CHEMICAL STUDIES OF ENZYME IMMOBILIZATION BY GLUTARALDEHYDE SPACING

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Two scriptures have fortified me during my academic career. The first reminded me that worrying was useless, to let my goals be known to God and his peace would sustain me (Phillipians 4:6-7). I read James 1:5 before difficult exams. Of course, there is the scripture which teaches that faith without (hard) work is dead. I thank God for making my boyhood dream to be a scientist a reality.

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NOMENCLATURE

CPG	Controlled-pore glass
CPG A	80/120 mesh particles, 1273 A pore CPG
CPG B	200/400 mesh particles, 1273 A pore CPG
CPG C	80/120 mesh particles, 530 A pore CPG
CPG D	80/120 mesh particles, 547 A pore CPG
CPG E	80/120 mesh particles, 156 A pore CPG
DACA	p - Dimethylaminocinnamaldehyde
FIA	Flow injection analysis
h	hour(s)
min	minute(s)
OTR	Open-tubular reactor
s	second(s)
SBSR	Single-bead-string reactor

CHAPTER I

INTRODUCTION

Continuous flow sample processing is not a new idea, applications of the forbearers of the modern science can be traced to the 1800's (1). The innovative application of sample processing in flowing streams holds great promise for the future and can be used in areas which are not strictly analytical in nature (2,3).

Modern continuous flow sample processing is regarded as having originated in the 1950's with Skeggs (4). Faced with an enormous backlog of analysis requests, Skeggs fashioned an assembly line within tubes. The automated system required less operator intervention and allowed a great number of repetitive samples to be processed in a relatively short time. Skeggs' system employed air bubbles to package each sample separately in the processing train. This type of segmented-flow system is widely referred to as Continuous Flow Analysis (CFA).

Interest in unsegmented flow systems has increased recently as exemplified by the publication of two monographs on the subject (5,6). Unsegmented continuous flow sample processing, popularly called Flow Injection Analysis (FIA), has been called the most advanced form of solution manipulation available to the analyst (6).

Both segmented and unsegmented systems utilize a pumping system to move carrier solution, reagents, and samples through a manifold composed of narrow tubes connecting various components, (e.g. sample injector, extractor, reactor, and detector). A sample solution is intercalated into the carrier stream in a manner which leaves the "plug" sandwiched between segments of the carrier in the moving stream. A great variety of processing components have been used in continuous flow sample processing. Psuedo-titrations, solvent extractions, distillations, dialysis, preconcentration by solid extraction, and ion-exchangers have all been implemented in FIA systems (6). FIA is a viable method for quickly processing a large number of repetitive samples in a convenient manner.

The use of enzymes, especially immobilized enzyme reactors in flow systems is rapidly becoming popular. Many enzyme preparations are expensive, this precludes their use in a great number of routine sample analysis schemes. The ability to reuse immobilized enzymes allows the cost (associated with a single determination) to be spread over a large number of samples. Enzyme solutions are not usually stable for long periods; appreciable loss of enzymatic activity with time can be observed in soluble enzyme preparations. The longevity of enzymatic activity is usually much greater in immobilized preparations as compared to soluble preparations (7). These reasons (lower cost,

ability to reuse, and greater stability) have led investigators to explore the use of insoluble or immobilized enzymes as analytical reagents (7). Enzymes are valuable analytical reagents and their use in the immobilized state makes the analytical methods developed with them cost effective.

If an enzyme is immobilized on material confined to a small section of tubing or other flow-through enclosure, such an enzyme reactor can be incorporated into a FIA manifold. This represents a convenient and simple way to fashion a method for catalytic substrate determinations. Considerable study has gone into the design and subsequent use of enzyme reactors in unsegmented continuous flow systems (7-9). Substrate solutions entering the reactor come in contact with the immobilized enzyme and are converted to product(s) which can be detected by monitoring some change in solution properties (pH, absorbance, etc). The conversion process is often selective enough to allow mixtures to be processed without prior separation.

When immobilized enzymes are used in FIA, the analytical method posses the attributes inherent with insoluble enzymes (high selectivity or even specificity, cost effectiveness, increased reactor stability) coupled with those associated with FIA (high sampling rate, low operator involvement, controlled and reproducible processes). The studies undertaken by the author have the common theme of improving either the implementation or the

fundamental understanding of the physical design of reactors and the chemical synthesis of reactors. The results of two projects are described herein. The first study resulted in the fabrication of an improved immobilized enzyme reactor. The final study describes an analytical method to determine amino groups on silica surfaces. This method was developed to be used as a tool to gain a better understanding of the glutaraldehyde procedure of immobilizing enzymes.

Enzyme Reactor

Applications which required high substrate conversion have often been forced to sacrifice high throughput, low back pressure, and low background noise to achieve this goal (9). The enhanced surface area open-tubular reactor reported here has increased sensitivity and allows the attainment of higher substrate conversion with low back pressure, low electrochemical background, and high sample throughput. The most important aspect of the new reactor design to practitioners may be the quickness and ease of manufacture (10).

Determination of Amino Groups

Glutaraldehyde immobilization of enzymes has been used in the synthesis of reactors for some time. There is today a lack of understanding of the chemistry of the immobilization process, especially the type of bonding involved and the final product structures which are formed

when protein amines react with glutaraldehyde. Immobilization of amines is the first step in the glutaraldehyde method of enzyme immobilization. Surface bound amines have been nondestructively determined in a manner which (when combined with other existing methods) will allow the analyst to quantitate each step in the glutaraldehyde enzyme immobilization procedure (11). This quantitation may provide a better fundamental understanding of the mechanism of glutaraldehyde interaction with amines.

Chapter Preview

Chapter II presents background information on the reagent glutaraldehyde. This chapter presents the state of knowledge of glutaraldehyde chemistry at this writing. A review of the literature will outline the efforts to unravel the mechanism of glutaraldehyes action.

Chapter III is a historical overview of both topics to be addressed. The relative merits of several enzyme reactor configurations will be examined first. A brief literature review of existing methods determination of aminosilames on surfaces will follow.

The experimental apparatus, conditions, chemicals, and procedures used in this study are presented in chapter IV. The results and discussion of the studies are found in chapter V. This last chapter is divided into two parts. The results obtained using the new reactor are presented

first, then results and details of method development for determining silica bound amines are discussed.

CHAPTER II

THE CHEMISTRY OF GLUTARALDEHYDE INTERACTION WITH PROTEINS

Glutaraldehyde has a remarkable ability to crosslink and/or immobilize proteins under conditions which allow the preparations to retain the original molecule's biological activity. The utilization of glutaraldehyde in electron microscopy, enzyme immobilization, and protein crosslinking has led to important innovations in several scientific areas. However, the mechanism by which glutaraldehyde reacts with protein amino groups is still unclear. The success of glutaraldehyde in a great many applications has been enough to lead to its extensive use.

This chapter will briefly review the use of glutaraldehyde by the technical community. The reaction mechanisms proposed for the reaction of glutaraldehyde with amino groups in homogeneous systems and the corresponding proposed product structures will be presented. Finally, an evaluation of several proposed mechanisms as potential descriptions for immobilization of enzymes will be presented. A brief section on the lack of enzyme-

immobilization studies in heterogeneous systems is also included.

The five carbon straight chain saturated dialdehyde, pentanedial, is referred to as glutaric dialdehyde, glutaral, but more commonly as glutaraldehyde. The first reported synthesis was published in 1908 (12). It is a clear to straw colored liquid with the odor of rotting fruit. Wide use of this reagent started with the discovery of it's exceptional leather tanning ability. The nature of glutaraldehyde-protein interactions were first of interest to leather chemists who discovered its remarkable tanning properties (13).

Glutaraldehyde is produced commercially in a two step process which yields 25-50% aqueous solutions (Figure 1). After acrolein and vinyl ethyl ether react (reaction 1), mildly acidic water is added to the dihydropyran to yield glutaraldehyde (reaction 2). Ethanol and excess water are vacuum distilled and a 25% solution of glutaraldehyde, stable for up to a year, remains (14). Under suitable conditions, glutaraldehyde undergoes typical aldehyde reactions, forming bisulphite complexes, oximes, cyanohydrins, acetals, and hydrozones (15).

The first major biological use of glutaraldehyde was as a tissue fixative in electron microscopy (15-18). The mild conditions of fixation, which left the fixed material biologically active, allowed the location of various enzymes within the ultrastructure of tissue samples (17). The

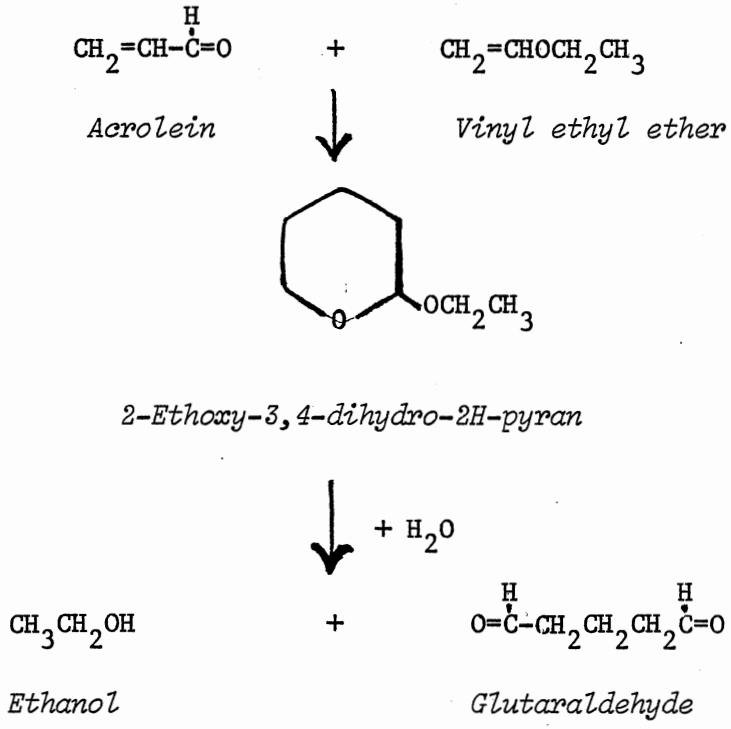


Figure 1. Manufacture of Glutaraldehyde.

following short review of glutaraldehyde's interactions with proteins has been separated into four groups: intramolecular, intermolecular, cellular, antimicrobial.

Intramolecular Crosslinking

Intramolecular crosslinking of proteins by glutaraldehyde can, with the judicious choice of conditions, yield solidified protein structures whose 3 dimensional configuration still retains biological activity (19). Quiocho and Richards used glutaraldehyde to form solid state single crystals of the enzyme carboxypeptidase-A (20). Inter and intra enzyme molecule crosslinks were used to prepare water insoluble trypsin (21) and glutamic dehydrogenase (22). Enzyme conjugates (enzymes internally crosslinked by glutaraldehyde) demonstrated higher resultant enzyme activity when immobilized than non-crosslinked preparations immobilized similarly (23). Performance of enzymes already immobilized may be improved by internal crosslinking. Stability of pyruvate oxidize immobilized by membrane entrapment was improved by subjecting the preparation to crosslinking by gaseous glutaraldehyde (24).

