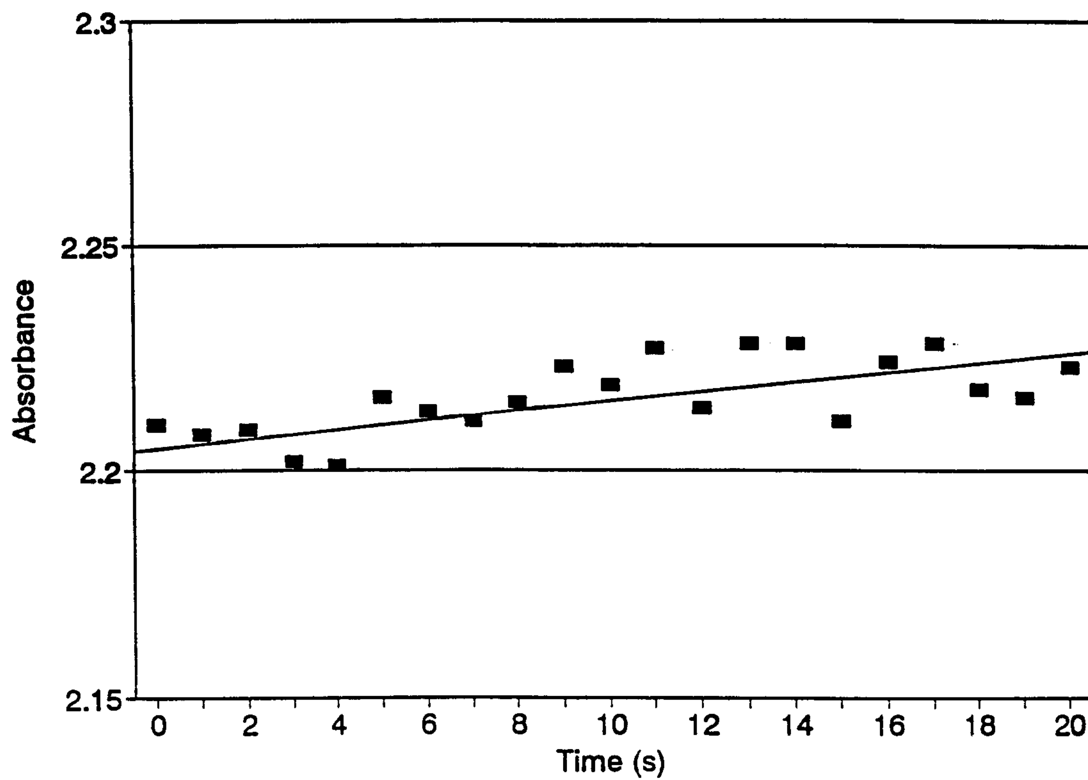


Figure 24) The resultant plot of covalently immobilized CHY at 80% methanol

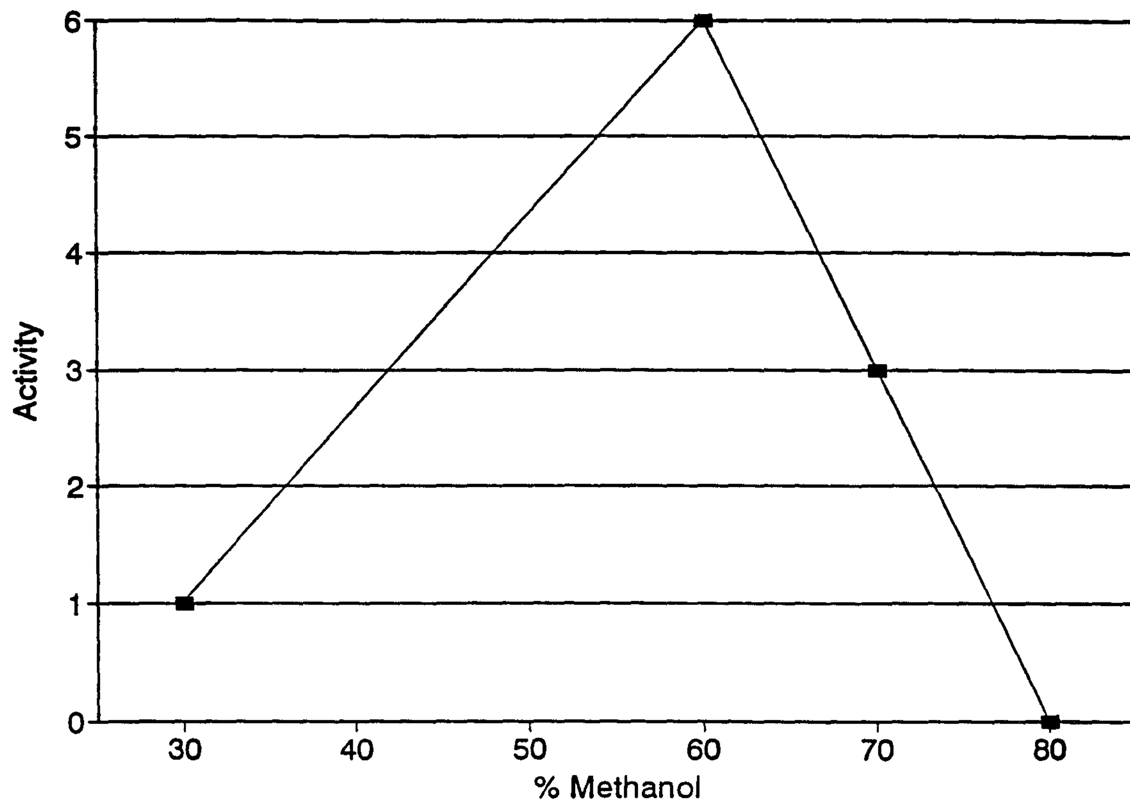


Figure 25) Plot of activity vs. % methanol for immobilized CHY.

Intermolecular Cross-Linking of Immobilized α-Chymotrypsin

Reagents: All reagents used in these experiments were prepared in the same fashion as those in the immobilized enzyme study except for what is listed below.

$5.4x10^{-3}$ M GLUT	2.16 mL of 25% (2.5 M) reagent GLUT was diluted to 1.0 L with deionized water and mixed 45 times by inversion.
$5.4x10^{-6}$ M GLUT	1.00 mL of the $5.4x\ 10^{-3}$ M GLUT was diluted to 1.0 L with deionized water and mixed 45 times by inversion.
$5.4x10^{-3}$ M GLUT	2.16 mL of 25% (2.5 M) reagent GLUT was diluted to1.0 L with 0.05 M Na_2HPO_4 buffer pH=7.8 and mixed 45 times by inversion.
$5.4x10^{-6}$ M GLUT in buffer	1.00 mL of the $5.4x\ 10^{-3}$ M GLUT was diluted to 1.0 L with the buffer at pH=7.8
$5.4x10^{-2}$ M GLUT in buffer	21.6 mL of the 25% reagent GLUT was diluted to 1.0 L with the buffer and mixed 45 times by inversion.
$5.4x10^{-4}$ M GLUT in buffer	10.0 mL of the $5.4x10^{-2}$ M GLUT was diluted to 1.0 L with the buffer and mixed 45 times by inversion.

Procedure: The immobilization procedure used here was identical to the previous method used to immobilize the enzyme. The only difference was that after the AGE was formed it was washed with a small portion of buffer and then placed in a GLUT cross-linking solution. This is described well below.

At this point, instead of packing the AGE into the syringe, $5.4x10^{-6}$ M GLUT was made from the 2.5 M reagent GLUT by successive dilutions.

To intermolecularly cross-link the immobilized CHY, 20.0 mL of the 5.4×10^{-6} M GLUT was pipetted into a beaker and used to wash the AGE from the filter paper to the beaker. Once in the beaker, the reaction was allowed to proceed for one hour. After one hour the AGE-GLUT (AGEG) was washed with 500mL of deionized water and packed into a 1cc. syringe in the same fashion as described previously in the immobilization procedure. The column was then analyzed following the same method as discussed in the immobilization analysis procedure. All samples were prepared as before. The samples were run through a blank column of CPG only and then through the immobilized and cross-linked enzyme column. Activity was monitored using the same equipment and run parameters. The following graph is a spectra of a 60% methanol assay run (Figure 26). As Figure 26 illustrates, the change in absorbance is flat thereby signifying the absence of activity. Additional runs with this column were not continued. This column was discarded and another prepared as before. This time the 5.4×10^{-6} M GLUT was prepared in 0.05 M Na_2HPO_4 buffer pH=7.8 instead of deionized water. The AGEG was also washed with 500 mL of this buffer prior to being packed in the column. This column was run by the same procedure as above. Figure 26 shows the spectra at 60% methanol. It is flat just like the one in Figure 27.

A calculation of the number of molecules of enzyme with respect to the number of molecules of GLUT in the cross-linking solution revealed that the number of GLUT molecules may not have been enough to effectively cross-link the immobilized CHY. The concentration of GLUT was therefore raised to 5.4×10^{-4} M. Two columns were made up and cross-linked by following the same method as described above. One was cross-linked with 5.4×10^{-4} M GLUT while the other was cross-linked with the 5.4×10^{-2} M GLUT solution. These two columns were analyzed in the same fashion as the initial immobilized and cross-linked enzyme column. The observed activity of these two columns was flat indicating no catalytic activity was present.

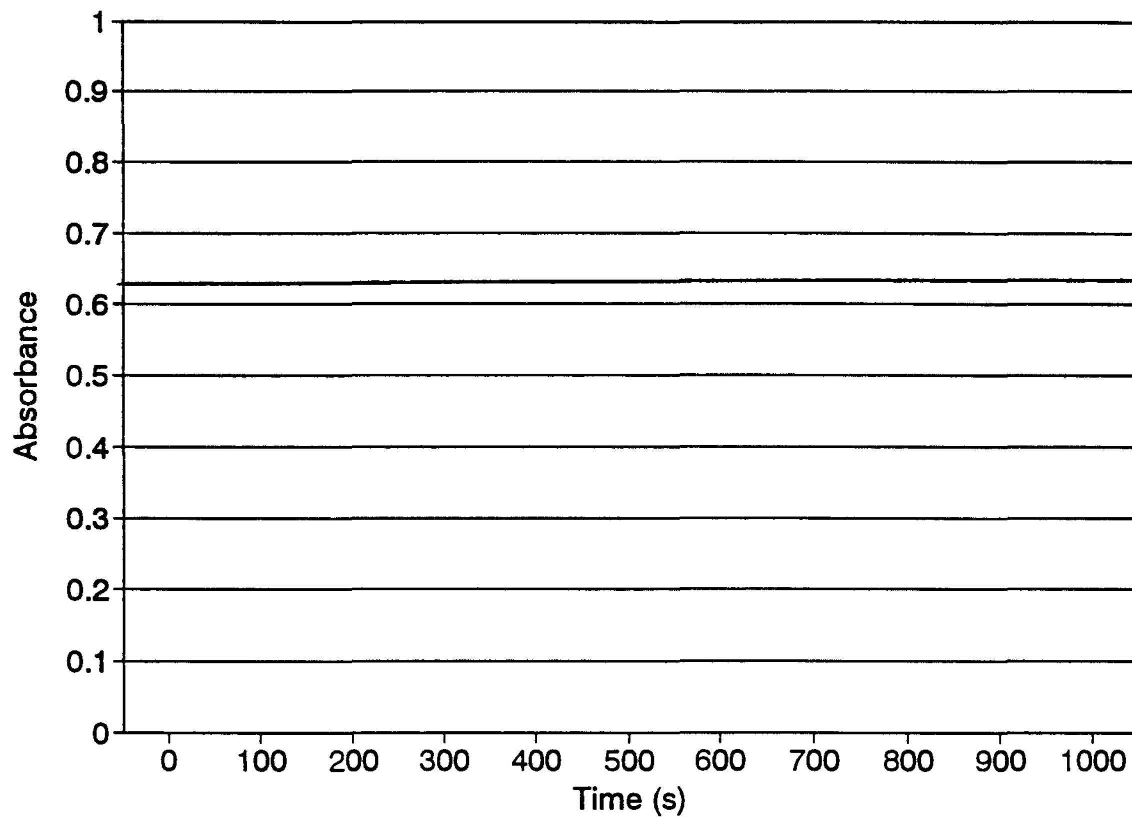


Figure 26) Flat scan observed at 60% methanol.

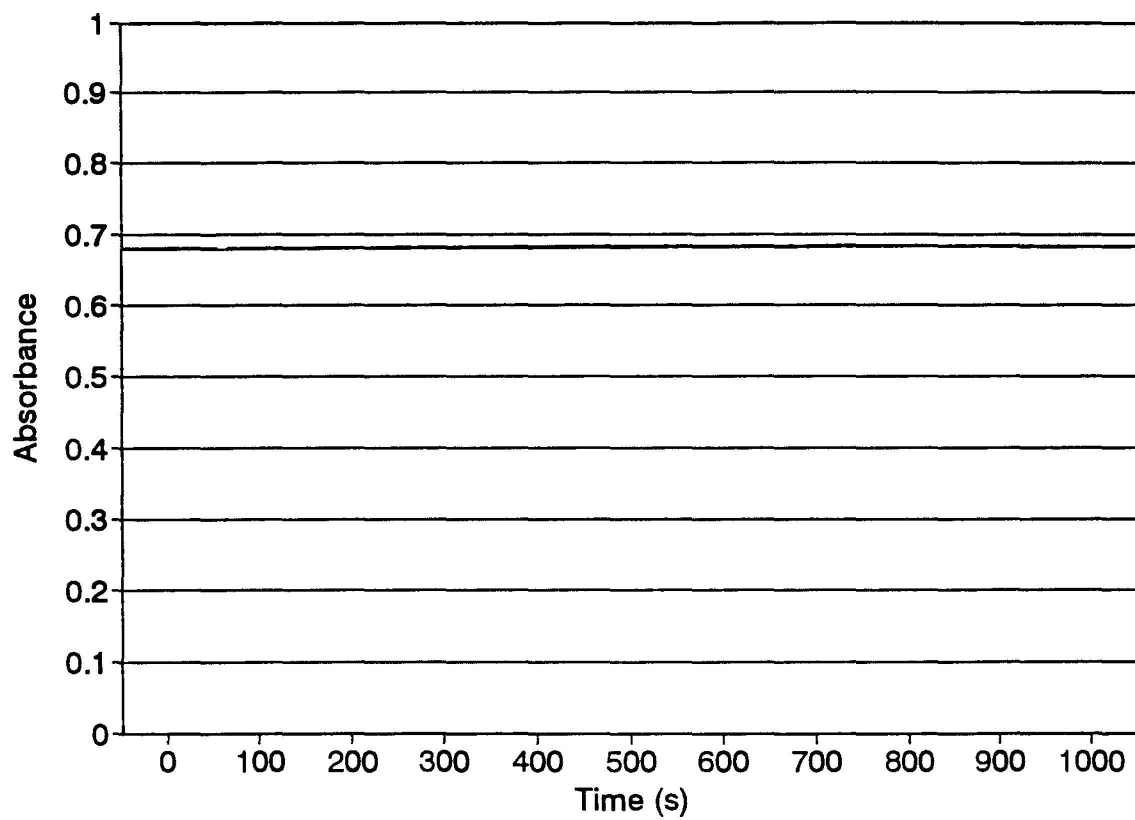


Figure 27) Flat scan as observed from the second column.

Cross-Linking of α-Chymotrypsin with Glutaraldehyde Prior to Immobilization

Reagents: All reagents used here were prepared in the same fashion as presented in the immobilized enzyme reagents list. All other solutions used in this method are described below.