Intermolecular Crosslinking

The linking of proteins with various supports and with other proteins is the basis for some significant biochemical techniques. Coupling of solid supports and biologically active proteins formed the basis of some column materials

for affinity chromatography (25,26). The first method to use a glutaraldehyde antibody-enzyme link was a microscopy technique to locate tissue antigens (27). ELISA (Enzyme Linked ImmunoSorbent Assay) uses glutaraldehyde to form active enzyme-antibody conjugates, and also to bind antibodies to solid phases (29). Of methods utilizing the protein binding abilities glutaraldehyde, this technique is currently receiving increased attention. Antibody immobilization by glutaraldehyde for ELISA methods have been reported on various solid materials including etched glass beads (29,30), aminated silochrome (31), methacrylate polymers (32), polypropylene (33), thermoplastic cellulose (34), polystyrene and nylon beads (35).

Use With Cellular Material

Viable (living) cells have been coupled to proteins and other materials by glutaraldehyde. Possible treatments for enzymatic deficiencies of pathological conditions led to investigation of coupling enzymes to red blood cells (36). Enzymes were also immobilized on microbial cell walls (37). Cells have been bound to various carriers to modify metabolism (38) or enhance amino acid production (39). Cell cakes and gels are prepared by treating the cellular materials with glutaraldehyde solutions (40,41).

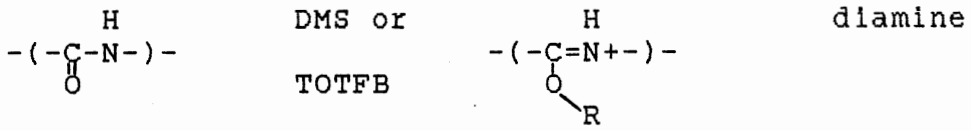
Antimicrobial Uses

Glutaraldehyde has been widely used as a biocidal

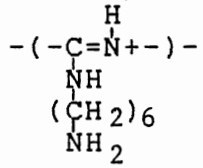
agent. The effectiveness of glutaraldehyde as an antimicrobial (42,43), bactericide (44,45), fungicide (46), sporicide (47) and viricide (46) has been reported. Glutaraldehyde possess special antimicrobial activity in comparison with other aldehydes and dialdehydes beyond hexanedial have virtually no sporicidal activity (15).

Enzyme Immobilization Using Glutaraldehyde

Glutaraldehyde is one of the most widely used coupling agents in enzyme immobilization. The immobilization procedures which use glutaraldehyde have mild conditions, are simple, fast, and can be applied to a large variety of enzymes (7). The introduction of amino groups onto the solid support prepares the surface for the reaction with glutaraldehyde. The use of glutaraldehyde immobilization of enzyme depends on the ability to first derivatize the proposed material to yield an aminated surface. The reactions shown below are for the immobilization of amino groups on (aminization of) nylon.



Nylon backbone



(1)

Nylon can be O-alkylated by either dimethyl sulfate (DMS), $(CH_3O)_2SO_2$, or triethyloxonium tetrafluoroborate (TOTFB), $(C_2H_5)_3OBF_4$. A diamine, typically 1,4 diaminobutane, is allowed to react with the derivatized nylon to yield free amino groups.

Silica surfaces can be aminated by reacting surface silanol groups with an aminoalkoxysilane. In the reaction shown below aminopropyltrimethoxysilane in 95% alcohol is reacted with a silica framework to give aminated glass.

$$|\text{-Si-OH} + (CH_3O)_3\text{-Si}(CH_2)_3NH_2 \longrightarrow |\text{-Si-O-Si}(CH_2)_3NH_2 \quad (2)$$

Once aminated, nylon or glass surfaces undergo sequential reactions with aqueous solutions of glutaraldehyde and enzyme to yield the immobilized preparation.

$$|\text{-}\{\ \}\text{-}NH_2 + O{=}\overset{H}{\underset{|}{C}}\text{-}(CH_2)_3\text{-}\overset{H}{\underset{|}{C}}{=}O \longrightarrow |\text{-}\{\ \}\text{-N} : \overset{H}{\underset{|}{C}}\text{-}(CH_2)_3\text{-}\overset{H}{\underset{|}{C}}{=}O \quad (3)$$

$$|\text{-}\{\ \}\text{-CH=O} + H_2N\text{-Enzyme} \longrightarrow |\text{-}\{\ \}\text{-CH} : \text{N-Enzyme} \quad (4)$$

The Nature of Aqueous Solutions of Glutaraldehyde

The glutaraldehyde-protein reaction controversy starts

with the solution chemistry, specifically the structural nature of the aqueous configuration of the compound by most investigators. Complicated polymeric, oligomeric, and cyclic structures are thought to predominate the forms in which the compound exist in aqueous solutions.

Richards and Knowles interpreted NMR studies to mean that glutaraldehyde solutions were oligomeric, containing significant quantities of α-β-unsaturated aldehydes (48). The proposed structure is the product of numerous aldol condensations (Figure 2). The presence of certain NMR peaks (4.8, 6.6 and 8.3) they say, show unequivocally the existence of α-β-unsaturated material (48). Richard and Knowles contended that most of glutaraldehyde was polymeric and consists of a mixture of dimer, cyclic dimer, trimer, and higher polymeric species. They confirmed their conclusions by freeze drying a sample, redissolving in D2O and obtaining an NMR spectra.

This paper fails to list detailed information necessary to compare it with other reports in this field. Unanswered are questions such as: what percent concentration was the glutaraldehyde, were any purification steps undertaken before the NMR spectra was obtained, and most importantly, what was the pH of sample.

In 1975 studies by Monsan's group (49-51) supported the findings of the original work of Richard and Knowles. These investigators argued that commercial solutions of glutaraldehyde were acidic (pH 3.1) and immobilizations

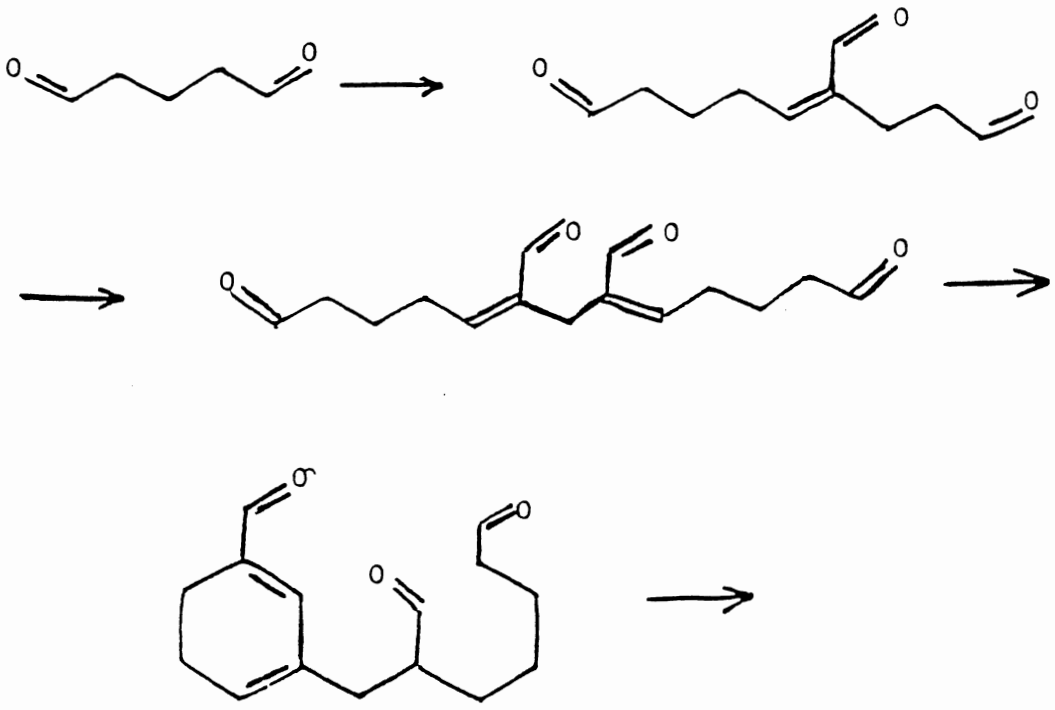


Figure 2. Aldol Condensation Products of Glutaraldehyde

generally occurred in neutral of basic medium. He used thin layer and gel chromatography, IR, and NMR to study glutaraldehyde solutions at pH 7.0 and pH 10.5 and found the α-β-unsaturated polymers suggested by Richards and Knowles. the amount of α- β-unsaturated material increases with pH until it finally precipitates at pH's greater than 11. Monsan analysis of 25% glutaraldehyde found little free dialdehyde (about 3%), a complex mixture of components (18%) of various molecular weights which easily revert to glutaraldehyde and 79% water.

Rydon, Nichols, and Hardy (52) reported finding α-β-unsaturation as evidence by light absorbance at 235 nm. They concluded contrary to Richards and Knowles, that it was a minor component of the organic content of commercially aqueous glutaraldehyde solutions (52). Ether extraction followed by distillation removed the unsaturated component from the aldehyde solution. These purified ether solutions had the simplied proton NMR of monomeric glutaraldehyde. Rydon et al. dissolved pure glutaraldehyde in deuterated water (D_2O) and observed the proton NMR of the solutions. Figure 3a shows the three hydrates proposed as well as the equilibrium thought to exist.