0.05 M Na_2HPO_4/THAM buffer pH=6.2	3.5 g Na_2HPO_4 was dissolved in 500 mL of deionized water and the pH adjusted to 6.2 with 6 M HCl
GLUT cross-linking solution	5.0 mL of reagent GLUT was added to 45.0 mL of the buffer at pH=6.2
GLUT solution for the APG	1.25 mL of reagent GLUT was added to 11.25 mL of Na_2HPO_4 buffer at pH=7.0
1 M NaOH	4.0 g of NaOH pellets were dissolved in 100 mL of deionized water

Since the immobilization of CHY followed by cross-linking yielded flat spectra the method of cross-linking was approached from a new angle. CHY was first cross-linked with a GLUT solution and then immobilized to the APG-GLUT complex. To accomplish this 0.1247 g CHY was placed in 50 mL of a 2.5% GLUT solution made by placing 5.0 mL of reagent GLUT in 45.0 mL 0.05 M Na_2HPO_4 buffer pH=6.2. This was stirred mildly and then set aside for two hours to react. In the mean time 0.1263 g APG was placed in 12.5 mL of 2.5% GLUT (1.25 mL GLUT reagent in 11.25 mL Na_2HPO_4 pH=7.0) for one hour. The white APG turned to a purple-wine color. The APG-GLUT (AG) was filtered on a Buchner funnel and washed with 500ml of deionized water. After washing, the AG was placed in 10.00 mL of Na_2HPO_4 buffer pH=7.0 with a Pasteur pipette. By this time the CHY-GLUT (CG) had formed a solid creamy white intermolecularly cross-linked precipitate. The CG was then filtered and washed on a

Buchner funnel with 500mL of Na_2HPO_4. In order to immobilize the CG to the AG, the CG needed to be solubilized. This was attempted by adding the CG to a solution of 0.5 mL of 1 M NaOH in 24.5 mL of Na_2HPO_4 buffer pH=7.0. This was unsuccessful so the CG was filtered and washed with 200 mL of the buffer and added to the 10.00 mL solution containing the AG. This solution was allowed to sit for four hours. After four hours no attachment had occurred so the experiment was discontinued. This experiment was repeated using a 0.05 M Na_2HPO_4 buffer pH=7.8 for the CG formation. This was done to keep the CG in solution as it cross-linked. The CG formed a trace amount of precipitate but not nearly as much as before. The APG was prepared with GLUT as before and washed. The AG was then placed in the solution containing the CG. At this time the CG precipitated out of solution and did not attach to the AG during the alotted reaction time. The experiment was terminated at this step.

Intramolecular Crosslinking[47] of Immobilized CHY

Reagents: The reagent solutions used for the preparation and analysis of the immobilized enzyme are listed below.

APG	Purchased from Sigma
6 M HCl	50.0 mL Reagent HCl diluted to 100 mL with water
0.05 M Na_2HPO_4 Buffer pH=7.8	3.5 g Na_2 HPO_4 dissolved in 500 mL H_20 and adjusted to pH=7.8
Enzyme Solution	0.2 g CHY dissolved in 2.0 mL buffer pH=7.8
2 M $CaCl_2 \cdot 2H_20$	55 g $CaCl_2$ $2H_20$ dissolved in 200 mL of water.
2.5% GLUT solution	2.50 mL 25% GLUT reagent in 22.50 mL of pH=7.8 buffer.
1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) in 0.02 M Na_2HPO_4 pH=8.2	1.6 g EDAC dissolved in 10 mL buffer pH=8.2.
0.02 M Na_2HPO_4 buffer pH= 8.2	1.5 g Na_2HP0_4dissolved in 500 mL of water and adjusted to pH = 8.2 with 6 N HCl
14.98 M Ethylenediamine	Sigma reagent
Tetramethylenediamine	Sigma reagent

Column Preparation: Two columns were prepared by following the Wheetall[8] method as described earlier. After the enzyme was immobilized, the AGE complex was washed with phosphate buffer and placed in a solution of 1.6 g EDAC/10 mL H_2O for one hour. Each column batch was washed with buffer and then placed in the respective

cross linking solution. For column 1, the batch was placed in a solution of 45 mL 0.02 M Na_2HPO_4 pH=8.2 buffer and 5.56 mL of 14.98 M ethylene diamine. This batch was allowed to react for one hour, and was then washed exhaustively with H_2O. Column 2 was placed in a cross link solution of 45mL 0.02M phosphate buffer pH=8.2 with 5.0mL of TMD solution (7.34 g TMD/5mL H_2O) and allowed to react for one hour. After one hour the column was washed exhaustively with H_2O. At this point both columns were packed into syringes and labeled.

Column Analysis: To analyze the stability of each column in increasing methanol concentrations, a starting point of 60% methanol was chosen. The water bath and instruments were turned on and allowed to warm up. A blank column of CPG was used to monitor the background reaction of the substrate. A BTEE solution was made by dissolving 2.4 g BTEE in 25 mL of methanol. The sample preparation consisted of 3.00 mL of 50% methanol/THAM Buffer, 1.50 mL BTEE and 1.50 mL methanol. This sample was circulated for three minutes before 1.50 mL BTEE was added. At four minutes, 1.5 mL BTEE was added and at four minutes, thirty seconds, 0.40 mL of 2M $CaCl_2$ was added. At five minutes, 1.5 mL BTEE was added. At the end of the blank run, column 1 was inserted and a scan performed with column 1 and the sample. This analysis showed that there was no column activity since the slope of the blank and assay runs were not significantly different.

Next, column 2 was analyzed in the same fashion and yielded similar results: The blank and assay slopes were essentially identical. At this point the analysis of these two columns was stopped.

Immobilized Enzyme Concentration Versus Substrate Concentration

Reagents: The reagents used for this set of experiments were the same as described under the immobilized enzyme preparation and methanol profile of the experimental section.

Column Preparation: Five individual columns were prepared, each with a different amount of enzyme immobilization. The enzyme concentrations ranged from 100-2000 units. All columns were prepared by following the Wheetall[8] method. The following table denotes the preparation of these columns:

Table 3: Immobilized enzyme column preparation.

Column Number	g APG	g Enzyme	Units of Enzyme
1	0.2007	0.0414	2000
2	0.1996	0.0202	1000
3	0.1960	0.0093	500
4	0.1996	0.0042	250
5	0.2000	0.00092	100

Once these columns were prepared, the lines of the analysis system were changed to 1/16 inch I.D. Teflon tubing, and several different substrate solutions were prepared. These substrate solutions ranged from the original assay concentration of 1.18 mM up to 14.16mM in three-fold increments of the 1.18 mM solution. Table 4 below lists how each was prepared.

Each column (1 through 5) was run against each different column (1 through 5) for a total of 25 runs. This allowed for a profile of enzyme activity in relation to the amount of enzyme immobilization as well as in relation to the substrate concentration. The hydrolysis of substrate was monitored at 256 nm and a run time of five minutes.

Table 4: Substrate preparation scheme.

Substrate Concentration		g BTEE	mL MeOH	mL H_2O
1	1.18 mM	0.0093	15.85	9.15
2	3.54 mM (3x)	0.0278	15.85	9.15
3	7.08 mM (6x)	0.0555	15.85	9.15
4	10.62 mM (9x)	0.0833	15.85	9.15
5	14.16 mM (12x)	0.1110	15.85	9.15

Since the lines were replaced with smaller ones, the respective volumes for each component in the sample were the same as those used in the initial free enzyme assay (1.42 mL THAM buffer, 0.40 mL BTEE, 0.08 mL 2M $CaCl_2$). The samples were circulated through the column and the scans were started. A typical scan showed an increase in absorbance (slope) that would usually last 60 seconds. Figure 28 illustrates the resultant spectra representative of these 25 runs. The activity of each column against each substrate was calculated from the most linear portion of the slope and also from equation 15.

$$\frac{\text{units}}{\text{mg attached enzyme}} = \frac{\text{A256/min} \times 100 \times 50}{(0.964)\ (\text{mg of attached enzymes})} \qquad (15)$$

Table 5: A compilation of the raw data obtained from the 25 runs:

	Activity				
Substrate Concentration	**Column 1**	**Column 2**	**Column 3**	**Column 4**	**Column 5**
1.18mM	14.4	30.1	7.8	157	933
3.54mM	42.1	77.4	11.9	560	1828
7.08mM	**169**	**285**	**484**	**970**	**3715**
10.62mM	289	132	883	801	3648
14.16mM	336	75.8	874	658	3433

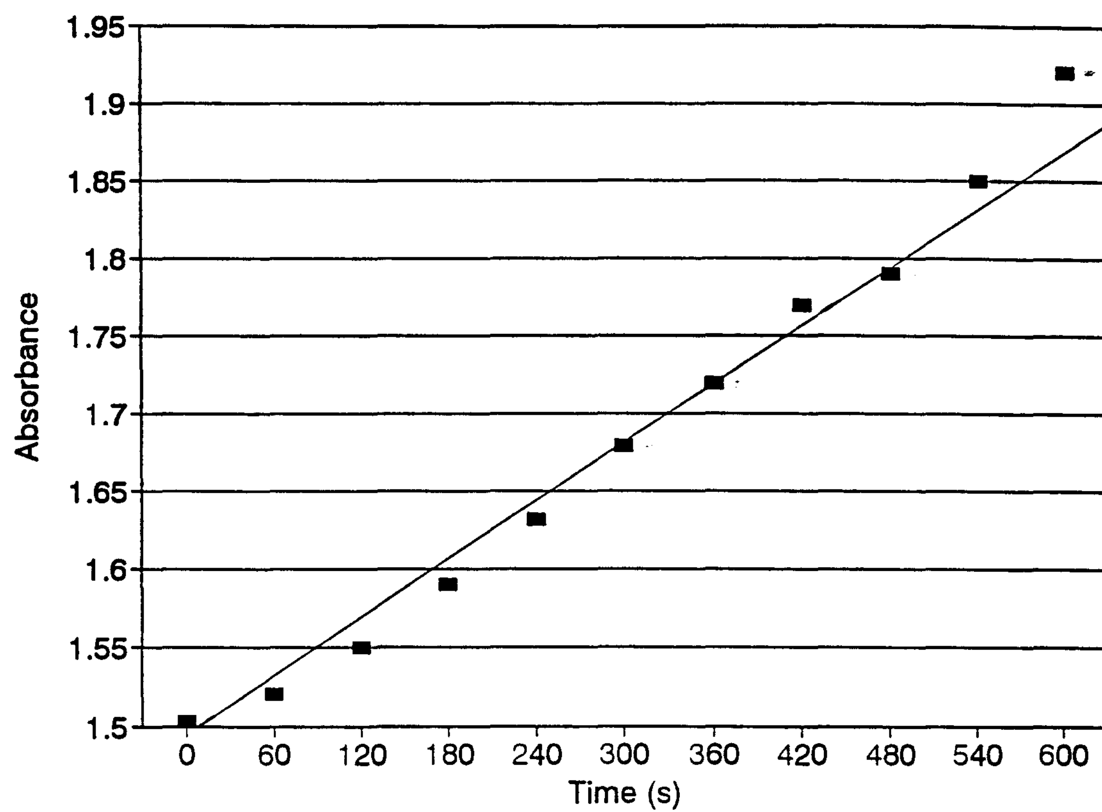


Figure 28) Typical resultant scan of the immobilized enzyme column's conversion of the substrate over a certain time period.

Each column's activity plot is illustrated in Figures 29-33. They all have similar characteristics in that they appear to follow the general Michaelis-Menton principles of enzyme kinetics concerning enzyme saturation. The actual trends of each column will be discussed in detail in the next chapter.

An interesting relationship resulted from a plot of the activity of each column for the third substrate concentration shown in bold in the above table. This plot revealed that large amounts of enzyme used in the immobilization process yielded the least amount of activity and that columns prepared with smaller amounts of enzyme exhibited greater activity levels. Figure 34 illustrates this relationship. This graph also yields a strong premise as to why all of the previous immobilized enzyme column experiments showed no activity. The amount of enzyme used in the previous experiments was five times more than that of column 1 which yielded a very low level activity.

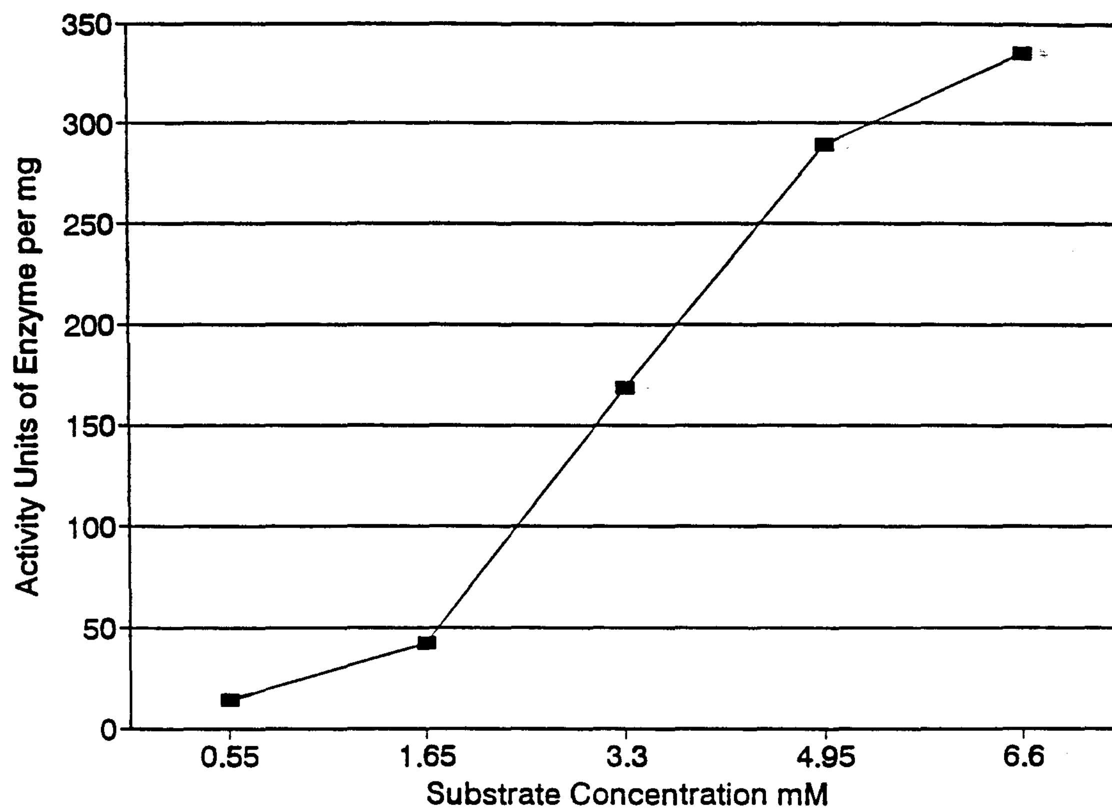


Figure 29) Column 1 activity versus substrate concentration.

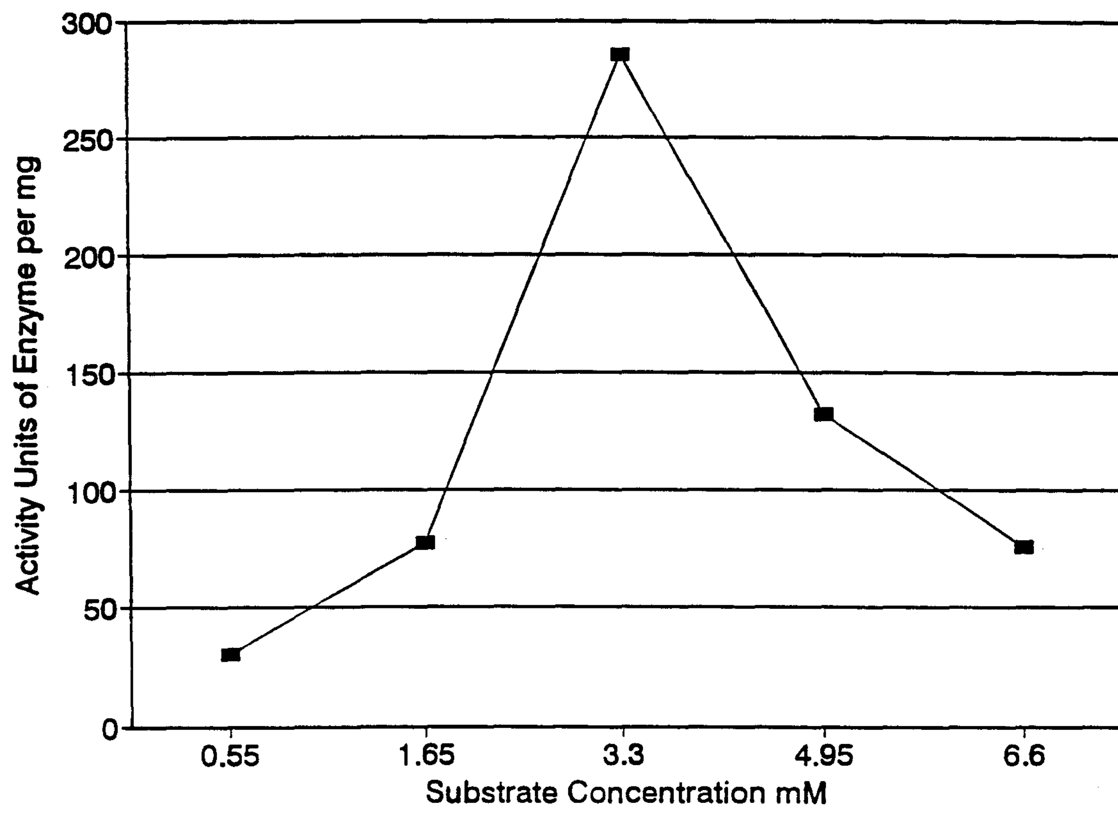


Figure 30) Column 2 activity versus substrate concentration.

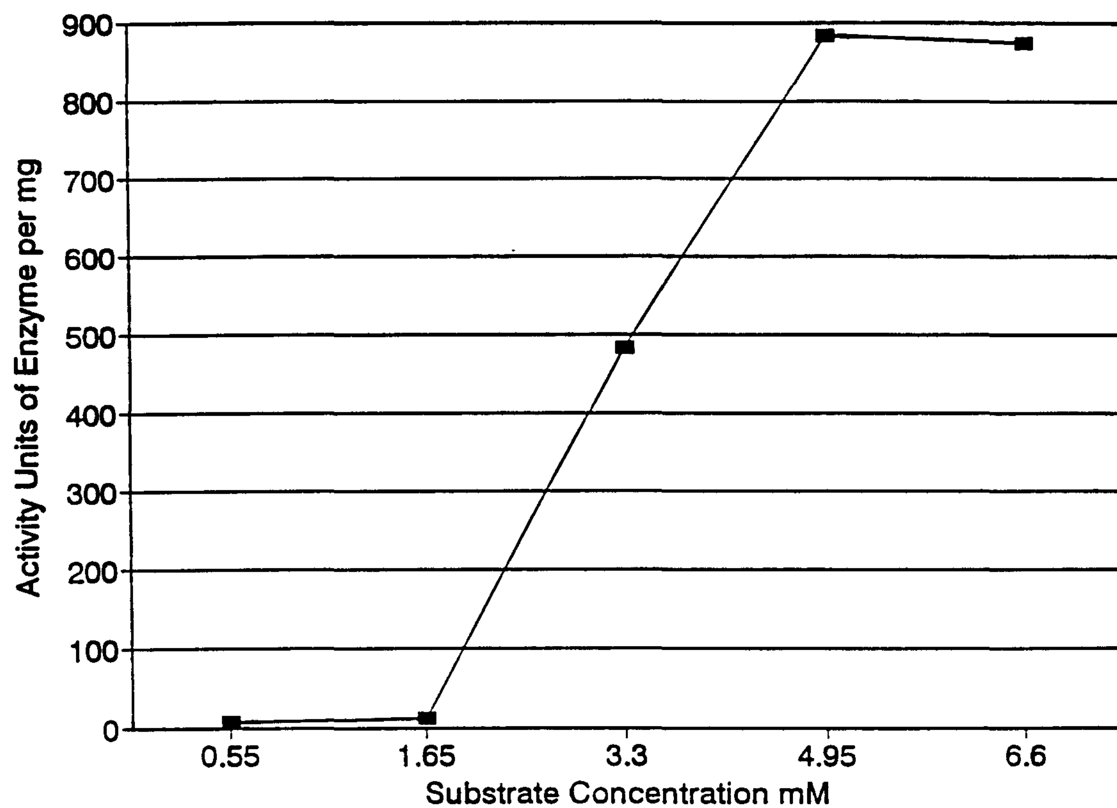


Figure 31) Column 3 activity versus substrate concentration.

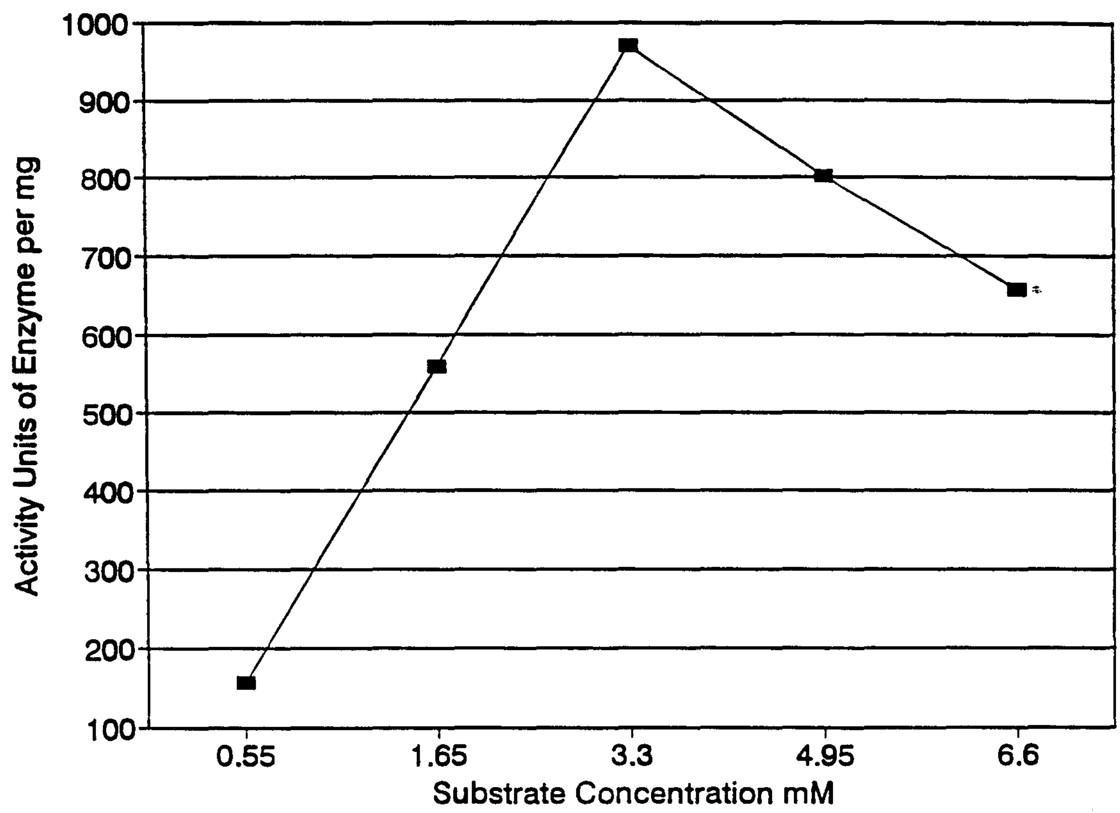


Figure 32) Column 4 activity versus substrate concentration.

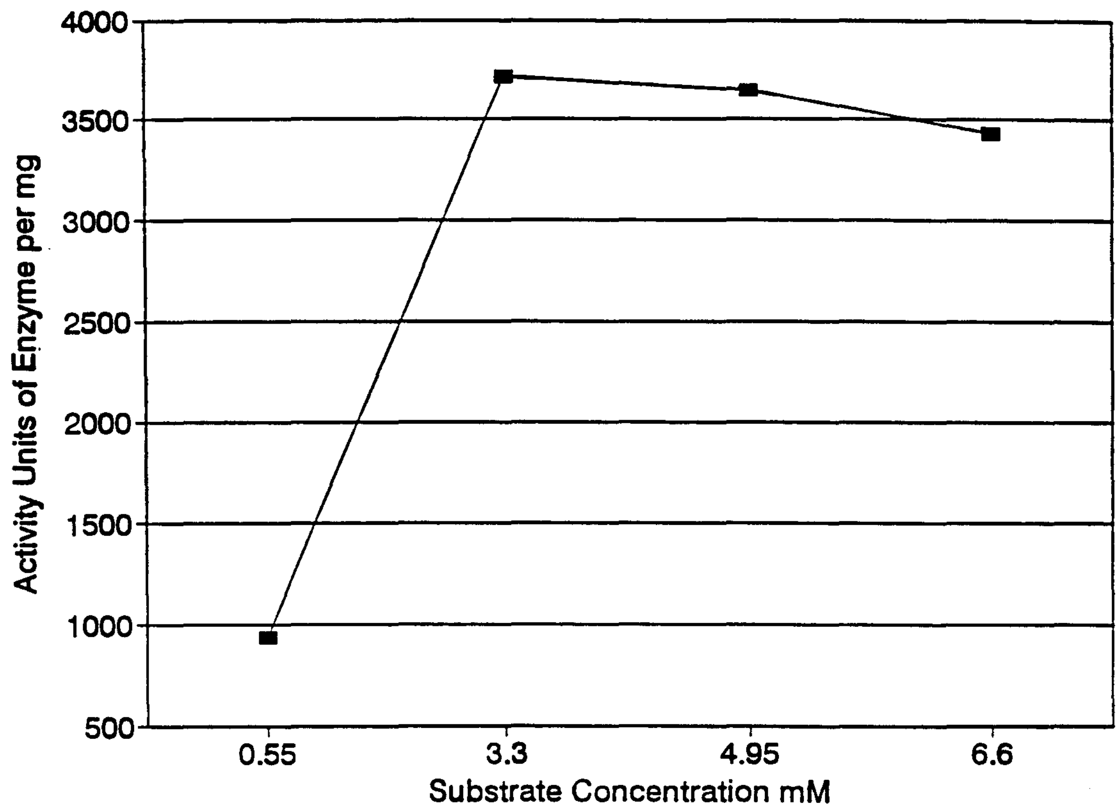


Figure 33) Column 5 activity versus substrate concentration.

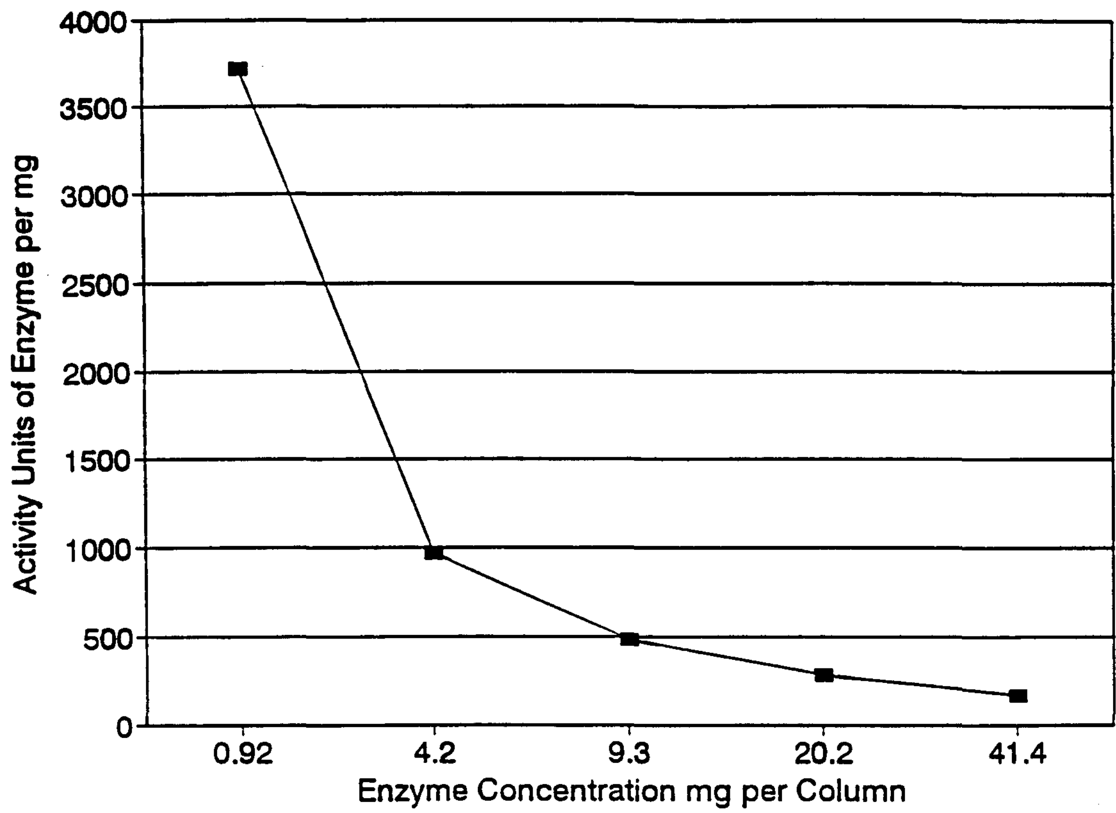


Figure 34) Activity versus amount of enzyme immobilized per column.

Intermolecular Cross-linked Immobilized Enzyme Columns

Reagents: The reagents used for this set of experiments were the same as described under the immobilized enzyme prep and methanol profile section of the experimental chapters. Any additional reagents used here are listed below.

1.0×10^{-4} M GLUT in buffer	0.04 mL of GLUT reagent in 1 L of phosphate buffer pH=7.0
80 mM Ethylene diamine	5.50 mL of 14.98 M reagent ED in phosphate buffer
80 mM Tetramethylene diamine	7.34 g reagent TMD in phosphate buffer

Column Preparation: In this experiment, six columns were prepared by following Wheetall's [8] method through the immobilization step. Once the AG was in the enzyme solution and the reaction had occurred (immobilization step), the AGE was packed into six columns. The columns consisted of two different sets enzyme concentration, the highest and lowest from the pervious experiment (2000 units and 100 units of enzyme), and also three different cross-linking agents. These two sets of enzyme columns (3 in each set) contained as cross-linking agents GLUT / THAM, GLUT / ED, and GLUT/ TMD.

After being packed into the column the AGE was washed with 0.05 M phosphate buffer pH=7.0 to remove any adsorbed enzyme. For the GLUT / THAM column, 100 mL 10^{-4} M GLUT was run through the column to waste, then 50 mL THAM pH=7.8 was run through to cap off any free, unreacted GLUT. This GLUT column was prepared using the highest and the lowest enzyme concentrations.

The next set of columns (highest and lowest enzyme concentrations) were made in the same fashion as the GLUT / THAM columns except instead of running THAM buffer

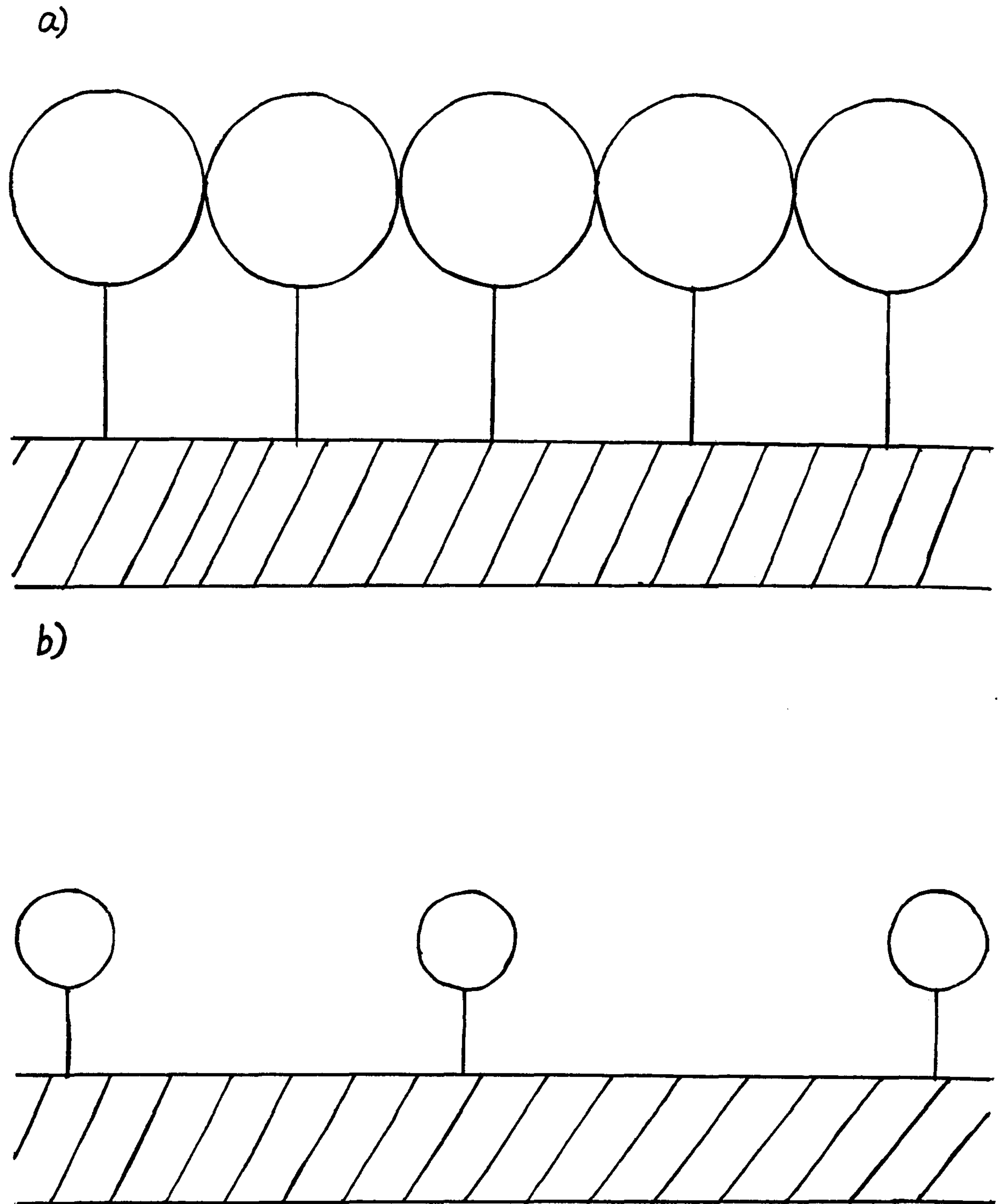


Figure 35) Description of the surface conditions of each column. a) column 1 with 2000 units, and b) column 5 with 100 units