The NMR of these solutions remained stable for several weeks. No re-appearance of absorbance at 235 nm and no NMR peak at 6.6 were presented as proof that unsaturation was not returning to the material. Therefore according to Rydon et al. polymerization of neutral solutions was at best very

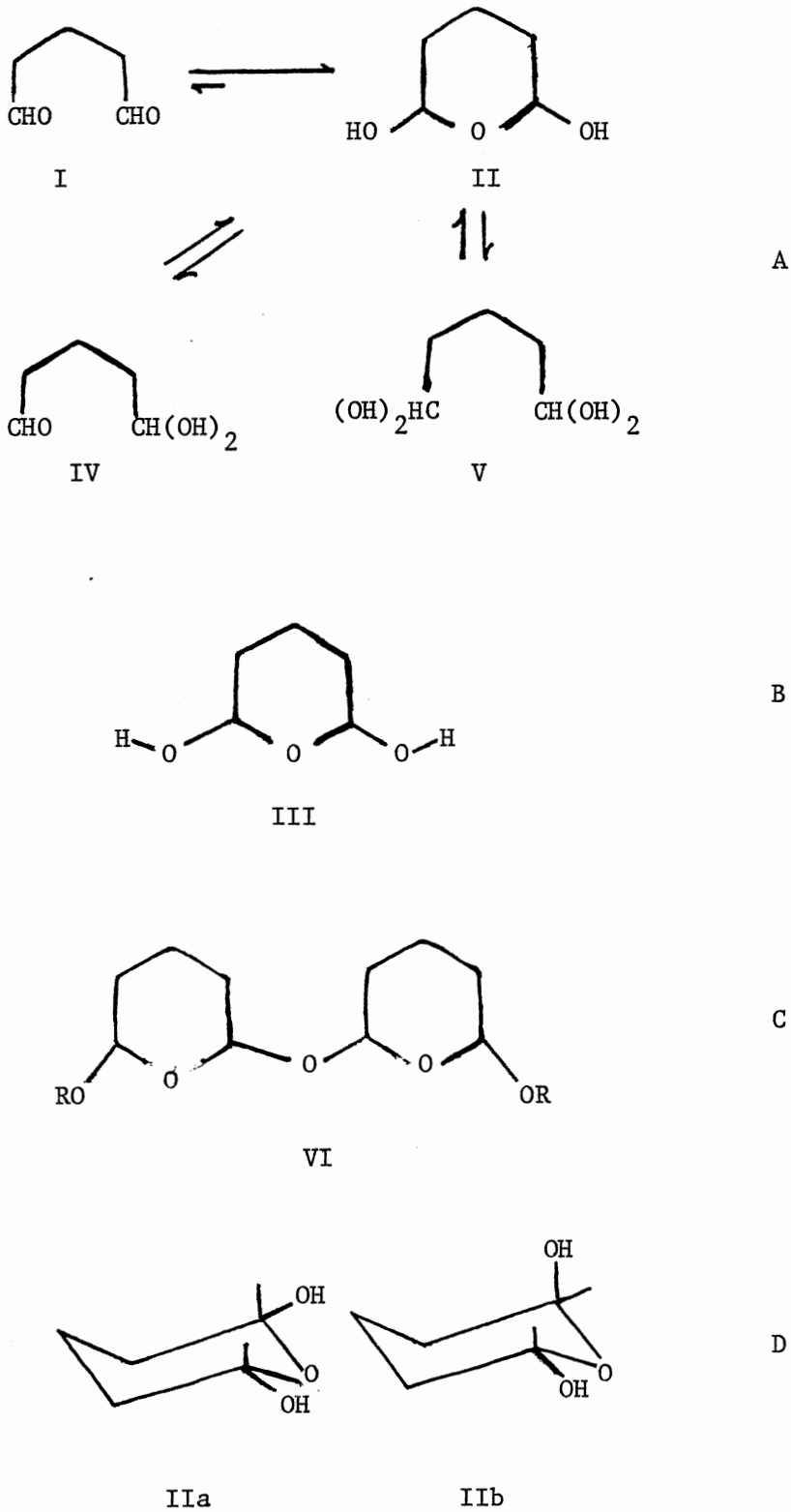


Figure 3. Proposed Structures for Aqueous Glutaraldehyde

slow. They concede that in alkaline solutions (pH 8) glutaraldehyde does polymerize and in those conditions the structures proposed by Richards and Knowles may be involved in the reaction with protein.

Korn, Feairheller, and Filachione (53) agreed with Rydon and his co-workers that the UV absorption peak at 235 nm is due to an unknown impurity in commercial samples of glutaraldehyde. This impurity, Korn et al. state, may even be the proposed aldol condensation polymers of Richards and Knowles. Korn and coworkers also sided with Rydon et al.'s work in saying that this component was minor (<1%).

The effect of temperature on the composition of glutaraldehyde solutions was studied by obtaining NMR at temperatures from 25 °C to 90 °C. The chemical shifts of most peaks remained constant, however the relative intensities did change indicating a shift the equilibrium between the various forms. Free glutaraldehyde was present at all temperatures and indeed dominated at 90 °C. At 75 °C and 90 °C, based on the NMR spectra, the dihydrate and hemihydrate could not occur. At 50 °C the spectra could not preclude the possibility that the dihydrate and hemihydrate were present in solution, but in the view of the investigators, inclusion of those forms is not necessary to explain the spectra.

Studies by Korn et al. concluded that the reagent was 15% free dialdehyde (I) and 85% in the form of structures II and III (Figure 3b) at room temperature, with no

contributions from other forms. This differs sharply with Rydon's group who state that form II, IV and V predominate as solution components with no, or at best trace amounts, of free dialdehyde (I). Korn et al. postulate that the free dialdehyde form may be responsible for the reaction with proteins. The existance of species with structure III, was suggested when pure glutaraldehyde was acetylated and the product shown in Figure 3c was obtained.

Whipple and Rutta (54) used carbon-13 NMR to refine the conclusions of Rydon's group. Their conclusion: the hemihydrate is far less abundant then Rydon and co-workers asserted. They estimated, for neutral solutions at 230 C, free dialdehyde (I) 4%; IV 16%; V 9%; IIa 35% and IIb 36%. Structures IIa and IIb are geometric isomers of II as shown in Figure 3d.

They came to these conclusions using three main arguments. First, both isomers should be present in about equimolar amounts based on conformational energy considerations. The relative chemical shifts of carbons 1-3 were consistent with effects observed in cyclic systems. Finally the relative intensities of the principal patterns do not change with varying concentrations of water. It should be expected that in higher concentrations of water the formation of the dihydrate would be favored. The concentration of the geometric isomers (cis and trans) would not be affected by water concentration.

Whipple and Rutta's temperature study confirmed the trend observed by Korn et al.; at higher temperatures the contribution of free diadlehyde increases.

The three studies above (52-54) used proton and ^{13}C NMR to arrive at generally the same conclusion that glutaraldehyde solutions are a mixture of dialdehyde, hemihydrate, dihydrates and cyclic oligomers. They differ mainly in how the equilibrium concentration of those forms are distributed (Table I). The studies that follow point to some aspect of the nature of the aqueous reagent without assigning new equilibrium concentration levels for each form.

Holloway and Dean (55) were interested in the pH dependence of antibacterial activity of glutaraldehyde solutions. Their carbon-13 NMR studies of the reagent were performed from pH 1 to 8. Within the pH range of 3-5 sharp resonances were observed. Above and below pH 3-5 the line broadening which occurred was interpreted as indicating fast interconversion between the various forms outside the pH 3-5 range. Equilibrium concentrations of the various forms of glutaraldehyde (dialdehyde, hemihydrate, dihydrate, and cyclic hemiacetal) did not change with pH according to this study (69). This study did not mention α-β-unsaturated forms which had been reported to occur at around pH 8.

A capillary gas chromatography - mass spectroscopy study was conducted to identify the components of aqueous glutaraldehyde solutions. The study found free dialdehyde,

TABLE I

COMPOSITION OF AQUEOUS GLUTARALDEHYDE

STRUCTURE	I	II		III	IV	V
		a	b			
RYDON et al.	-	32%			41%	27%
KORN et al.	15%	(85% II + III)				
WHIPPLE & RUTA	4%	35%a	36%b		16%	9%

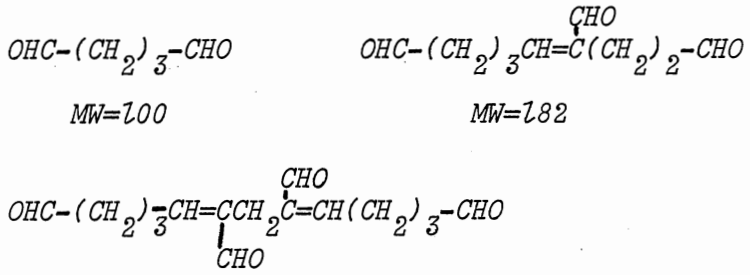
several products formed by aldol condensation and products formed by hydration-polymerization (56). The study did not propose any equilibrium concentration levels but simply reported the results of the mass spectra interpretation. Figure 4 lists the structures proposed by Hashimoto et al.

Kirkeby, Jakobsen, and Moe (57) recently compared "pure" and "impure" glutaraldehyde preparations by HPLC, UV, proton and ^{13}C NMR spectroscopy. They proposed that the two glutaraldehyde solutions give different products when reacted with protein animes. Pure glutaraldehyde gave one UV absorbance peak at 280 nm. Commercial glutaraldehyde shows an additional peak at 235 nm which has been correlated to the presence of α-β-unsaturated species. The HPLC of the "impure glutaraldehyde had two peaks not present in the "pure" sample. They concluded that less than 5% of the "impure" glutaraldehyde was composed of the unsaturated species. The ^{13}C NMR they obtained were similar to those reported by Whipple and Rutta (54) and Holloway and Dean (55).

Reaction of Glutaraldehyde with Protein in Homogeneous Solutions

The reaction of glutaraldehyde with proteins has been the subject of study for a number of years. The following are general observations which characterize this reaction and which any proposed reaction scheme should explain. The reaction proceeds rapidly at room temperature. The reaction

Aldol Condensation



Hydration-polymerization

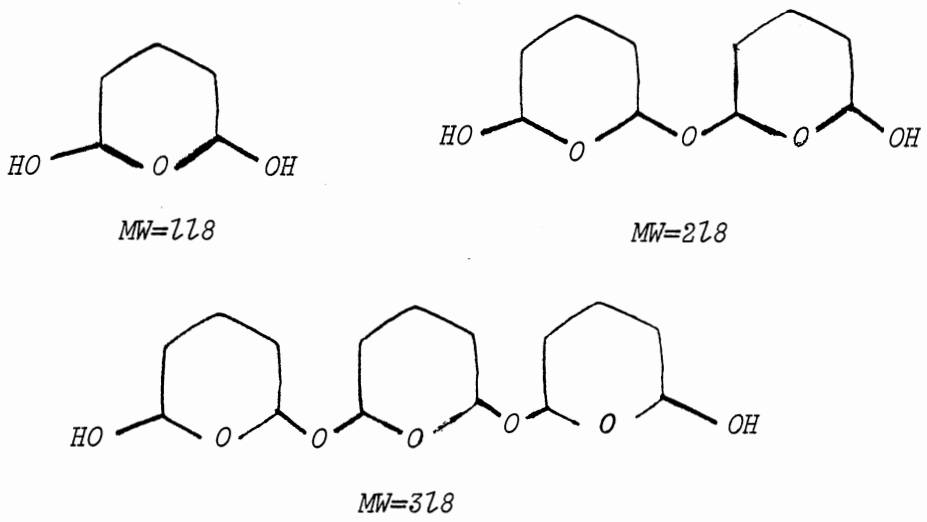
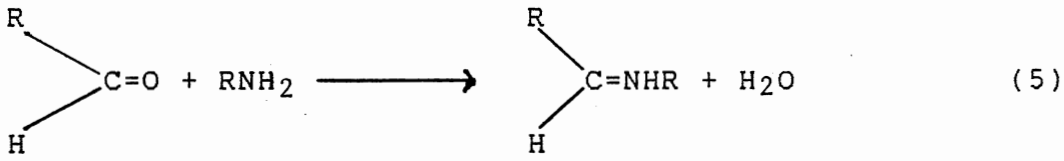


Figure 4. Structures of Compounds Proposed by GC-MS Study

proceeds to completion in aqueous buffers. As the product forms a yellow coloration develops, this coloration becomes pink, then darkens with time. The reaction is essentially irreversible, the product is stable under a wide range of conditions including aqueous media, acid hydrolysis, changes in pH, temperature, and ionic strength. Importantly, some enzyme activity is unaltered by the reaction. The usual reaction of aldehydes and primary amines produces imines (equation 5).