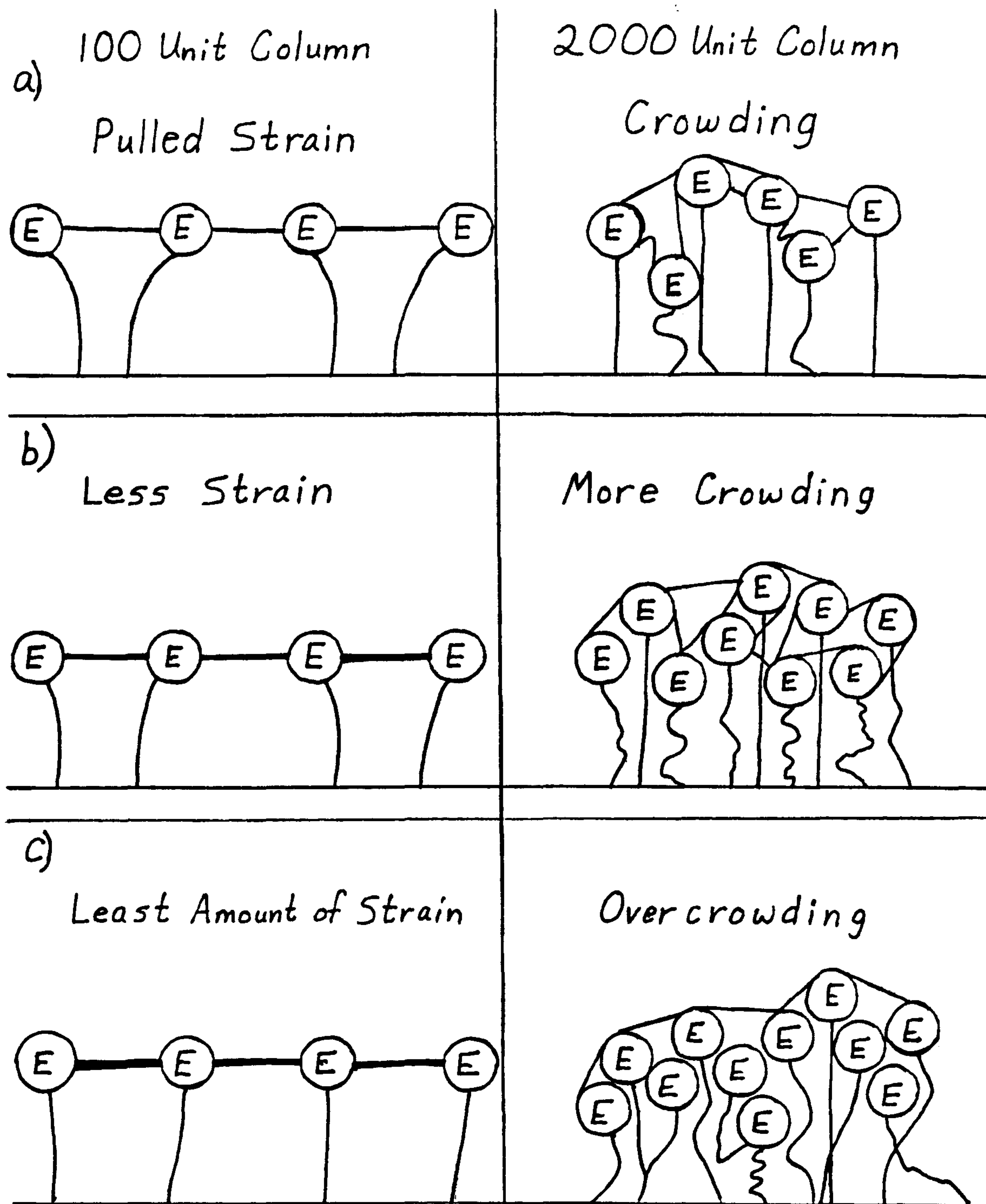


Figure 36) Surface conditions resulting from intermolecular cross linking the immobilized enzyme: a) GLUT-THAM provide the shortest linkage, b) GLUT-ED is a medium length bridge between enzyme molecules, and c) GLUT-TMD yielded the longest connection.

through the columns, 80 mM ED in phosphate buffer was used in place of THAM for the second set of columns and 80 mM TMD in phosphate buffer was used in place of THAM for the third set of columns. All columns were rinsed with a final aliquot of THAM buffer.

Each of the six cross linked columns were run through the same substrate concentration range as mentioned in the previous experiment of assay concentration through 12 times the assay concentration, thus resulting in 30 analysis runs.

Figures 37-42 plot the activity summaries of these columns. The resultant spectra for each substrate run from which column activity was calculated were similar to Figure 28. The activity was also calculated by using equation 15 which was used for the previous experiment.

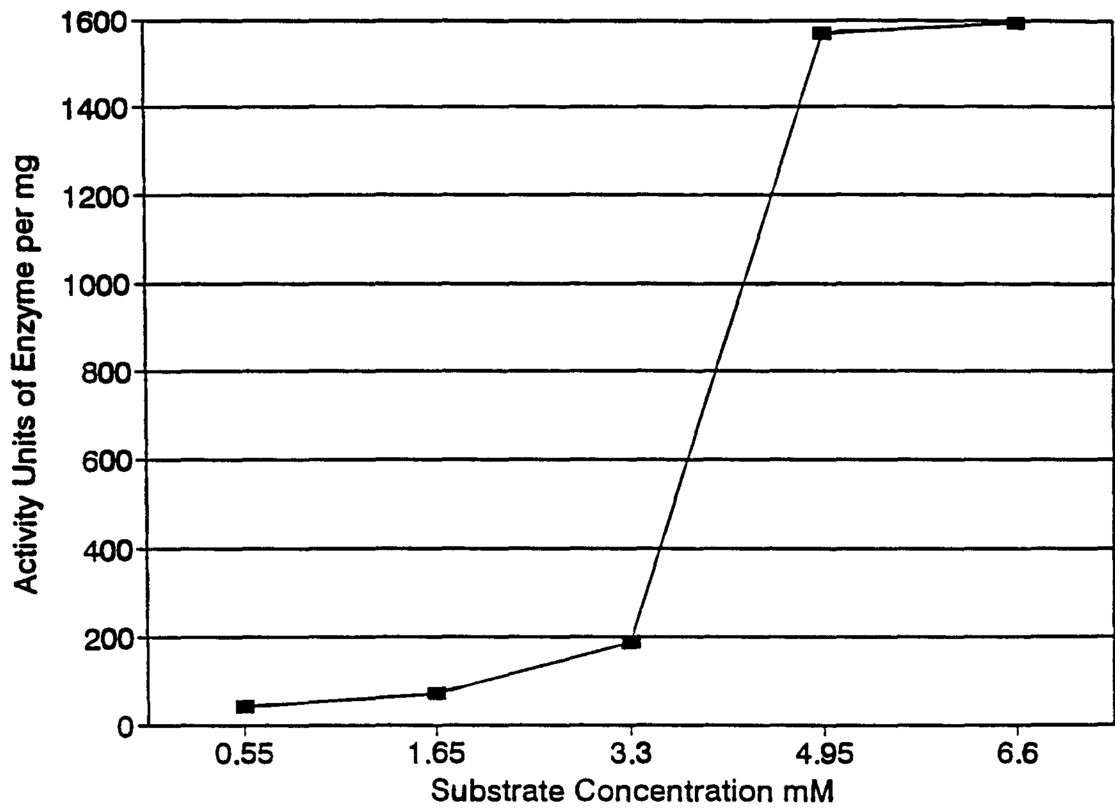


Figure 37) Column 1 cross-linked with GLUT/THAM activity versus substrate concentration plot.

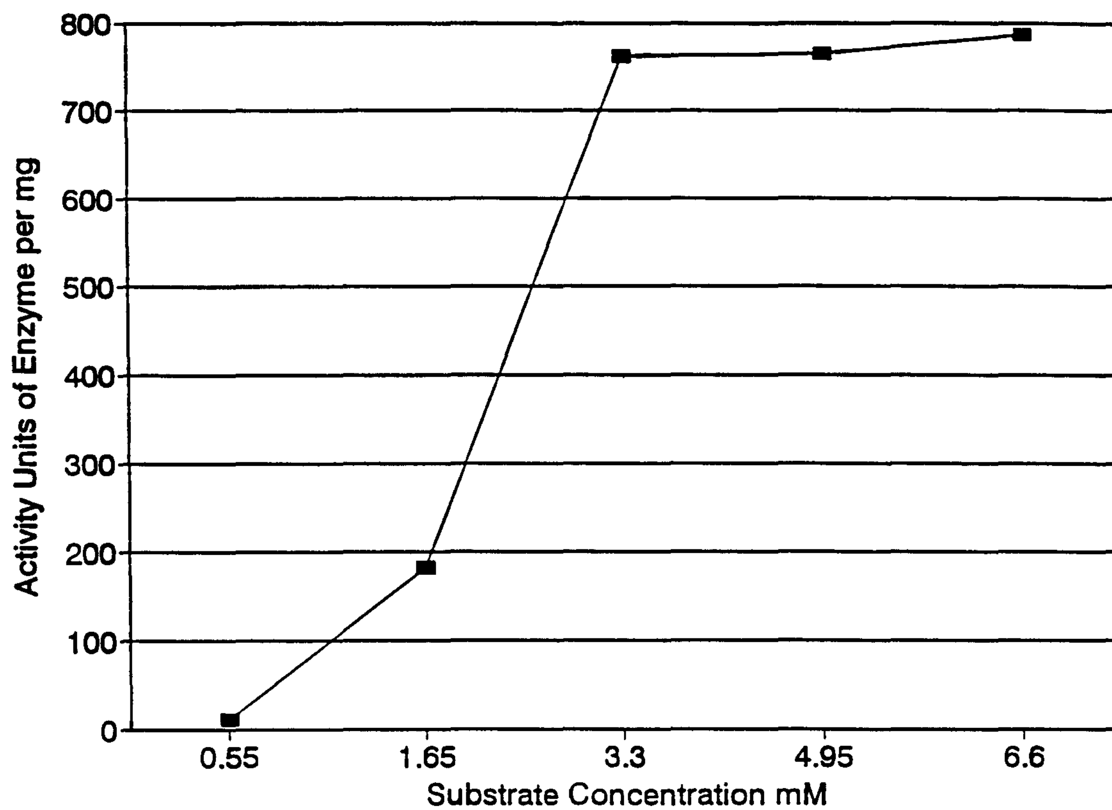


Figure 38) Column 1 cross-linked with GLUT/ED activity vs. substrate concentration plot.

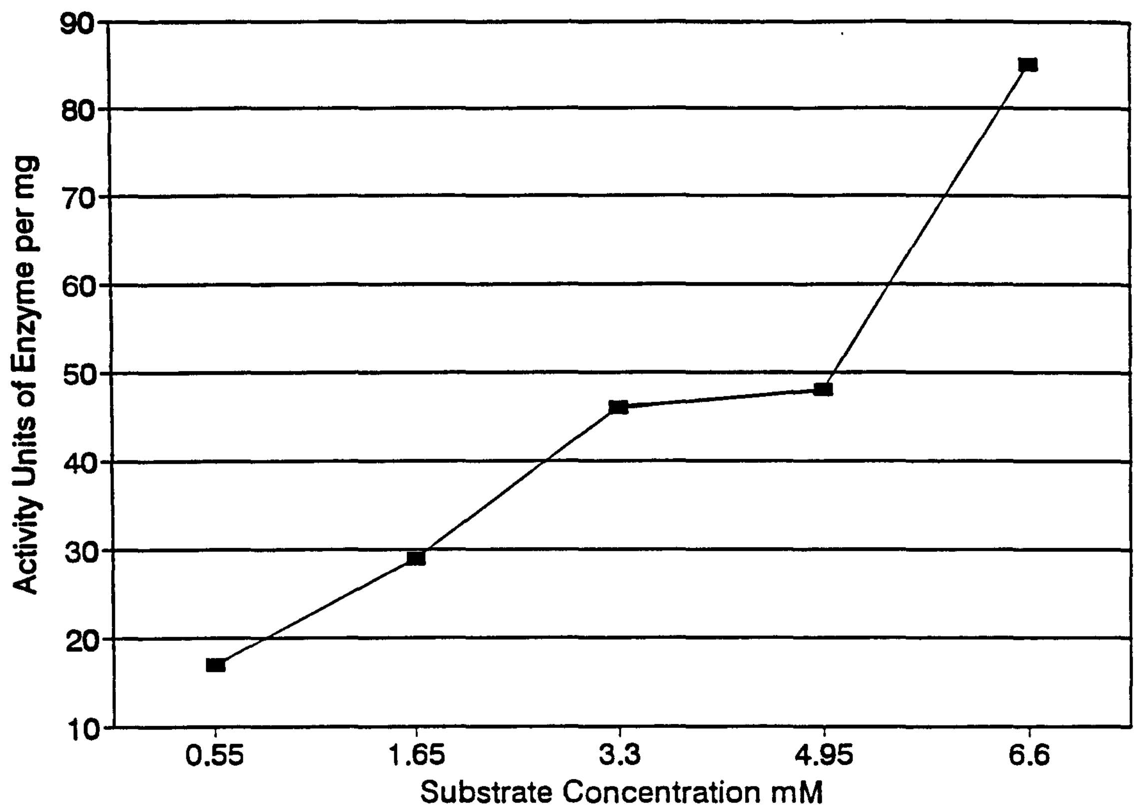


Figure 39) Column 1 cross-linked with GLUT/TMD activity vs. substrate concentration plot.

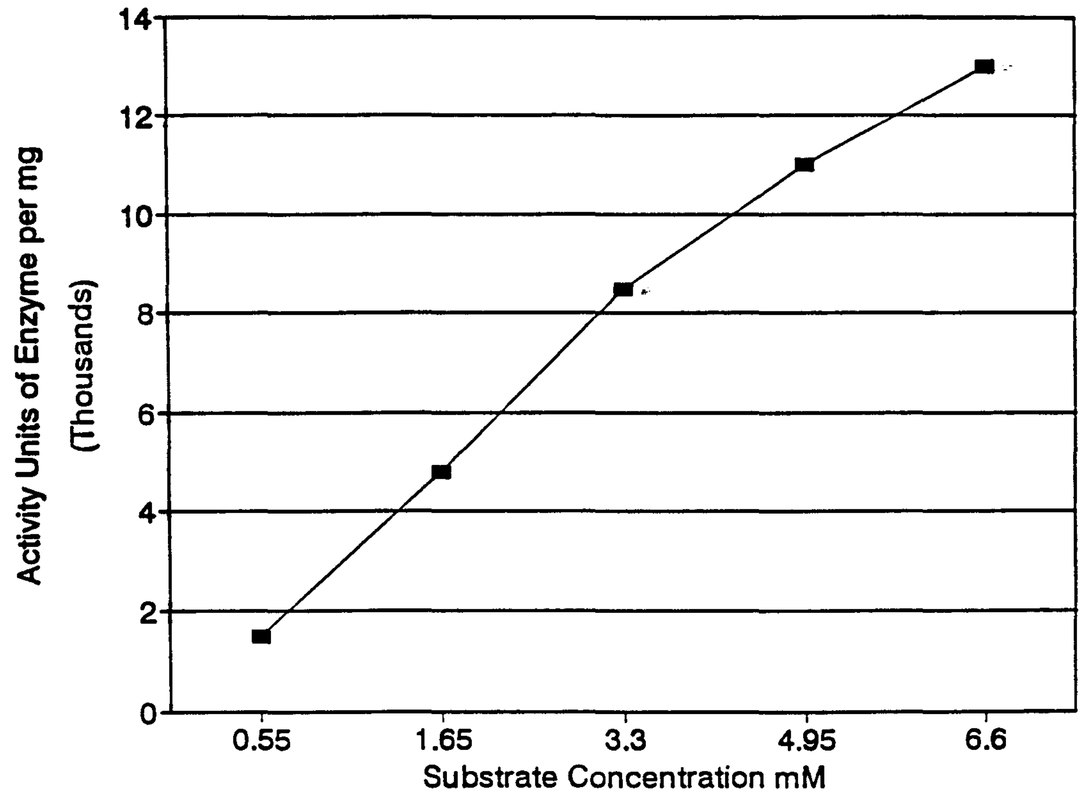


Figure 40) Column 5 cross-linked with GLUT/THAM activity vs. substrate concentration plot.

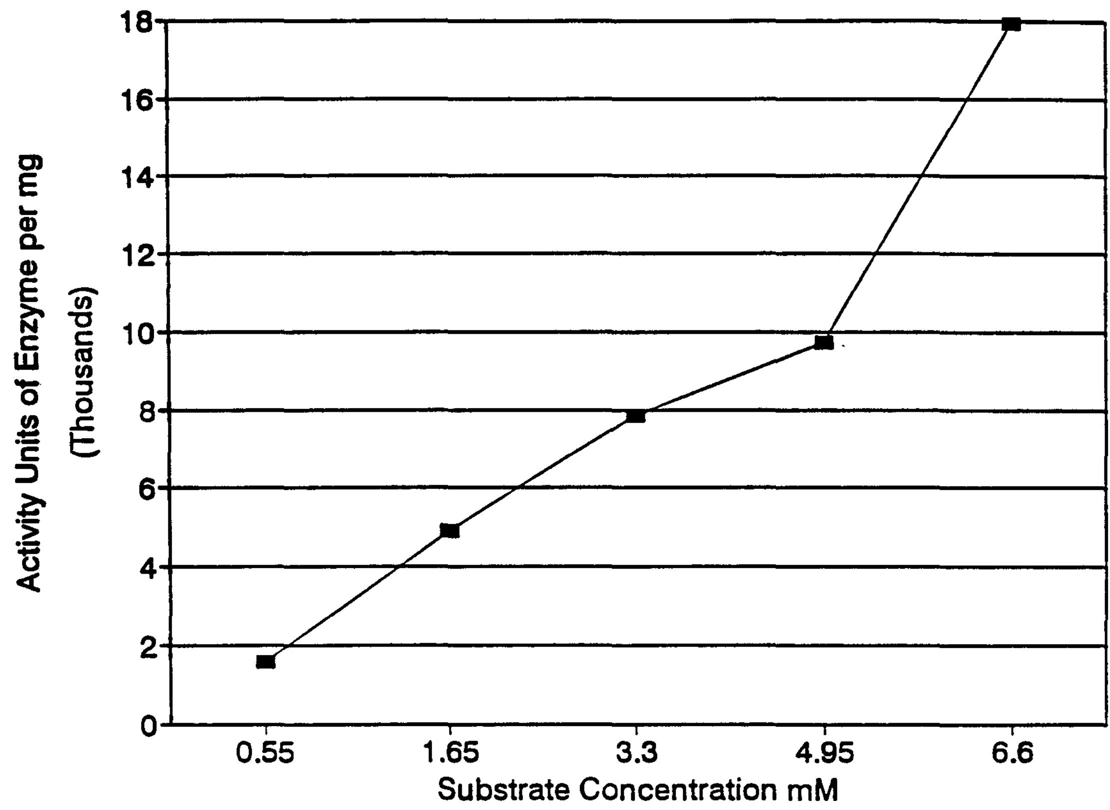


Figure 41) Column 5 cross-linked with GLUT/ED activity vs. substrate concentration plot.

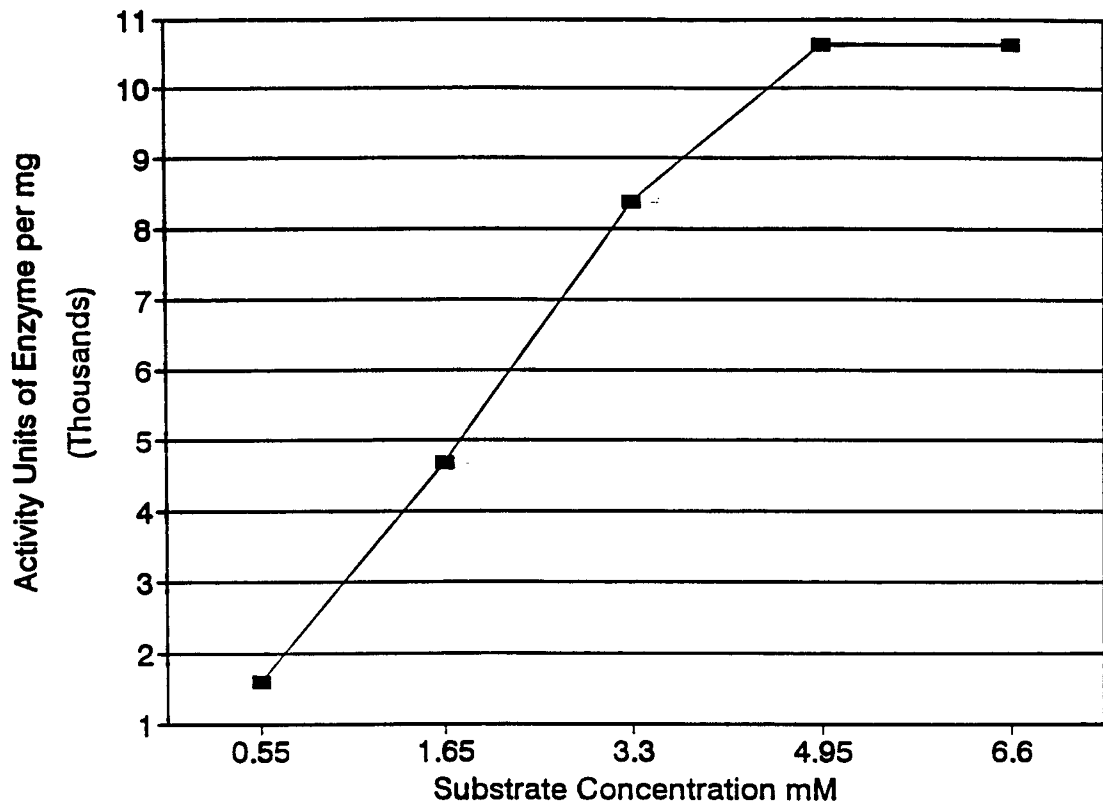


Figure 42) Column 5 cross-linked with GLUT/TMD activity vs. substrate concentration plot.

Enzyme Column and Cross-linked Enzyme Column Methanol Profile

Reagents: The reagent solutions used in the methanol profile and their preparation are listed below:

Reagent	Preparation
APG	Purchased from Sigma
0.05 M Na_2HPO_4 buffer pH=7.8	14 g Na_2HPO_4 in 2 L H_20, pH adjusted to 7.8 with 6 M HCl
6 M HCl	50 mL Reagent HCl in 50 mL H_20
2 M $CaCl_2$	55 g $CaCl_2$ in 200 mL H_20
2.5% GLUT solution	20 mL 25% GLUT reagent in 180 mL 0.05 M Na_2HPO_4 buffer pH=7.8
THAM buffer pH=7.8	19 g THAM in 2 L H_20, pH adjusted to 7.8 with 6 M HCl
1.0×10^{-4} M GLUT	0.04 mL 25% GLUT reagent in 1 L Na_2HPO_4 buffer pH=7.8
80 mM Ethylenediamine (ED)	2.7 mL ED reagent in 500 mL Na_2HPO_4 buffer pH=7.8
80 mM Tetramethylenediamine (TMD)	1.5 g TMD reagent in 200 mL Na_2HPO_4 buffer pH=7.8
20% Methanol BTEE	0.009 g BTEE in 6.42 mL methanol and 8.58 mL H_20
30% Methanol BTEE	0.009 g BTEE in 9.63 mL methanol and 5.37 mL H_20
40% Methanol BTEE	0.009 g BTEE in 12.84 mL methanol and 2.16 mL H_20
50-100% Methanol BTEE	0.0444 g BTEE in 70 mL Methanol
100% Methanol THAM buffer pH=7.8	9.6 g THAM in 1 L Methanol, pH adjusted to 7.8 with 6 M HCl

Column Preparation:

Two sets of columns were prepared. One set contained 2000 units of enzyme per column and the other set contained 100 units of enzyme per column. These two sets were consistent with the highest and lowest enzyme concentrations used in the enzyme concentration versus substrate concentration experiments. Both sets were prepared in bulk by following the Wheetall[8] method as described earlier. For the highest enzyme concentration columns, 0.8 g of APG was required, along with 0.17 g of enzyme. This batch yielded enough immobilized enzyme to prepare four columns. For the lowest enzyme concentration columns, 0.8 g APG was required and also 0.003 g of enzyme. From this batch four columns were packed.

Eight columns were prepared all total, of which six were cross-linked. One column from each set was left uncross-linked. One column from each set was cross-linked intermolecularly in an "on-line" fashion by rinsing with 50mL Na_2HPO_4 buffer, followed by 100 mL $1.0x10^{-4}$ M GLUT, followed by 50 mL THAM buffer. A second column from each set was cross-linked "on-line" by rinsing with 50 mL Na_2HPO_4 buffer, followed by 100 mL $1.0x10^{-4}$ M GLUT, followed by 50 mL 80 mM Ethylenediamine, followed by 50 mL THAM buffer. A third and final column from each set was cross-linked by rinsing with 50 mL Na_2HPO_4 buffer, followed by 100 mL $1.0x10^{-4}$ M GLUT, followed by 50 mL of 80 mM Tetramethylenediamine, followed by 50 mL of THAM buffer.

The initial 50 mL phosphate buffer rinse was done to remove any adsorbed enzyme on the column. The GLUT was used to react with free amine groups within the structure of the enzyme. The THAM, ED, TMD were used to complete the bridge between enzyme molecules. THAM, ED, and TMD are of different lengths to account for the different distances between enzyme molecules on the glass surface.

Column Analysis:

The samples consisted of 2.84 mL THAM, 2.80 mL BTEE, and 0.16 mL $CaCl_2$. The initial assay procedure used a total of methanol concentration of 30.6%. To calculate the respective concentrations, the total sample volume of 5.80 mL and milliliters of methanol to give a particular percentage of methanol in the sample were used to make the appropriate percentage methanol/BTEE solutions. The following table lists the percentage of methanol in the total sample, and the other parameters required.

Table 6: Percent Methanol and Sample Compositon

	BTEE Prepared			
% Methanol	**mL Methanol**	**mL H_20**	**THAM (aq)**	**THAM (100%MeOH)**
10	3.20	11.80	2.80	0.00
20	6.42	8.58	2.84	0.00
30	9.63	5.37	2.84	0.00
40	12.84	2.16	2.84	0.00
50	15.00	0.00	2.84	0.00
60	15.00	0.00	2.16	0.68
70	15.00	0.00	1.58	1.26
80	15.00	0.00	1.00	1.84
90	15.00	0.00	0.42	2.42
100	15.00	0.00	0.00	2.84

The diode array spectrometer was blanked with a sample of non-hydrolyzed substrate and each enzyme column was profiled at these different methanol concentrations. A 10% methanol sample was the starting point, since BTEE was not soluble in water. The 10% methanol sample showed a substrate precipitation as did the 20% methanol sample. The profile thus had to begin at 30% methanol. The experiment

consisted of eight columns run from 30-100% methanol (64 runs). The activity of each run was calculated in per equation 15 and the resultant plots can be seen in Figures 43-50. Tables 7 and 8 list the activity values obtained from the 64 runs.

TABLE 7: Column 5 (100 unit) activity summary.

		C5 Activity		
% Methanol	**Noncross-linked**	**GLUT**	**ED**	**TMD**
30	71	100	380	224
40	163	196	264	334
50	234	288	274	295
60	435	400	135	589
70	345	196	209	685
80	96	200	150	123
90	64	158	78	35
100	20	40	53	70

TABLE 8: Column 1 (2000 unit) activity summary.

		Cl Activity		
% Methanol	**Noncross-linked**	**GLUT**	**ED**	**TMD**
30	15	5	6	106
40	12	3	42	61
50	28	3	62	30
60	31	14	12	14
70	40	18	17	20
80	12	3	15	5
90	4.3	5	12	5
100	1.5	2	3	1

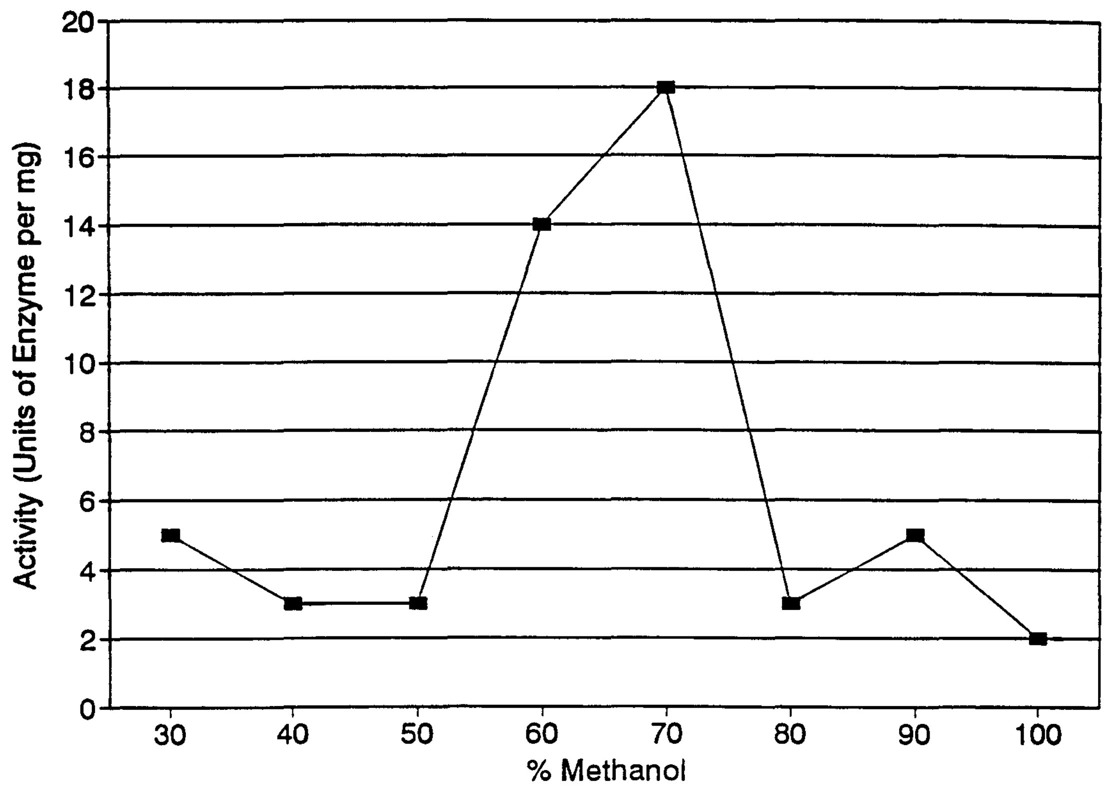


Figure 43) Column 1 activity with no cross-linking.