(5)

An analogous imine formation was thought to occur between proteins and glutaraldehyde until the work of Quicho and Richards (20). Their study of single enzyme crystals formed by crosslinking with glutaraldehyde help establish that the predominant amino acid reacting with glutaraldehyde is lysine. Other amino acids are known to react reversibly and regenerate the amino acid in hydrolysis conditions (58). Quicho and Richards treated the crosslinked crystals with high concentrations of a semicarbazide and noted the lack of any reaction. The work of Cordes and Jencks has shown that Schiff-type bases are rapidly attacked by simicarbazide (59) and water (60). Thus the protein-glutaraldehyde product is something other than a simple imine, however reports in the literature still refer to the glutaraldehyde-enzyme link as analogous to Schiff base formation.

Stoichiometry is one point, as one examines the literature, where there is no general agreement among workers in the field. Measuring the amount of glutaraldehyde lost from solution and the amount of lysine plus hydroxylysine consumed, four moles of glutaraldehyde per mole of lysine has been reported (53). Bowes and Cater found a 3 to 1 ratio by reacting [14C] glutaraldehyde with collagen (61). Ford reported 2 moles of [3H] glutaraldehyde reacted with every mole of bovine-albumin lysine consumed (62). These ratios are probably the correct values returned by the methods and conditions used. If a consensus existed it would be a simple matter to screen proposed mechanisms against the known stoichiometry.

Studies in this area have been grouped into two broad headings by the author. All the investigators who have published to date proposed either α-β-unsaturated imine products or cyclic pyridin type products. There is no pattern of evolution in thought, time (and more studies) have not proved or disproved either theory. It is noted that, with one peripheral exception, all studies reported here were performed in homogeneous media. Even researchers specifically studying the nature of heterogeneous enzyme immobilization used homogeneous solutions. A second note is that the use of model compounds is widespread. The complexity of the problem dictates the use of simplified systems in order to extract some information.

α-β-unsaturated Imines Products or Precursors

Homogeneous Reaction of Protein Amines via Michael-Type Addition

Richard and Knowles (48) pioneered the work in elucidating the reaction mechanism between glutaraldehyde and proteins in solution. In 1968 Richards and Knowles enumerated additional reasons why simple Schiff base formation does not occur between proteins and glutaraldehyde in solution. Imine formation is reversible and the equilibrium in aqueous solutions is unfavorable for imine stability (60). Imines generally do not survive acid hydrolysis, simicarbazide treatment and wide ranges of temperature and pH. The reaction of proteins with glutaraldehyde is not reversible and the product survives acid hydrolysis, aqueous solutions, and wide ranges in pH and temperature. The pKa of imines was expected to be no more than 5, the pKa of glutaraldehyde modified lysine was found to be about 8.

Using proton NMR and tirations, they postulated that aqueous glutaraldehyde solutions exist mainly as α-β-unsaturated aldehydes formed by numerous aldol condensations. Protein amines would eventually add across the conjugated carbon-carbon double bond in a Michael-type addition. These type products, they reasoned, would be stable to acid hydrolysis, in aqueous solutions, and possess the appropiate apparent pKa's. These characteristics are

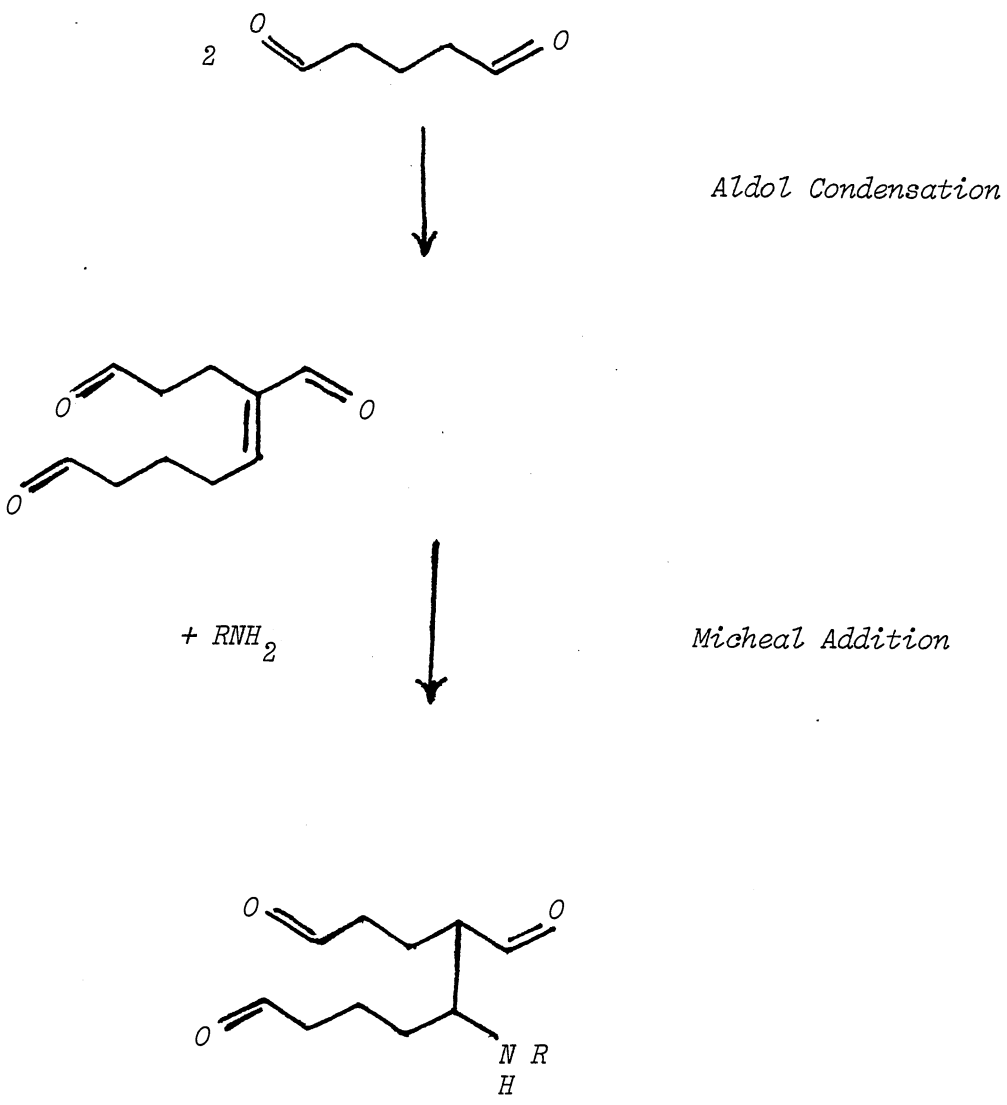


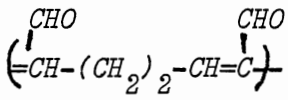
Figure 5. Reaction Scheme Propsoed by Richards and Knowles

not present in a simple imine linkage. Based on the stability of the proposed Michael addition products, they developed the reaction scheme seen in Figure 5. Titrations were the only experimental work performed on the glutaraldehyde-protein product. The purity and pH of the glutaraldehyde used in this work was not reported and has been the subject of concern by other workers.

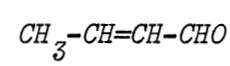
Homogeneous Reaction of Protein Amines via Conjugated Imines

According to Monsan et al. (49-51) glutaraldehyde solutions contain no stable form of the dialdehyde but are complex mixtures of compounds of various molecular weights which easily revert to glutaraldehyde. Monsan noted that commercial preparations of glutaraldehyde are acidic (pH 3.1) but the reactivity of the reagent (with proteins) increased with increasing pH. They studied the structure of glutaraldehyde in highly alkaline solutions, since the reagent is generally used in the neutral to alkaline pH range. Polymeric forms of the dialdehyde are more important at higher pH's and glutaraldehyde reactivity increases with pH, thus they hypothesized that the exceptional stability of the glutaraldehyde link was caused by reaction with the polymeric form.

Monsan et al. analysis of alkaline (pH 8-10) glutaradlehyde samples led him to propose a polyglutaraldehyde structure similar to the one proposed by



Polyglutaraldehyde



Crotonaldehyde

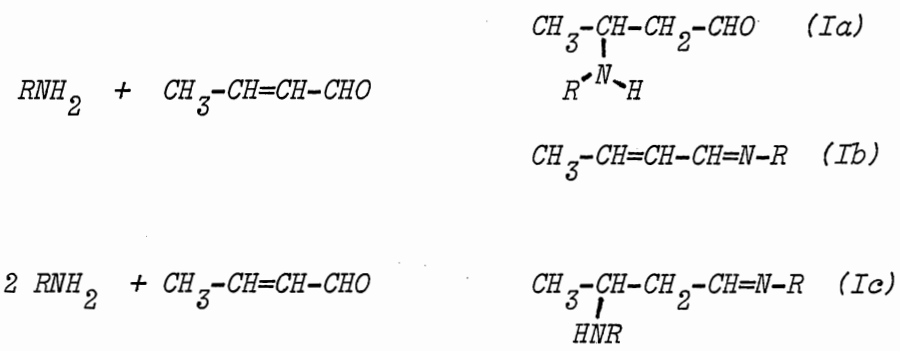


Figure 6. Monsan's Model Compound and Reactions

Richards and Knowles. Crotonaldehyde was proposed as a molecular model which contained the essential repeat units of polyglutaraldehyde (Figure 6). By studying the NMR spectra of the products of a reaction similar to the glutaraldehyde-protein reaction Monsan hoped to elucidate the mechanism of the bonding. Stoichiometric amounts of the model compounds, isopropyl amine, and crotonaldehyde were reacted with the two possible pathways (Ia and Ib) shown in Figure 6.

Pathway Ia corresponds to the Michael type addition proposed by Richards and Knowles (48). The results of Monsan's NMR study concludes that the product formed is Ib and not Ia. They asserted that primary amines react mainly with the aldehyde groups and not with the double bond. They did however observe di-addition (reaction Ic, Figure 6) in excess amine and conceded that this pathway may also play a small role when the reaction involves macromolecules such as proteins. It should be noted, however, that immobilization usually occurs in excess amine.