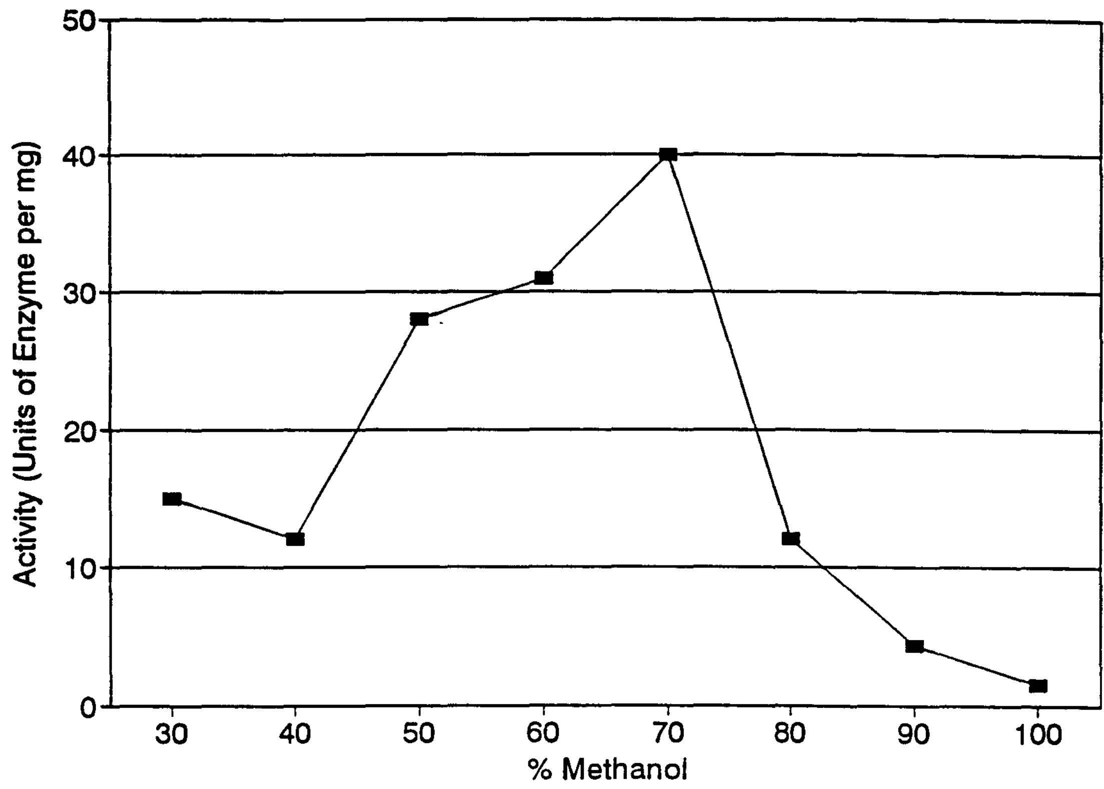


Figure 44) Column 1 activity with GLUT-THAM cross-linking.

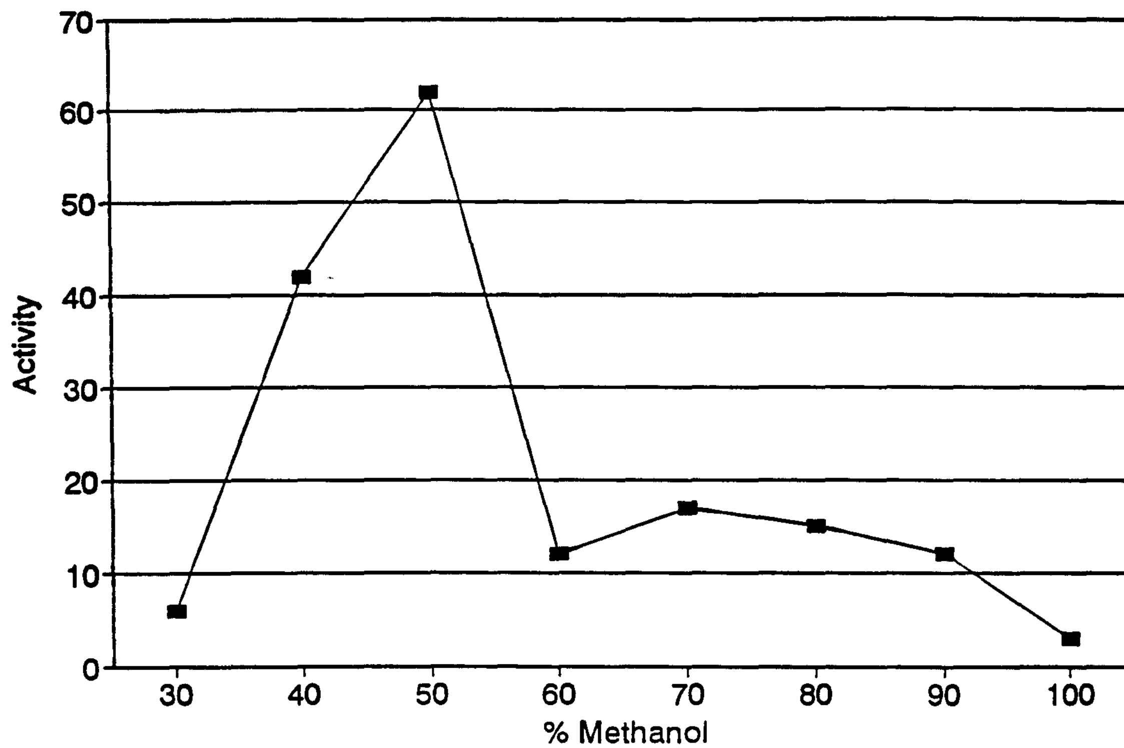


Figure 45) Column 1 activity with GLUT-ED cross-linking.

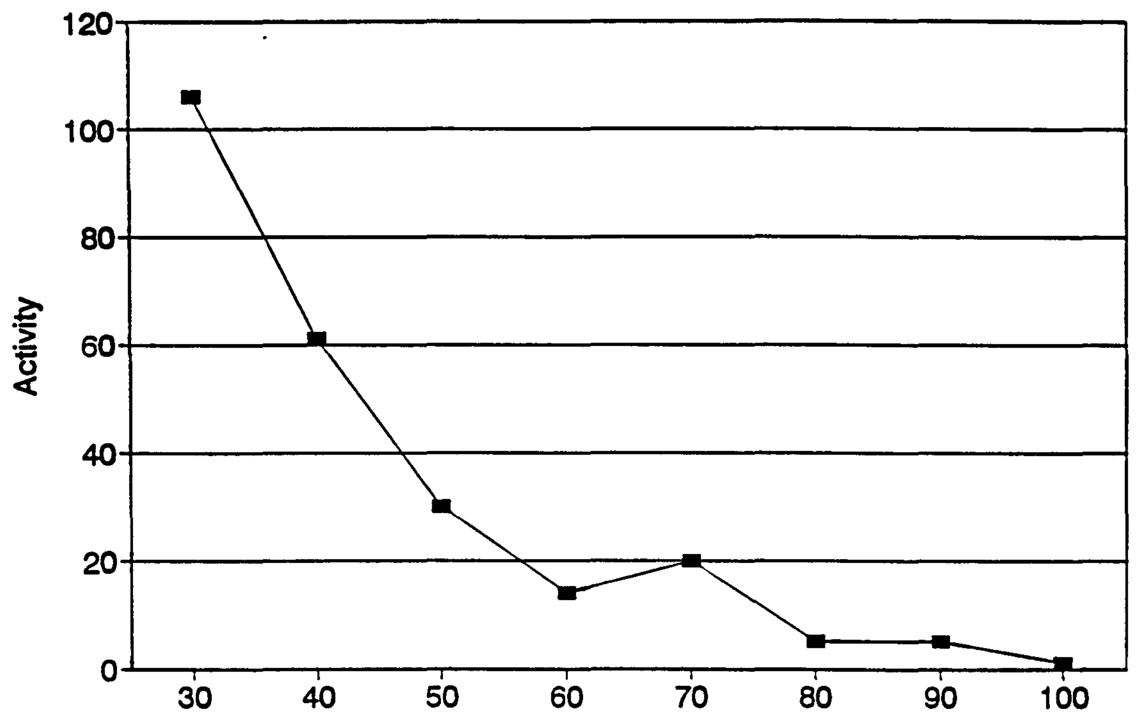


Figure 46) Column 1 activity with GLUT-TMD cross-linking.

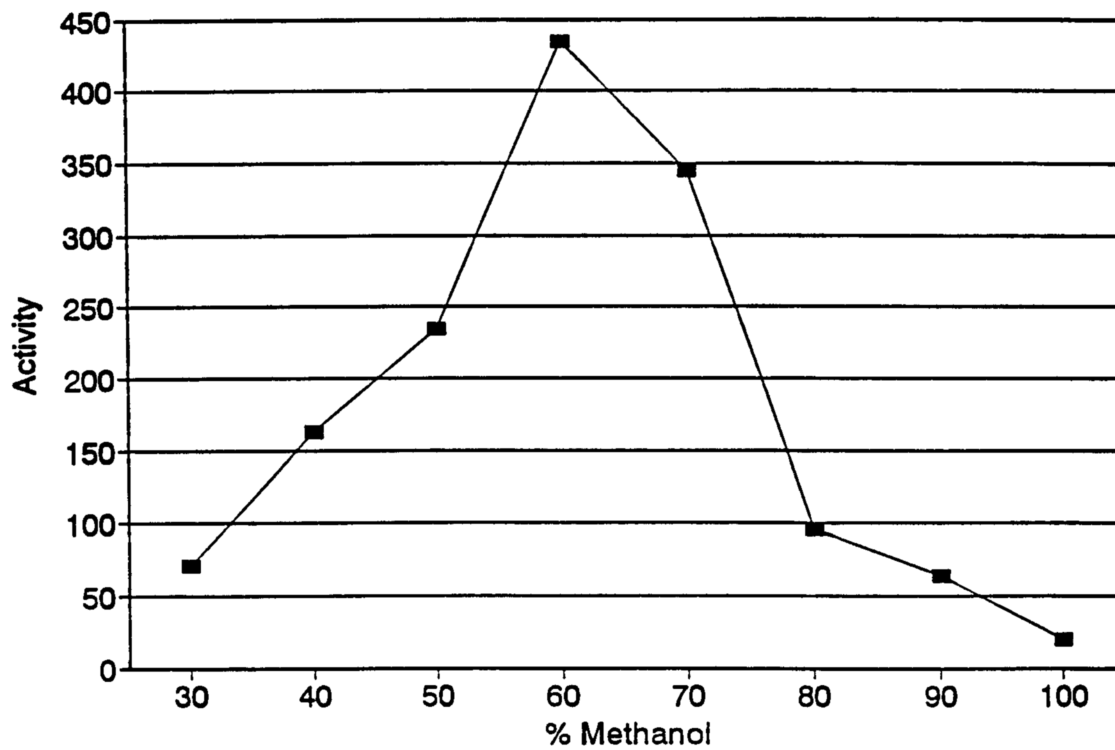


Figure 47) Column 5 activity with no cross-linking.

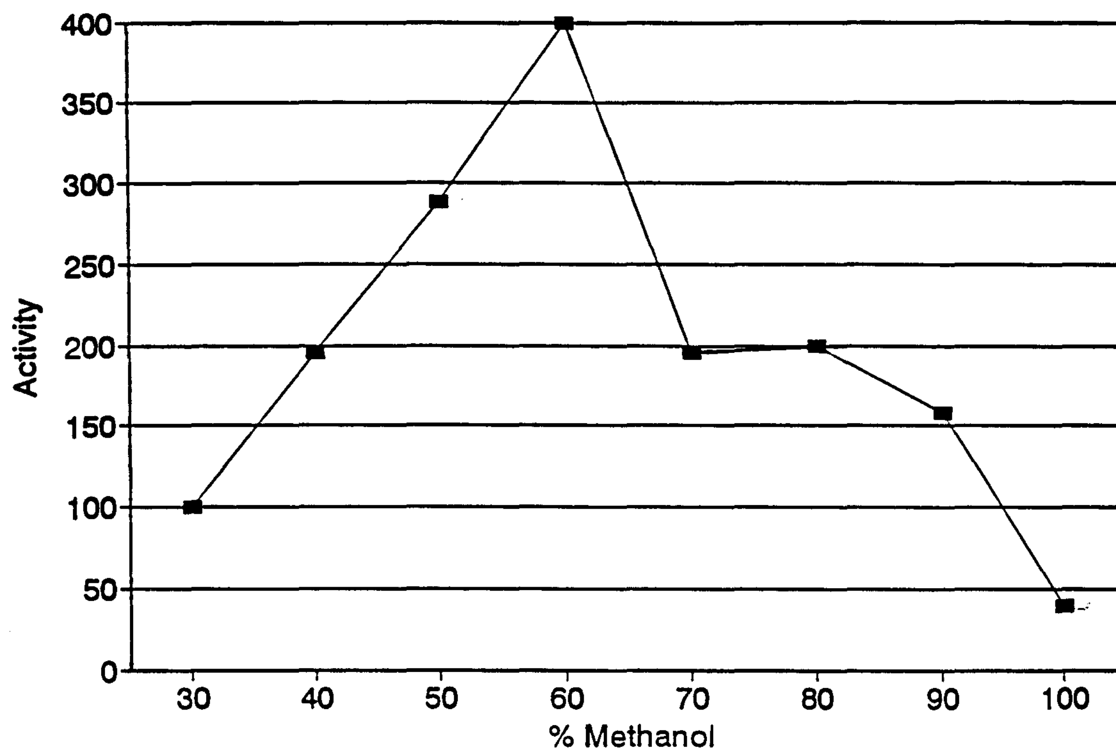


Figure 48) Column 5 activity with GLUT-THAM cross-linking.

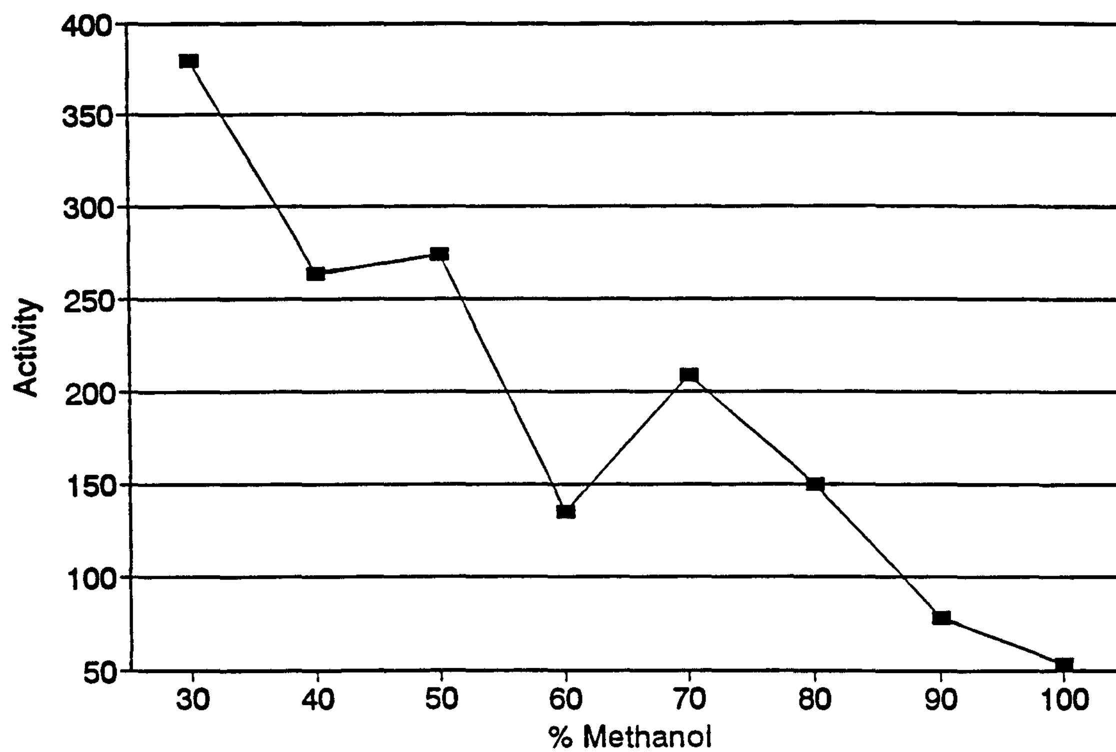


Figure 49) Column 5 activity with GLUT-ED cross-linking.

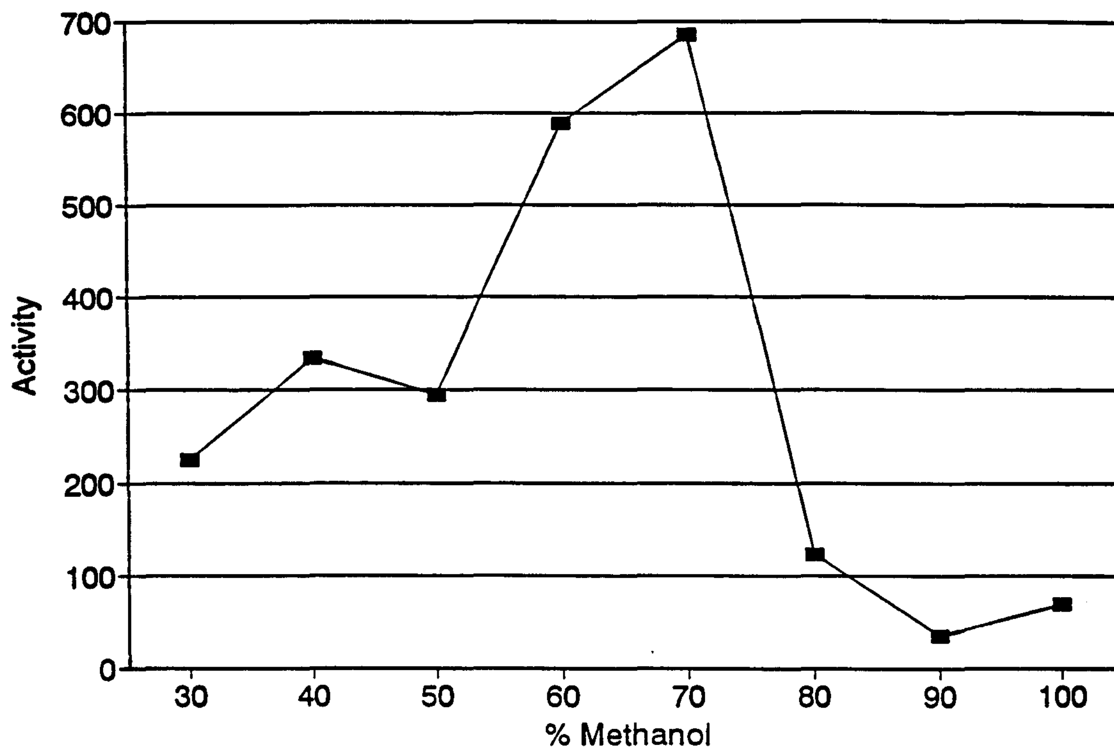


Figure 50) Column 5 activity with GLUT-TMD crosslinking.

Chapter 6

Results and Discussion

In chapter 5, all of the experiments performed and data recorded were outlined. In this chapter, the data has been tabulated, correlated, and interpreted. These comparisons, and their applied insights provide a meaningful representation as to the success or failure of these experiments. This study was grouped into three primary sections with several sub-sections within each section. The first group was a "Free" enzyme series of tests, the second was immobilized enzyme column analyses, and the third was immobilized and cross-linked enzyme column experiments. This chapter follows the sequence of chapter 5, beginning with the Free enzyme study.

Free Enzyme Study:

Once the enzyme was chosen, it needed to be assayed for activity, which provided the foundation for all other work in this project. The enzyme was purchased from Sigma Chemical Co., St. Louis MO, who also supplied an assay procedure that allowed for the calculation of units of enzyme per mg of sample. This assay was a quality check of the purchased product, and also served as a method of providing a base number of units of enzyme per mg of sample for future experiments. Figures 15 and 16 illustrate typical results of the assay procedure. The enzyme activity was calculated from equation (4) which required a blank scan to be performed. The blank scan was actually the non-enzymatic hydrolysis rate of BTEE at the conditions of the experiment. When this background reaction was subtracted from the enzyme action on the substrate an accurate depiction of the enzyme activity was shown.

Moving on from the assay, a methanol profile of the free enzyme was performed to establish the stability of the enzyme in the solvent environment. In the original assay, the methanol concentration was 30%. From this and from the aliquot of substrate solution used in the sample, it was determined that a 50% methanol sample could be

obtained with a substrate solution prepared from 100% methanol. The beginning of the profile started at 1% methanol since the substrate was not soluble in water. The methanol profile scans were similar to those in Figures 15 and 16. The only difference being that as the methanol concentration increased the rate of change in absorbance units decreased to the point where the blank slope and assay slope were almost equal. As a result, Figure 17 illustrates the stability of free chymotrypsin in increasing concentrations of methanol. Figure 17 was plotted as percent of activity versus percent methanol. Since the methanol in the 1% sample would have little denaturing effects on the enzyme, thus allowing it to perform efficiently, the activity at this point was dubbed 100% activity. All other activity values in the plot were done in reference to this level of activity. The denaturing effects of methanol on free chymotrypsin caused the enzyme to have a minimal activity at 50% methanol.

This information is very useful not only for comparison purposes later, but also as a base for the immobilized enzyme column studies. Since the free enzyme "died off" at about 50% methanol, it would be expected that stabilization upon immobilization should allow the enzyme to display catalytic activity at higher methanol concentrations.

The next series of experiments defined what research is all about. A set of experiments that did not work, followed by some insight, followed by a study that worked well.

After obtaining a good assay and methanol profile, the next step was to immobilize the enzyme, pack it into a column, and test its stability and activity levels.

Immobilized Enzyme Column Methanol Profile:

As described in the Experimental section, the enzyme was immobilized using a well established technique by Wheetall [8]. A portion of enzyme (0.2 g) was reacted with 0.2 g of support. The resultant column and sample placed was into a 37^{0}C water jacket for the assay. The problem with this analysis was that the column contained 8000 units of enzyme (6000 times more enzyme than in the free enzyme assay). To account for this,

the substrate would have to be increased. To increase the substrate to a comparable level would cause solubility problems, even at 30% methanol. To begin the analysis, 60% methanol was the required starting point. Precipitation of the substrate was discovered in the lines of the system and in the column. A new column and sample had to be used, only the sample had to be prepared in an unusual fashion. A blank column had to be used to prepare the sample. Substrate had to be added in repetitive aliquots to the circulating sample until the needed concentration was obtained. At this time, the enzyme column was inserted into the system and the run proceeded. Precipitation did not occur, but very little activity was exhibited. Since there was some activity present, a 70% and an 80% methanol sample were prepared. Figures 20-24 illustrate the activity scans at each methanol level. Figure 25 shows that some activity was obtained with its maximum at 60% methanol. Six units of enzyme were active at this point which corresponded to 0.1% of the enzyme immobilized remained intact and active. While this experiment yielded some activity it was obvious that there were problems. Also, the successive addition of substrate was not a very good way to prepare a sample for analysis, since in the final application of this column, for example an HPLC/PCR, successive addition of substrate would not be possible.

From this experiment the next logical step appeared to be a cross-linked experiment, since it was thought that the enzyme was readily denaturing on the surface of the support. Diluted GLUT was used to form links between the immobilized enzyme molecules. This intermolecular technique yielded entirely flat scans thus showing a "dead" column. The GLUT cross-linking may have affected the enzyme's activity in three ways. First, the GLUT may have formed a blanket over top of the immobilized enzyme thereby preventing the substrate from fitting into the active site. Second, the GLUT may have formed intermolecular cross-linkings that were short enough to pull the enzyme molecules apart, and thus, the GLUT may have reacted with amino groups near

the active site on the enzyme and then folded over thus denying substrate penetration. It was likely that a combination of all three effects had occurred.

Since GLUT cross-linking proved to be ineffective with enzymes that were already immobilized, it was thought that the enzyme could be cross-linked before the immobilization procedure, followed by immobilization. The enzyme was placed in solution and GLUT was added. The result was a milky white precipitate of enzyme and GLUT. The enzyme-GLUT solid could not be redissolved so the immobilization step was useless to try. A second enzyme solution was made in 0.05 M buffer with a pH=7.8, which allowed the enzyme and GLUT to cross-link and remain in solution. The attachment of the enzyme-GLUT matrix was performed as in the previous experiments, but no attachment had occurred since the chymotrypsin-GLUT precipitated at this point.

As a result, the approach to cross-linking the immobilized enzyme was redirected. Instead of cross-linking the enzyme intermolecularly, an internal or intramolecular experiment was conducted. This method formed the cross-links on the inside of the enzyme molecules, thereby allowing immobilization to occur from the outside. For chymotrypsin this was a complex and intricate procedure because the reactive amino acid residues of the enzyme had to be prepared by attaching carbodiimide units to them. This step alone has been shown to decrease the activity of the enzyme threefold. After the diimides were in place, a bifunctional reagent, or mixture of such, were used to form the links between the more reactive diimide groups. Once intramolecularly cross-linked, the enzyme (which remained in solution) was attached to the APG support. The two columns prepared were assayed in the same fashion as all other previous column types. The resultant spectra from these two columns yielded no change in absorbance thus indicating both columns contained inactive enzyme molecules.

Those immobilized enzyme experiments just mentioned did not work. However, they were essential learning experiences for properly redirecting the rest of the project. The key was the fact that if 0.2 g of support was used, then 0.2 g enzyme was also used.

This corresponded to 8000 units of enzyme which was an extremely large amount. The question was posed: Could the enzyme be denaturing itself during the immobilization procedure since such a high concentration was being used? This question governed the next experiment which showed an interesting relationship between enzyme concentration, substrate concentration, and activity.

Immobilized Enzyme Concentraton versus Substrate Concentration:

The scope of this study was to immobilize a range of enzyme units that was much lower than that previously used. Since no activity was obtained with the previous columns of 8000 enzyme units, the working range used here was set from 2000 enzyme units to 100 enzyme units (Table 4). Five columns were prepared as described in Chapter 5, each with the different enzyme concentrations. These five columns were assayed for activity using five different substrate concentrations ranging from 1.18 mM to 14.16 mM in threefold increments. Table 5 in Chapter 5 displays the substrate scheme used. Figures 29-33 of Chapter 5 illustrate the resultant activity plots, which appeared to follow the typical kinetic pattern of increasing steeply and then leveling off. Figure 29 shows a nice example of the described pattern. In Figure 30 the last two substrate runs (4.95 mM and 6.6 mM) were much lower than expected. The most probable explanation is partial substrate precipitation. The substrate was insoluble in water and when a substrate concentration of 9 and 12 times the assay substrate concentraton was used, it was possible that the substrate may have built up inside the column. While this trend was the most significant for column two, it was also present in columns 3 through 5. The activity at 3.3 mM appeared to be the inflection point in the graphs of Figures 29-33, so they were plotted as activity versus enzyme concentration in Figure 34. This graph revealed a trend that showed why significant activity was not obtained in all the previous immobilization experiments. The plot showed that the more enzyme used for immobilization, the less activity the column would display. Assuming 100% attachment of the enzyme, the 2000 unit column, at this substrate concentration, exhibited only 8.45% activity. When

applying this trend to the 8000 unit columns it now seems conceivable that 0.1% activity would be observed. The remaining studies used a two set column approach - the highest and lowest enzyme concentration columns would be used for comparison purposes.

Now that a base of activity versus enzyme concentration had been established, it was time to cross-link. The cross-linking approach also took on a new angle. This was based on the fact that 2000 units of enzyme and 100 units of enzyme, when immobilized on the same amount of support material, would be distributed across the support's surface differently. The intermolecular distance in the 2000 unit column should have been much smaller than in the 100 unit column. When this idea was tied together with intramolecular cross-linking techniques, not much could be done, but when intermolecular concepts were introduced, a whole new study could be performed. By using the same initial idea as in the failed experiment of placing a bifunctional reagent between enzyme molecules, a series of experiments were devised. Inserting a bifunctional unit between the enzyme molecules now seemed promising, but the distribution of the enzyme on the support surface was still unknown. To accomodate for this problem three different lengths of cross-linking molecules were used. GLUT had to be used in each one to react with the protruding amino groups of the enzyme. Something then had to be placed between the two GLUT molecules. THAM, ethylenediamine, and tetramethylenediamine were used as the inserts.

Intermolecular Cross-Linking Immobilized Enzyme Columns versus Substrate Concentrations:

Six columns were made - three 2000 unit and three 100 unit. These two sets of columns were cross-linked using GLUT/THAM, GLUT/ED, and GLUT/TMD. These six columns were then assayed by following the same substrate concentration profile as described in the previous experiment. Figures 35 and 36 illustrate the enzyme distribution on the support surface and the effects of cross-linking with variable length molecules. Figures 37-42 show the resultant activity plots for the six columns. They

were all similar in shape with respect to each other and also with the non cross-linked activity plots.

When the activity of the noncross-linked 2000 and 100 unit columns were compared to their respective three cross-linked columns the following table was formed:

Table 9: Activity summary of the two columns. The substrate concentration ([Sub]) below is represented as millimolar. The noncross-linked columns are listed as NOXL.

	Column 1 (2000 units)				Column 5 (100 units)			
[Sub]	**NOXL**	**GLUT**	**ED**	**TMD**	**NOXL**	**GLUT**	**ED**	**TMD**
1.18	14	42	11	17	933	1500	1586	1604
3.54	42	71	183	29	1828	4800	4904	4673
7.08	169	188	762	46	3715	8500	7875	8382
10.62	289	1569	765	48	3648	11000	9754	10627
14.16	336	1582	787	85	3433	13000	17922	10741

The general trend when comparing the noncross-linked, GLUT, ED, and TMD results for each substrate concentration for column l showed an increase from noncross-linked to GLUT (the maximum), a decrease from GLUT to ED, and a decrease from ED to TMD. The general trend from chapter 5 showed an increase from noncross-linked to GLUT, an increase from GLUT to ED, and a decrease from ED to TMD.

For column l at the assay substrate concentration of 1.18 mM, GLUT, the shortest cross-linked agent yielded the most activity, at the next to higher concentrations the ED column yielded the highest activity levels, and at the highest two substrate concentrations the GLUT yielded the maximum activity.

For Chapter 5 (in Figure 38) the maximum activity of the assay substrate concentration was displayed by the ED column 1, at three times the assay substrate concentration, the ED column had the highest activity. At the next two substrate concentrations column 1 yielded the highest activity levels, and at twelve times assay substrate concentration the ED column activity was also the highest.

Although one type of cross-linking did not provide superior activity for all the substrate concentrations in the profile for column l, cross-linking did enhance the activity of the column when compared to the non cross-linked column. The only exception to this was for the column cross-linked with TMD. This column had the least amount of activity of all the 2000 unit columns and this was most likely due to the the length of the GLUT/TMD linking agent. For the 100 unit column, cross-linking with ED proved to be most beneficial since it yielded the highest activity for all substrate concentrations in the profile. The TMD column gave the second most active column, the GLUT was third, and the noncross-linked column showed the least amount of activity of all four columns.

Now that some insight had been gained as to column behavior with different amounts of enzyme, substrate, and cross-linking, the stability of these columns in methanol were studied.

Enzyme Columns and Cross-Linked Enzyme Columns in the Methanol Profile:

In this stability study, eight columns were made; four each of the 2000 unit and 100 unit enzyme level. One column from each group was left uncross-linked, while the other three from each group were cross-linked with GLUT/THAM, GLUT/ED, and GLUT/TMD. For the analysis it would have been ideal to start at 10% methanol, but 30% methanol had to be the starting point since the water insoluble substrate precipitated out of solution in the 10% and 20% samples. Thirty percent methanol was an acceptable starting point since all other assays and profiles were performed at 30% methanol. The activity curves for the eight columns were plotted as Figures 43-50 of chapter 5.

For the 2000 unit column the activity curves all followed a trend that can be described as parabolic or bell-shaped. Each column started off with a certain level of activity, then as the percent of methanol increased, the activity would peak and fall back down. In the non cross-linked column the peak activity was observed at 70% methanol. At 80% methanol the activity was comparable to the activity at 40% methanol. The GLUT/THAM column in Figure 43 shows no significant loss of activity from 30-50%

methanol, followed by the maximum activity at 70% methanol. The 80 and 90% values were again similar to the 30-50% values. For the GLUT/ED column, the bell shape was still present, but the maximum activity was observed at 50% methanol instead of 70%. The 60-90% methanol activity levels all remained close together. Finally, in the GLUT/TMD column the general trend is again broken away from. This column begins with its highest activity level at 30% methanol and regularly drops as the percent of methanol increased.

The general bell-shaped pattern was also evident in the 100 unit columns. The non cross-linked column in Figure 47 exhibited its maximum activity at 60% methanol. Again the 80 and 90% methanol activity levels were similar to the 30% methanol level. Figure 48 in chapter 5 was a plot of the GLUT/THAM column. The activity from 30-60% methanol was relatively linear. The maximum activity displayed by this column was at 60%methanol with the 70-80% activity levels being comparable to the 30-40% methanol levels. As in the 2000 unit columns, one column here broke the bell-shaped trend and showed a curve with the maximum activity level at 30% methanol. When looking at the GLUT/TMD column the bell-shaped trend was back and the maximum activity this time was not at 60% methanol but at 70% methanol.