The stability of the protein-glutaraldehyde products to acid hydrolysis could be explained by the resonance of the conjugated imine bond. The isopropylamine-crotonaldehyde product prepared by this research group posses some of the characteristics usually found in glutaraldehyde treated proteins. Monsan's investigation of immobilized enzyme stoichiometry and optimization generally confirmed his theory, but did not rule out others (49).

In 1977, Richards reviewed gluatraldehyde as a crosslinking agent and accepted most of Monsan's conclusions. Peters and Richards (63) echoed the view that the glutaraldehyde-protein reaction product was a conjugated imine. The facts, according to Richards and Peters, which were explained by the conjugated imine product theory follow: 1.) A yellow color is produced as glutaraldehyde reacts with a protein or simple amine. 2.) Amino acid analysis of crosslinked proteins showed only lysine was consumed irreversibly. 3.) The pKa of the product function is about 1 pH unit more acidic than the original primary amine.

In his review of other work in the field, Richards rationalized that in acidic pH ranges (about pH 3) the hydrates proposed by Rydon's group (52) predominate. At neutral pH and higher α-β-unsaturated polymers predominate. The originally proposed Michael type addition products can be understood in light of Figure 7. Under conditions where di-addition was achieved, amines adding across the double bond were stable under acid hydrolysis and those attached to the aldehyde were not. When mono-addition occurs, the conjugated imine is stable to acid hydrolysis. By 1977 Richards had relegated the Michael addition product to a minor role and only in cases where there is high local amine concentration.

Cheung and Nimni investigated the mechanism of crosslinking proteins with gluraraldehyde by studying the

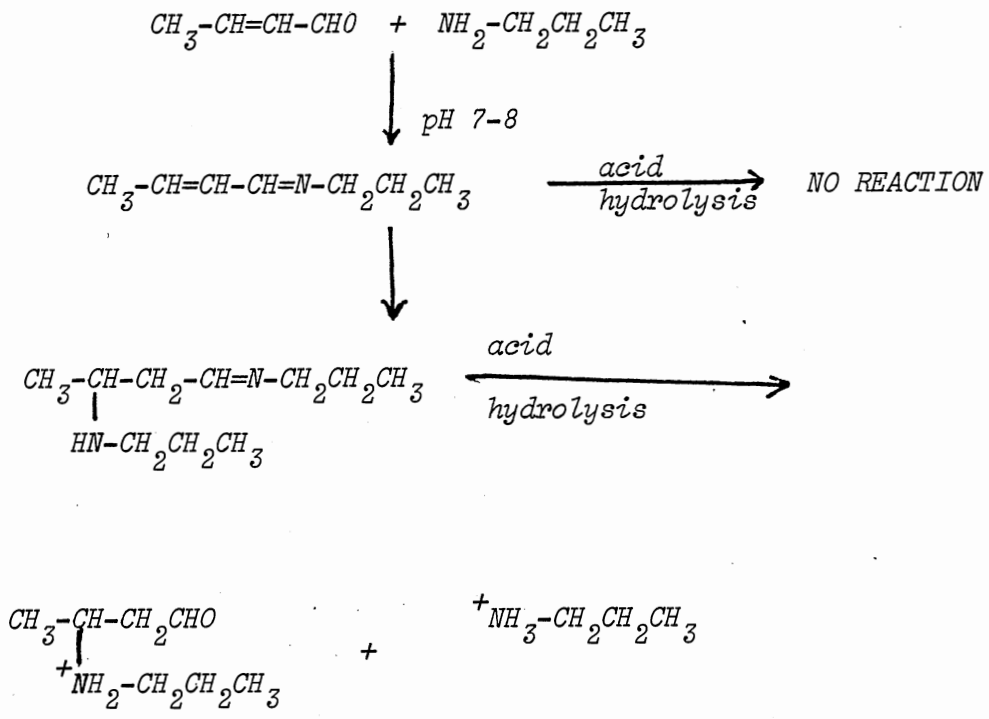


Figure 7. Stability of Michael Addition Products to Acid Hydrolysis

reaction with model compounds (64) and various forms of collagen (65). Cheng and Nimni work focused on reasons why they dissagreed with publications (66) proposing cyclic or pyridinium products.

Cheung and Nimni reacted 6-amino hexanoic acid with glutaraldehyde and noticed, as have others, the development of an absorption peak at 265 nm. They report that the 265 nm chromophore decreased with time (10 h) as a new peak (325 nm) grows into the spectrum. Cheung and Nimni proposed two consecutive products. The first is the 263 nm absorbing material which they believe is a precursor to the final stable product, the 325 nm chromophore. Experiments conducted to elucidate the product structure found that α-β-unsaturated aldehydes or polymeric glutaraldehyde reacting with amines produced 325 nm absorbing material. Saturated aldehyde or monomeric glutaraldehyde when reacted with an amine did not produce any 325 nm absorbing material.

An argument used to advance the pyridinium-product theory was that product material isolated from the reaction of glutaraldehyde with amines absorbed at 265 nm (66). Cheung and Nimni argued that isolation procedures which include acid or peracid hydrolysis could produce 265 nm chromophores. As proof, they present data which suggest that acid hydrolysis of glutaraldehyde-amine reaction products may produce 265 nm absorbing material as a by-product. Also, borohydride reduction of pyridinium compounds yields a strongly absorbing material with a peak

above 300 nm. When the glutaraldehyde-amine product, they studied, was subjected to the same treatment it did not produce this peak.

Cheung and Nimni supported the theory that glutaraldehyde was partially polymerized as a result of catalytic action of primary amines. These α-β-unsaturated aldehydes on the polymer then undergo a reaction with amines which yields conjugated imines as the final product. As evidence, they present the result of molecular weight studies performed on the reaction product of [3H] glycine and glutaraldehyde. A wide range of product molecular weights was found (200-4000 units). The upper molecular weight of 4,000 daltons is much larger than the crosslinking entity envisioned by proponents of cylic products (>500). In another experiment, when a reaction was allowed to continue to apparent completion, adding more amine did not alter the maximum absorbence at 265 nm. Conversely, adding more glutaraldehyde to a "completed" reaction caused the presence of more 265 nm absorbing material in the reaction product.

Moe et al. (57) compared practical glutaraldehyde and electron microscopy grade glutaraldehyde. The products obtained from reacting each of the two solutions with simple amines were different, based on UV absorption spectra. The product of glutaraldehyde reacting with lysine was spectrally different from the product of glutaraldehyde reacting with other amino acids. These authors contend that

both the α-β-unsaturated glutaraldehyde polymers and the cyclic pyridin-like structures could cause the absorption seen at 265-285 nm. The authors then developed the reaction scheme seen in figure 8 based on the following: α-β-unsaturated imines (220 nm) can be formed from aldimines. Substitution will cause the peak maximum to shift around 230 nm. Aldol type condensation can take place to provide this substitution. The reaction scheme pictured in Figure 8 is for "pure" glutaraldehyde reacting with lysine.

Cyclic Products of Precursors

Homogeneous Reaction with Protein Amines to Form Pyrdinium Salts

Rydon's studies (52) of the composition of aqueous glutaraldehyde found α-β-unsaturated material to be a minor contaminant. Results from his experiments suggested that "pure" and "impure" glutaraldehyde posses almost identical reactivity, thus he reasoned, the crosslinking ability was not dependent on the intial presence of α-β-unsaturated compounds. Rydon et al. (66) investigated the reaction of glutaraldehyde with model compounds and a generic structure was proposed from the purified product of glutaraldehyde and 6-amino-hexanoic acid (Figure 9a).

Amino hexanoic acid was reacted with glutaraldehyde and the reaction mixture was evaporated, solidified, then subjected to repeated column chromatographic separation. The authors note that the crude product was a mixture of at

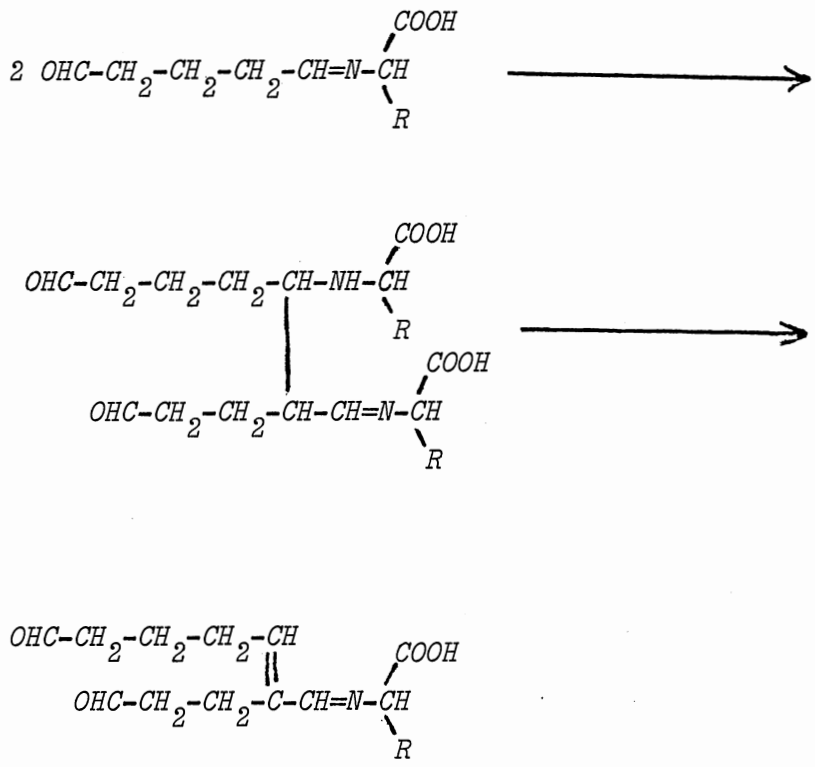
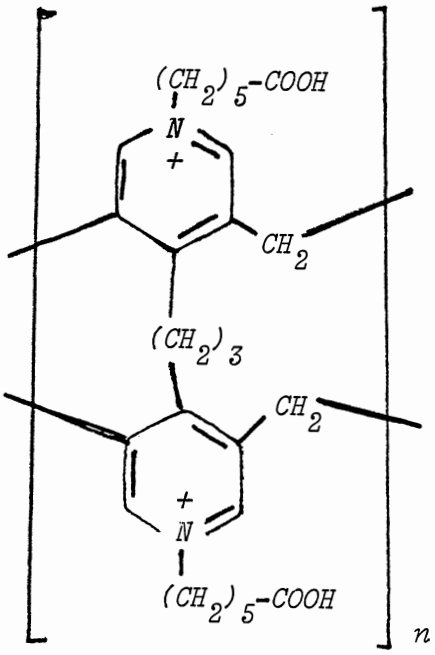
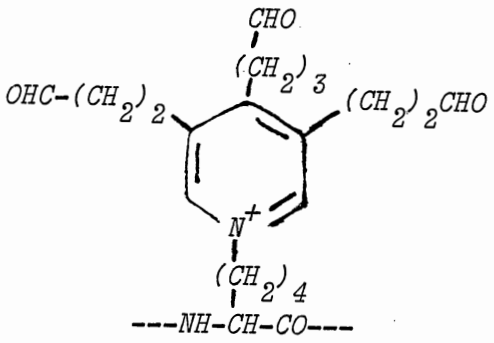


Figure 8. Reaction Scheme Proposed by Moe et al.

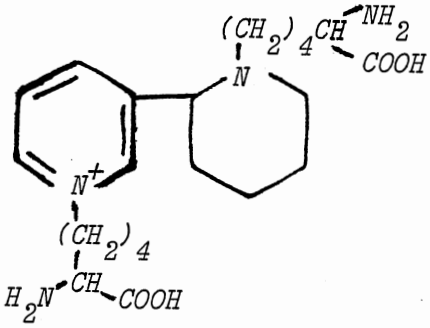


A



VII

B



C

Figure 9. Compounds Isolated by Rydon's Group

least 12 components. The most stable peak or compound (overall yield about 10%) was further analyzed by potentiometric titration, UV, IR, and proton NMR.

Figure 9b illustrates the product isolated from the model compound reacting with glutaraldehyde. The actual protein product structure was assumed to be a mixture of closely related compounds which differ in the value of n (Figure 9a). Rydon states that aromaticity is indicated by characteristics of the UV and NMR spectra. Titration and ion exchange chromatography with amino acid analysis support the assertion that the amino group disappears and is replaced in the product by a strong basic group. The isolated product possess chemical characteristics of pyridinium salts including sensitivity to hydroxide, hyride and fluorescence when treated with Dragendorff's reagent. The isolated product absorbed UV with a peak maximum at 265 nm. The progressive darkening of glutaraldehyde treated proteins was explained by noting that the existence of free formyl groups in the structure give the potential to undergo further crosslinks.

The progress of the reaction was envisioned as three molecules of aldehyde and one of lysine reacting to form VII. Further reaction with two aldehyde molecules and another lysine could make the crosslinks visible Figure 9a. The final protein - glutaraldehyde product is a complex matrix consisting of these and many other possibilities. The authors acknowledged that it would be unwise to assume

that this type of crosslinking was the only one present in glutaraldehyde treated proteins. In a later paper (67), Rydon postulates that the precursor to the pyridinium salts may be anabilysine as shown in figure 9c.

Anabilysine was the first crosslinking entity to be isolated from glutaraldehyde-treated proteins. Other isolated products were from studies using model compounds for either glutaraldehyde, protein, or both. Anabilysine was isolated from the acid hydrolysis of glutaraldehyde-treated ovalbumin. It's structure was confirmed when 13C NMR, chromatography, and elemental analysis were indistinquishable from the synthesized compound. The authors envisioned crosslinking by various closely similar analogs of anabilysine, differing in oxidation state and substituent pattern. Parenthetically, this paper also reports the only isolation of the product of lysine immobilization by the glutaraldehyde method. Anabilysine was recovered from aminated glass that had been reacted with acetyl-lysine.

Homogeneous Reaction with Protein Amines to Form Polymerized 1,4 Dihydropyridine

Ford and Pesce (62,68) are the investigators who more recently have proposed a mechanism. They reviewed the work of others in this area and dismissed the previous conclusions for the following reasons (among others) 1) stoichiometry (which they maintain is 2:1), 2) the use of

model compounds which cannot duplicate the special reactivity of glutaraldehyde with proteins.

The mechanisms presented thus far, they argue, would predict that a monoaldehyde would react in a similiar fashion as glutaraldehyde. They allowed equal aldehyde concentrations of glutaraldehyde and the monoadlehyde butanal to react with bovine serum albumin. The glutaraldehyde reacted with up to 8 times as many lysine residues as butanal. This, according to Ford and Pesce, shows that the dialdehyde possess special reactivity not adequately explained by previous authors (Richards and Knowles; Monsan; and Rydon). The use of model compounds was also challenged. The reaction kinetics and the stoichiometry of model compounds reacting with glutaraldehyde was found to be at variance to reactions involving actual proteins. The authors conclude that the reaction products would also differ and thus the use of model compounds invalidates the results obtained.

[3H] glutaraldehyde was reacted with proteins amines to establish the stoichiometric ration of 2:1. The stoichiometry of polyglutaraldehyde reacting with lysine is near 1:1 and not 2:1 (62). Mechanisms which require prepolymerization (Richards and Knowles, and Monsan) fail since aqueous solutions are slow to polymerize. Amines may be thought to catalyze the polymerization , if so increasing the concentration of glutaraldehyde should cause more

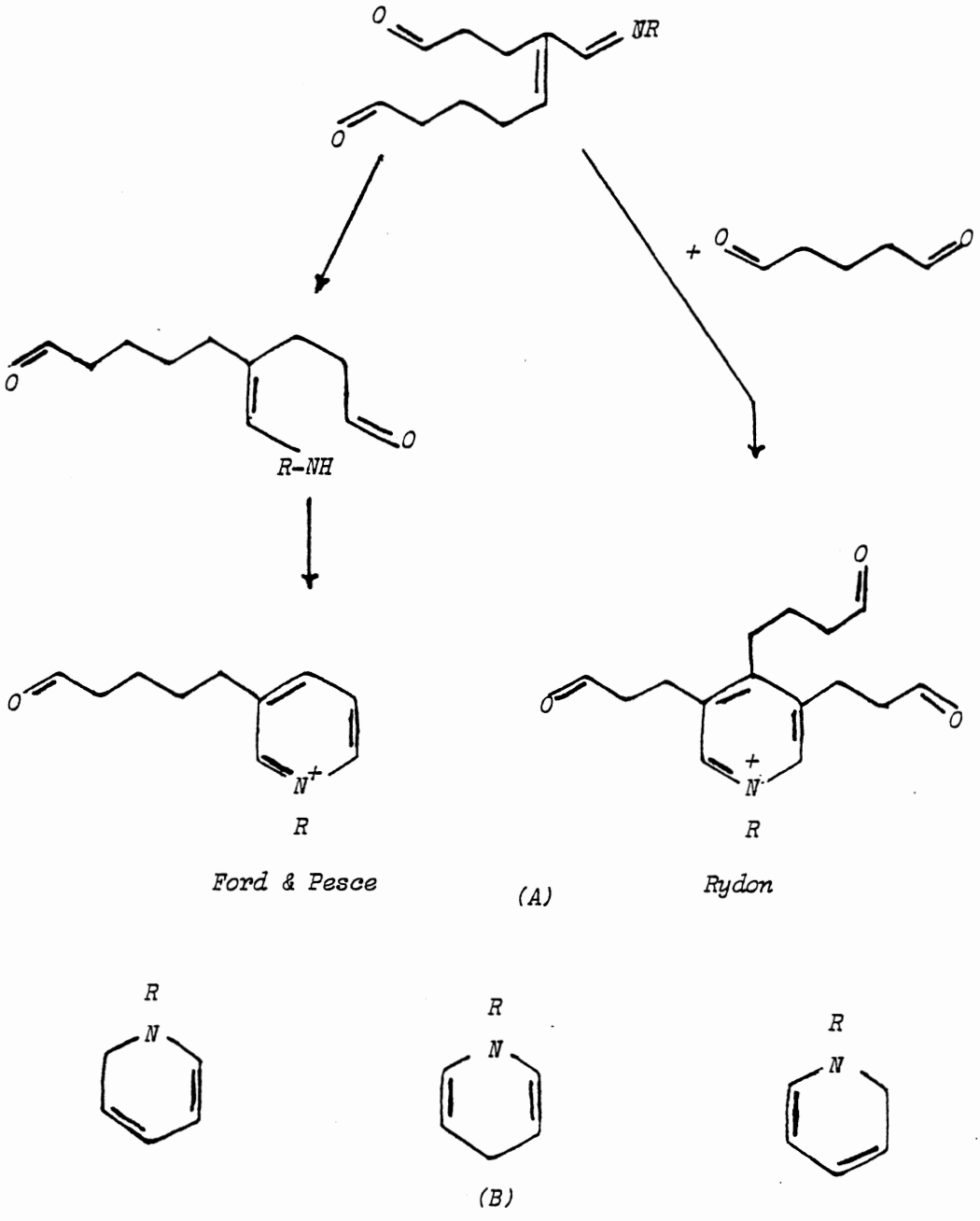


Figure 10. Proposed Ring Forming Reaction Schemes

polymerization and alter the stoichiometry. This, however, was not found to be the case by Ford and Pesce.

Ford and Pesce observed in their studies that tritium used to label the carbonyl carbon of glutaraldehyde, was released to aqueous solutions from the dialdehyde-protein product. Of the mechanisms previously proposed, only Rydon's model could account for this fact, however Rydon's model requires a 3:1 ratio of reactants and thus does not fulfill the stoichiometry requirements.

Ford and Pesce also studied the coloration of solutions as glutaraldehyde and primary amines react. None of the existing models, according to the authors, could adequately explain the (430 nm) yellow to deep red color change that proteins treated with glutaraldehyde undergo. They presented a variation of Rydon's mechanism which they felt beat explained most of the observed reaction characteristics (Figure 10a).

The model presented in Figure 10a has a stoichiometric ratio of 2 to 1, the bond is stable in aqueous solutions, and to acid hydrolysis. The ability of glutaraldehyde to form a stable ring system in one step should give it a special reactivity which monoaldehydes cannot duplicate. Dihydropyridines react with amines to form first yellow and then red derivatives. Tritium release can occur during equilibrium between the resonance forms (Figure 10b).

The model that Ford and Pesce present is by their own admission a precursor. The final glutaraldehyde-protein

product is thought to be much more complex. The pathway to the final product and the structure of the final product remains unclear. The Ford and Pesce model does not lead to a simple explanation of enzyme immobilization. Proteins treated with glutaraldehyde in the absence of excess reagent are able to bind to other proteins. Aminated surfaces which have been reacted with glutaraldehyde, then rinsed free of reagent, can bind proteins. If the Ford/Pesce precursor is correct it must undergo extensive crosslinking or polymerization to form the final network. The precursor adheres (by design) to their stoichiometric restraints (2:1), but the final crosslinked matrix may not.

Mechanism of the Enzyme Immobilization Reaction

Optimization studies

It may seem reasonable that an examination of published optimized immobilization conditions will reveal some information about the details of the immobilization chemistry. The conditions reported for optimum glutaraldehyde activation of solid supports do not show any pattern. A literature review shows the optimum conditions for glutaraldehyde activation may vary for each enzyme. The best activation conditions also change for each type of support and the intended application of the immobilized preparation.