Column Comparison by Group:

In comparing the noncross-linked 2000 unit column with its cross-linked counterparts, Figure 51 resulted. This plot of all four columns shows that the noncross-linked column yielded the largest amount of activity at 70% methanol than any of the cross-linked columns. This was the percent of methanol where each column exhibited maximum activity in their bell-shaped trend. All the cross-linked columns converged at 60% methanol and remained similar in activity through 100% methanol. The noncross-linked column joined the similar activity levels of the cross-linked columns at 80, 90, and 100% methanol.

When comparing the activity of the noncross-linked 100 unit column with the 100 unit cross-linked columns in Figure 52, the noncross-linked column exhibited the second highest activity levels at 60 and 70% methanol. The TMD cross-linked column had the highest activity levels at the 60-70% methanol bell-shaped maximum. All of the columns converged, in a similar fashion as the 2000 unit column, from 80-100% methanol.

Column Activity at 100% Methanol:

For the 2000 unit column, very little activity was seen at 100% methanol. From 2000 units immobilized, only 1.5 units remained active in the noncross-linked column which corresponded to 0.075% activity (assuming 100% immobilization). When considering the activity of the column at 30% methanol, the column lost 90% of its activity at 100% methanol. The GLUT/THAM column was not much better at 100% methanol with 0.1% activity. This column did not lose as much activity as the noncross-linked column in that comparing the 30% run and the 100% run, only 60% of the activity was lost. The highest level of activity at 100% methanol was demonstrated by the GLUT/ED column. This column had 0.15% activity at this methanol concentration. Also in comparing the 30% methanol activity with the 100% methanol activity of this column, 50% of the initial activity was lost. The last column in this group did not demonstrate the bell-shaped pattern and also yielded the least amount of activity (0.05%) at 100% methanol. This GLUT/TMD column did however yield the most activity at 30% methanol. In considering the stability of the column from 30% methanol to 100% methanol, this column lost 99.1% of its activity. Figure 53 plots the 100% methanol activities for each of the 2000 unit columns. The activity values are plotted in order of increasing length of the cross-linking agent. The resultant Figure 53 also shows a similar bell-shaped plot with the GLUT/ED column being the most active column at 100% methanol.

When the 100 unit column activities at 100% methanol were plotted, the result was not a bell-shaped curve as the previous plots displayed. Figure 54 showed the

activity of each column at 100% methanol with respect to cross-linking agent length. The graph was almost completely linear with the noncross-linked column having the least amount of activity with 20% of the 100 units being active. When comparing the starting activity at 30% methanol to the 100% methanol value, the column lost 72% of its activity. The 2000 unit noncross-linked column lost 90% of its activity, so this was an improvement. The GLUT/THAM column retained 40 of the 100 units immobilized at 100% methanol. This column lost 60% of its activity as compared to its 30% methanol starting point. The 2000 unit column lost the same amount. By using GLUT/ED to cross-link the enzyme, the resultant 100% methanol run yielded 53 of the 100 units immobilized, but from the 30% initial run it lost 86% of its activity. This is 36% more lost activity than the 2000 unit ED cross-linked column. The GLUT/TMD column yielded the highest level of activity at 100% methanol by yielding 70 of the 100 units immobilized. When comparing the activity levels at 30 and 100% methanol, the loss of activity was 69% as opposed to 99% for the 2000 unit column. The overall plot in Figure 54 shows that the longest cross-linked agent used provided the most stable enzyme column in methanol. Also, the different activity values obtained for both column sets provided information about the distribution of enzyme molecules on the surface of the support material. Longer cross-linking agents for the 100 unit columns may provide for even more activity in 100% methanol.

Finally, in comparing the column activities back to the free enzyme methanol profile, it can be seen that where the free enzyme died at approximately 50% methanol, the immobilized enzyme and immobilized cross-linked enzyme columns were beginning to exhibit their maximum activity. The free enzyme proved to be the least stable, the 2000 unit columns were next, and the 100 unit columns were the most stable systems in the methanol profiles. Cross-linking provided even more stability and activity at higher methanol concentrations then noncross-linking.

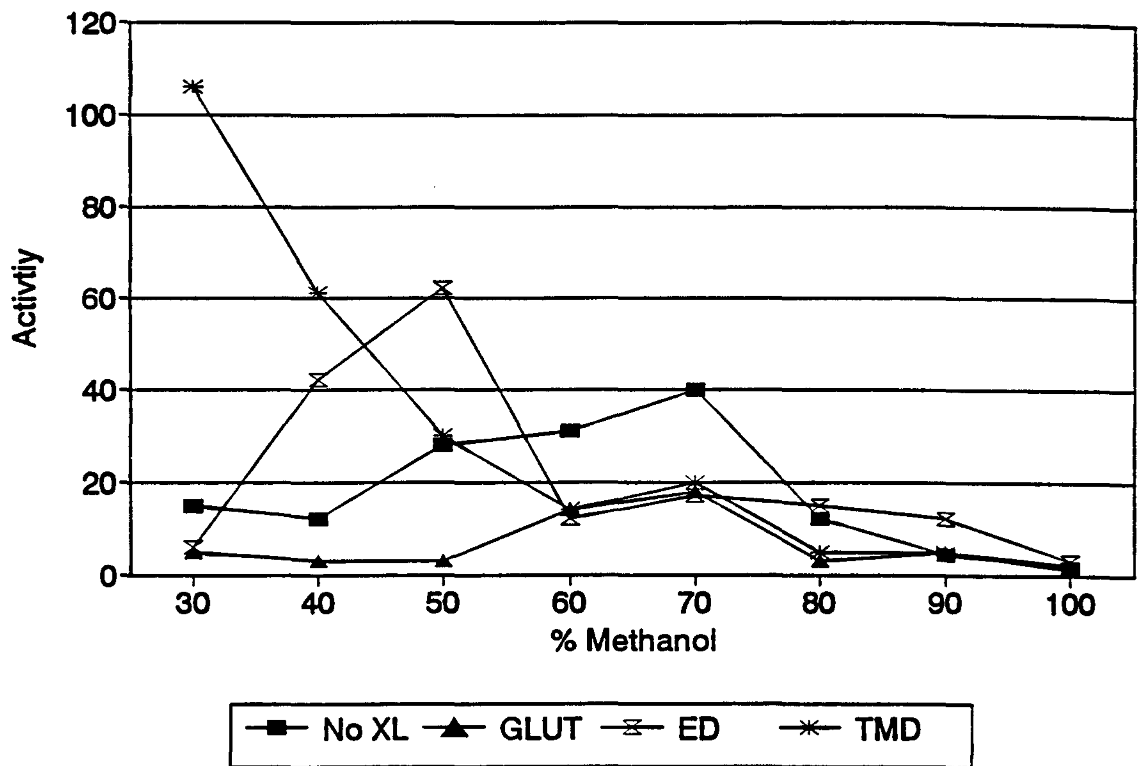


Figure 51) 2000 unit column plot of activity vs. no cross-linking vs. GLUT/THAM vs. GLUT/ED vs. GLUT/TMD.

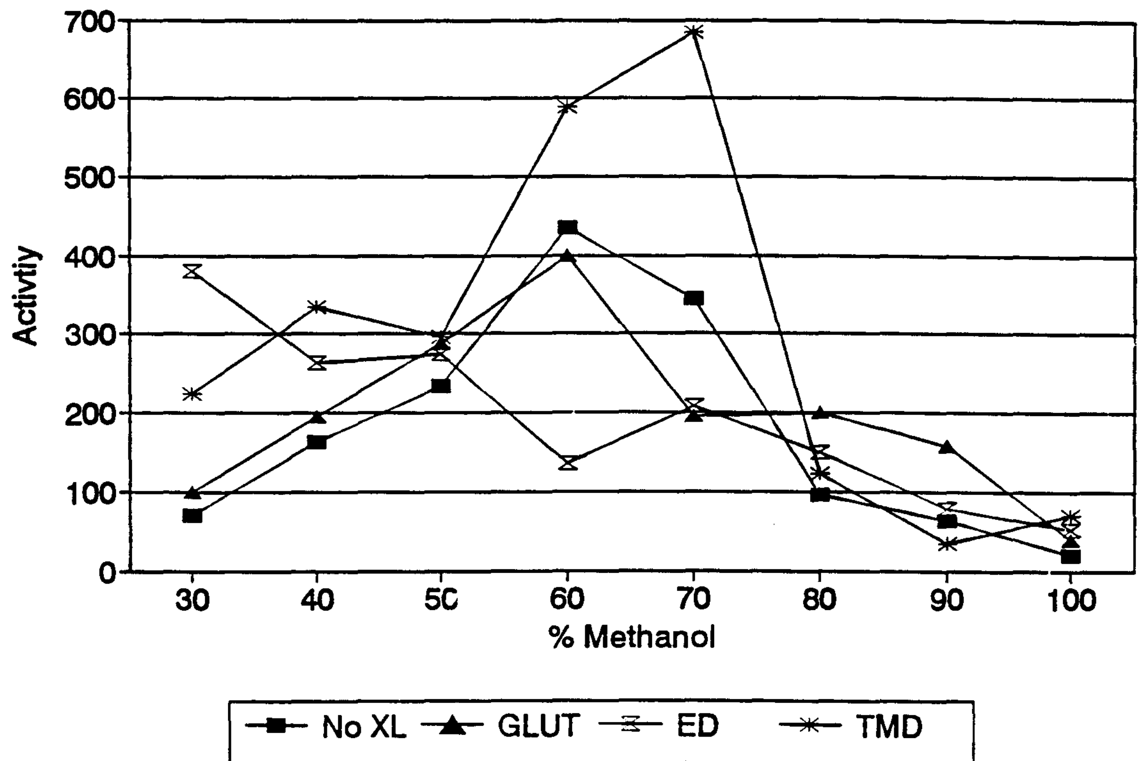


Figure 52) 100 unit column plot of activity vs. no cross-linking, vs. GLUT/THAM, vs. GLUT/ED, vs. GLUT/TMD.

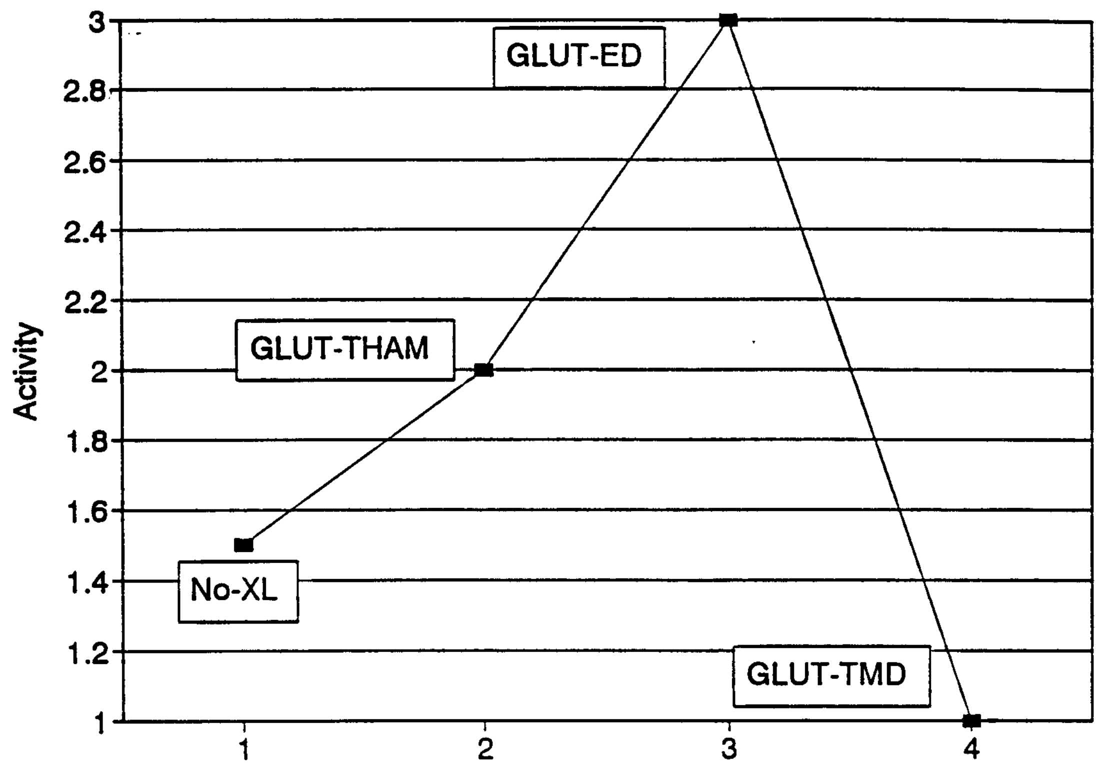


Figure 53) 100% methanol activities for each 2000 unit column.

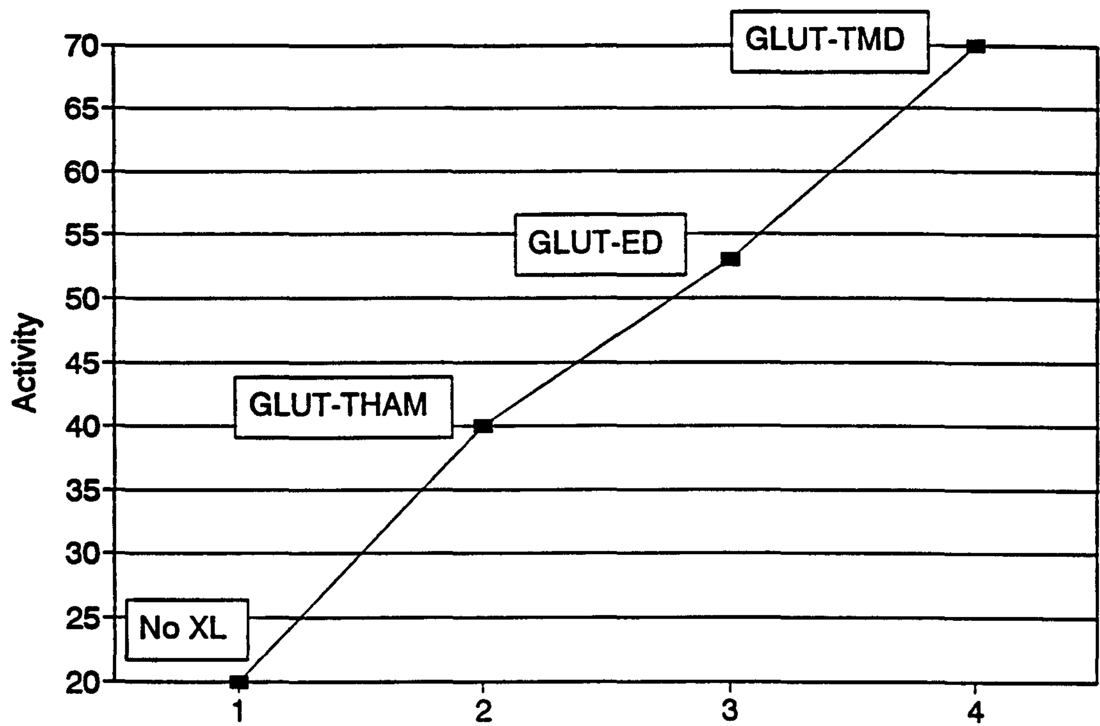


Figure 54) 100% methanol activities for each 100 unit column.

Chapter 7

Conclusions

Some important insights concerning immobilized enzyme columns were gained as a result of this study. First, and most significant, was the fact that larger amounts of enzyme yielded smaller amounts of activity. The optimum amount of enzyme to support probably depends on the enzyme being used. Smaller amounts, however, appear to work best. Second, while immobilizing an enzyme provided stability, cross-linking the immobilized enzyme properly led to even greater levels of stability and activity. The third factor arises from enzyme distribution on the support material. A densely packed layer of enzyme on the surface was demonstrated to be more inefficient than a layer of enzyme that had some distance between the molecules. This distribution also allowed for more uniform cross-linking.

Future investigations should involve the testing of other enzymes for the optimum amount to immobilize to yield maximum levels of activity. Since there are many different bifunctionals available, a more extensive study of their role in stabilizing immobilized enzymes should be conducted. A coimmobilization of the enzyme along with a potential stabilizing agent may yield similar results. Methanol is only one of several organic components found in HPLC mobile phases. In order to add even more depth to this project, the stability and activity of these immobilized and cross-linked enzyme columns should be tested in acetonitrile, tetrahydrofuran, etc. Finally, in order to prove that cross-linking did occur, to see what type of cross-linking is forming, and to what degree the cross-linking is occurring, studies should be performed to identify these dilemmas.

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