Onyezilli (69) studied glutaraldehyde activation of O-alkylated nylon tubes. He concluded activation with

glutaraldehyde favored alkaline pH, but the enzyme attachment step, the stability of the enzyme should be the major consideration when selecting a coupling pH. The progressive color changes (clear to yellow to red) often noted after supports were glutaraldehyde-activated were not accompanied by reduction of enzyme activity. Onyezilli postulated that the color changes were due to the presence of interconvertible species of aldehyde (Figure 3).

Mokeev, Mazurova and Strilets (70) studied the optimum conditions for modification of aminosilica with glutaraldehyde and the storage stability of the product obtained. An one hour reaction in a 20 °C aqueous solution modified more than 90% of the available surface amino groups. The report states that the amount of carbonyl groups bound to the surface was independent of glutaraldehyde concentration from about 2% to 20% (v/v). The derivatized silica was stable for 3 months with no detectable change in carbonyl content (70).

Marty et al. (71,72) applied simplex methodology to the study glutaraldehyde activation of a support for enzyme immobilization. The influence of reagent concentration, pH, and reaction time were studied for the immobilization on Ribonuclease A on amine-Spherosil. Marty found that between pH 4.0 and 9.0, the amount of immobilized ribonuclease A (as measured by carbon content), increases with increasing glutaraldehyde concentration and reaction time. Enzymatic activity behaved differently.

At pH 7.0, increasing glutaraldehyde concentration and decreasing the reaction time enhanced the observed enzymatic activity. Enzyme activity also increases as glutaraldehyde concentration decreases and reaction time is increased. The author contends that long contact times and high glutaraldehyde concentration represent conditions which produce a surface that binds the enzyme with "too many" bonds, altering the 3-dimensional structure and denaturing the enzyme. When both contact time and glutaraldehyde concentration are low, the reaction does not produce enough bonding sites on the surface to immobilize high enzyme activity.

The optimum conditions reported for this immobilized enzyme system (ribonuclease A on amine-Spherosil)are one hour reaction, with 8% glutaraldehyde solution, and at a pH of 6.4. Marty concludes maximum "enzyme" (protein measured by carbon content difference) immobilization occurs in basic pH, maximum specific activity (U/mg immobilized protein) occurs in acidic pH and maximum enzyme activity (U/mg support) at pH 7.0 (71,72).

Monsan (49) studied glutaraldehyde activation of amine Spherosil for the immobilization of trypsin. The amount of immobilized glutaraldehyde and enzyme was determined by carbon content measured before and after each reaction. He reports the optimum conditions for glutaraldehyde activation of amine-Spherosil as a one hour reaction at 25 °C with 2% glutaraldehyde solution at pH 8.6. Monsan also observed an

increase in bound glutaraldehyde with increasing pH. At very high pH (>10), according to Monsan, polymerization is so extensive that the pores of the support are clogged and some glutaraldehyde binding sites are unavailable for subsequent reaction with the enzyme (49).

Immobilization Mechanism

The studies by Monsan and coworkers (49-51) are the only ones which specifically address the mechanism of enzyme immobilization by the crosslinking agent glutaraldehyde. The other studies were mainly concerned with understanding the homogeneous reaction of proteins with glutaraldehyde. Since this report deals specifically with the immobilization mechanism of glutaraldehyde, the adaptation of the previously presented (48,50,51,66-68) crosslinking mechanisms to immobilization will be discussed here. The ability of activated supports to bind enzyme in the absence of soluble glutaraldehyde can be used to screen these models.

Rydon's proposed mechanism (66.67) involves the reaction of three glutaraldehyde molecules and one amine to form a conjugated pyridinium salt. Solid supports which are thoroughly rinsed after a reaction with glutaraldehyde are still activated for enzyme attachment. This observation is not easily explained by Rydon's model. The reaction sequence proposed may be employed to explain the reaction of glutaraldehyde with aminated surfaces. However for the

subsequent reaction with a soluble enzyme to occur. as Rydon envisioned it at least two molecules of glutaraldehyde must be present (in solution) to link a protein-lysine amino group to the activated solid.

Rydon points to free formyl groups in his structure as possible sites for further crosslinking. These groups must react to form products quite different from the ring structures Rydon presented. The proposed crosslinking between the separate rings of the pyridinium salts to form polymeric-like structures does not explain the ability to covalently bond to enzymes which have not been exposed to glutaraldehyde. Rydon's group was able to isolate the proposed product (anabilysine) from the hydrolysis of glass-immobilized lysine. The hydrolysis and isolation procedures as well as the product structure have been recently challenged (64,65).

The Ford-Pesce model (62,68) has similar arguments against it. The first step in their model is an aldol condensation of two glutaraldehyde molecules to form an α-β-unsaturated aldehyde. The second step is the cyclization of glutaraldehyde with an amine. The potential for glutaraldehyde to form this ring in one step gives it a special reactivity and is responsible for the success of this reagent in bonding with proteins. Their model explains several characteristics of the glutaraldehyde reaction but the aldehyde activation of an aminated support for enzyme

immobilization in the absence of soluble glutaraldehyde cannot easily visualized using the Ford-Pesce precursor.

The aldol condensation polymers proposed by Monsan et al. (49-51), Richard and Knowles (48), and Cheung and Nimni (64,65) can readily adapted to explain enzyme immobilization. The basic difference between the two groups is in where the enzyme attaches to the polymeric glutaraldehyde. Richards and Knowles, prior to adapting Monsan's view, proposed a Michael type addition at the carbon-carbon double bond. Monsan group argued that the addition of enzyme is to the carbonyl carbon to form a conjugated imine. The adaptation of these models can be visualized in general as the polymeric sheet of glutaraldehyde (proposed by Cheung and Nimni) forming at the aminated surfaces of supports. A bond between a surface amine and a carbon-oxygen or a carbon-carbon double bond at many points holds the sheet in place, while similar functional groups are available for attachment by enzyme molecules in solution (Figure 11).

A common practice of the studies involving the gluraraldehyde-protein reaction is the use of model compounds for protein amine to simplify the problem. Monsan utilized model compounds for both glutaraldehyde and protein amines. Monsan's studies to understand the heterogeneous reaction were done with homogeneous solutions of model compounds. Some workers in this field have suggested that extrapolations drawn from model compounds are invalid (62).

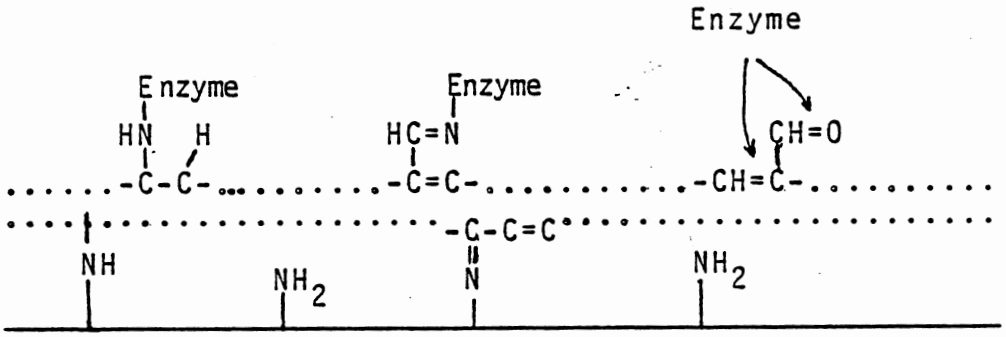


Figure 11. Enzyme Immobilization via Michael Addition or imination

Monsan did work with actual immobilized enzyme systems in his study of the optimization of solid support activation by glutaraldehyde. Monsan concludes from this study that the behavior observed is predicted by his model. The observations made by Monsan do not disproved his model, but cannot be used to rule out others presented.

The ease of visualization is not proof that these models are operating in glutaraldehyde immobilization of enzymes to solid supports. Indeed the Michael addition product is not currently thought to be an important pathway for crosslinking, even by one of the original proposers. At this stage of our understanding we cannot rule out the possibility of more than one type of bond between glutaraldehyde and amines. Indeed one type of reaction may redominate as glutaraldehyde reacts with surface bound amino groups, and another pathway operate as that activated surface contacts the enzyme solution.

Richards and Knowles concluded that the variety of possible products may explain the remarkable effectiveness of glutaraldehyde as a crosslinking reagent. Monsan, whose work followed Richards and Knowles, stated that when proteins instead of model compounds were reacted other types of bonding could occur. Rydon et al. formulated structures based on approximately 10% of the reaction products. Ford and Pesce cannot explain the reaction course after their envisioned precursors are formed. Most workers in this

field have acknowledged that the widely used glutaraldehyde chemistry is complex and poorly understood.

CHAPTER III

BACKGROUND INFORMATION AND LITERATURE REVIEW

Immobilized Enzyme Reactor Configurations

Immobilized enzymes have gained increasing acceptance as analytical reagents and their use in flow systems is rapidly becoming routine. A fair number of successful immobilization techniques have been developed (73) and a large number of matrics (polymers, gels, glass, etc.) have been employed to bind the enzyme (73,74). As flow systems need materials that are inert, incompressible, resistant to adsorption and chemical attack, recent reports show a greater use of glass and specialized glass products (controlled-pore glass, CPG) as the immobilization matrix.

A schematic of a typical immobilized-enzyme flow system is shown in Figure 12. Carrier and reagent solutions as well as the sample are propelled through the system by means of a pump (typically a peristaltic pump). Substrate reacting with the enzyme immobilized on the reactor surfaces is converted to product(s). A product of the enzyme-catalyzed reaction may be detected directly, derivatized by a merging reagent, or coupled to another (enzyme) reaction. Although colorimetry dominates the detection systems being used, flow methods need not be limited to this mode of

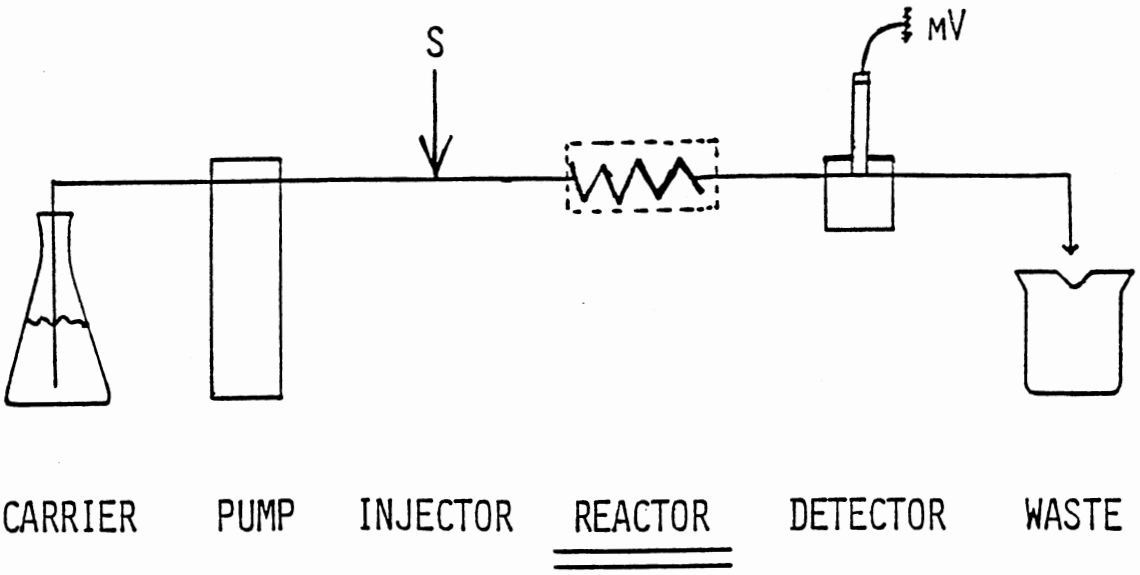


Figure 12. Typical FIA Experimental Setup Utilizing Immobilized Enzyme Reactors

detection (3). Detection systems that minimize the volume in which the analyte is contained should be preferred to those that may cause sample dilution by merging reagent streams.

Color development reactions as well as other derivatization reactions may be restricted to post enzyme-reactor merging streams because of absorption of the dye and other hydrophobic materials on the immobilization layer. This absorption contributes to enzyme inactivation and its partitioning aspects lead to signal dispersion. Direct detection of the product of the enzyme reaction electrochemically or by other methods bypasses these problems. The technology for these systems (merging reagent streams) has developed to a point where dispersion is minimized.

Figure 13 shows the visual differences between packed reactors, open tubular reactors (OTR), and single-bead-string reactors (SBSR). Packed and open tubular reactors have been used since the infancy of immobilized enzymes in flow systems (74,7). SBSRs constitute a relatively new configuration (75) with comparatively few adaptations to immobilized enzyme systems.

Packed Reactors

Packed reactors are constructed by restricting small-diameter enzyme-immobilized particles to a short length of a narrow tube. The reactor is typically 5 to 10 cm long with

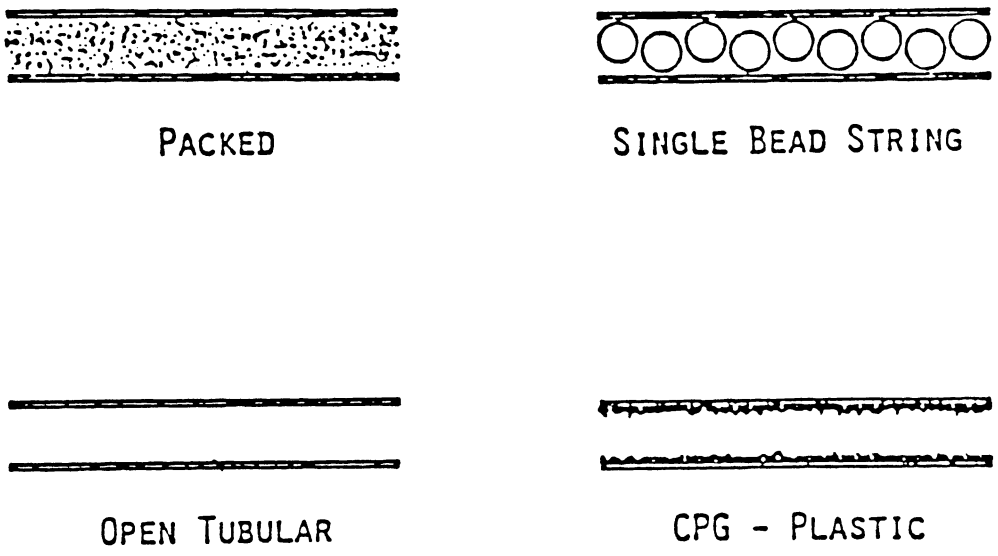


Figure 13. Schematic Representations of Major Reactor Configurations

an inner diameter of about 0.2 to 0.5 cm and is packed with 100-μm particles. The particles disturb the established parabolic profiles of laminar flow within the reactor, the carrier and analyte being forced into numerous tortuous routes throughout the packed bed. These routes act as numerous mixing channels increasing the mass transport of the analyte to the particle surfaces where the enzyme reaction can occur.

Packed reactors have the largest surface area per unit length of the three configurations of reactors. This allows large enzyme loading and nearly complete conversion of substrate to product.

Packed reactors suffer from a high pressure drop. Particle size and reactor dimensions must be judiciously selected to avoid producing detrimental pressures in the flow-system pumps (typically peristaltic). The wide use of packed reactors has afforded the experience necessary to minimize this difficulty. Colorimetric detection can be successful at the relatively high pressures involved. When electrochemical detection is considered, the case is somewhat different. Both the signal and the baseline in electrochemical detection can be extremely noisy because of two attributes of packed reactors: a) high back pressure and b) numerous channels. The limit of detection (in electrochemical detection) is adversely affected if these aspects are not addressed.

The elevated pressures create conditions that transmit pump pulsations throughout the system to the detector. This is true for most peristaltic pumps, and flow propagation by other less pulsed means (syringe pumps, gas pressure, and gravity flow) may be unfeasible because of the pressure constraints. The large number of mixing channels in the packed bed causes an increase in streaming potentials and thus the ionic strength (specific conductivity) of the carrier solution becomes important (76,77).

Single-Bead-String Reactors

Single-bead-string reactors (SBSRs) are constructed with solid beads having a diameter about 65% that of the tube. SBSRs are reported to increase mixing greatly by inducing secondary flows and radial mixing, while at the same time minimizing axial dispersion (75). These reactors have been mainly used in flow systems as mixing devices to facilitate homogeneous interactions, solvent extractions and chemical reactions (6). SBSR have recently been used as immobilized enzyme reactors in flow systems (7,78). Enzyme immobilized on the beads alone may form a good reactor (7) and enzyme immobilized on beads and tubing has been made the basis for determination of penicillin (78).

SBSRs also act as damping devices for pump pulses. Beads alter flow paths so as to induce mixing and enhance the transport of analyte to the reactor surfaces. SBSRs have less surface area per unit length than packed reactors,

consequently reactors one meter in length are common as compared to 10 cm for packed reactors. SBSRs, however, have good flow characteristics, allow a greater sample throughput, are not prone to clogging, and offer lower back pressure.

Since SBSRs are a relatively new immobilization configuration there are few formal studies on their kinetics, dispersion, and comparison with packed reactors (8). SBSRs have been shown to promote very efficient mixing (7) while the integrity of the sample plug is preserved and dispersion is minimized (75).

Open Tubular Reactors

Open tubular reactors (OTRs) are made by attaching an enzyme to the inner wall of a suitable tubing. OTRs with enzyme immobilized on plastic tubing were first reported by Hornby and Sundaram (79), nylon having been the plastic of choice (74). Recently the use of glass capillaries have been reported (80,81). Since OTRs are essentially "open tubes", sample throughput is highest and operating back pressures are extremely low. This low-pressure environment permits the use of alternative modes of pumping, many of which produce little or no pulsation, particularly gravity flow and flow driven by pressurized gas. Coiling of the reactors induces secondary flows, radial mixing, and reduces dispersion due to parabolic profiles and the length of travel.

Recently two new reactor geometries were described. The so called 3-D coil, is a variety of open tubular reactor (82). This "tight knotted" geometry compares favorably with that of coiled OTRs and SBSRs (83) but has not been used with immobilized enzymes. Dasgupta's group has reported enhanced performance in a variation of the open tubular configuration which involves entrapment of the enzymes in Folded microtubes (84).

Unaltered OTRs have a low surface area per unit length (8). This has been remedied in various ways. The surface area of plastic tubes coated with a monolayer of immobilized enzyme is usually increased by partial hydrolysis or pitting (73,74). The area of glass capillary surfaces can be enhanced by the process of "whisker" growth (80,81). This etching with ammonium bifluoride can increase the surface area by two to three orders of magnitude. Glass provides an inert matrix which is resistant to adsorption by organics and to long term erosion by fluid flow.

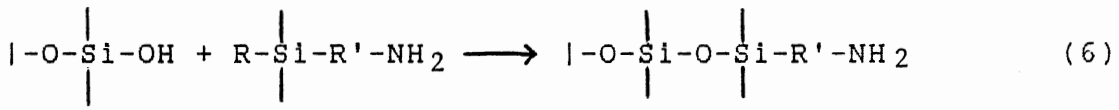
Open tubular reactors have the lowest back pressure, can achieve the highest sample throughput, and possess, when coiled, acceptable low dispersion. The low conversion efficiency per unit length of these reactors is perhaps its greatest drawback. An enhanced-surface-area open-tubular reactors which possessed all the advantageous characteristics of OTRs might be a nearly ideal reactor in some applications. While it reduces dispersion due to length of travel, substrate conversion would increase

because of higher enzyme loading. Part One of this work describes an innovation in immobilized enzyme OTRs which addresses the problem of limited surface area.

Determination of Reactive Amino Groups Immobilized on Silica Surfaces

Reaction of silica frameworks to generate reactive amino groups on the surface of solid supports is a widely used first step in the immobilization of enzymes on glass-surface reactors. Analytical accounting of the reactive groups generated is of interest in method optimization and in reactor design when these materials are used as analytical reagents.

Chemical modification of silica surfaces to attach reactive amino groups is generally accomplished by reaction with an amino-alkoxysilane and can be represented as shown below:



(6)

The modified surface can be directly used for analytical purposes [e.g. x-ray fluorescence determination of orthophosphate (85), ion-pair extraction of uranium in mining samples prior to x-ray fluorescence determination (86), preconcentration of metal ions also prior to x-ray fluorescence determination (87), and chromatographic separations (88)] or can be modified by attachment of

enzymes (7) or chelating agents (89) to be further used as analytical reagents. Performance of chemically immobilized reagents and chemical reactors depends on the effectiveness of attachment procedures; this, in turn, may be improved by gaining quantitative understanding of each reaction step in the immobilization sequence. In covalent attachment of enzymes via glutaraldehyde spacing (one of the simplest, most gentle, rapid and efficient methods for chemical immobilization of enzymes), procedures are available to determine reactive aldehyde groups (90) and protein (91) immobilized on silica materials.

Reaction 6 has been the subject of physical as well as chemical studies to characterize and/or quantify surfaces modification. Methods such as ^{13}C and ^{29}Si solid-state NMR, calorimetry, Fourier transform infrared spectrometry, and photoacoustic spectrometry have been used (92,93). These methods have been ruled out because they require physical destruction of the sample and thus cannot be used if (a) the physical form of the modified surface (by extension the overall reactor) need to be kept intact, and (b) "reactive" groups are to be measured in a manner that does not disqualify them for further immobilization purposes. When these "nondestructive" requirements are considered, one attractive alternative is the use of "on/off" chemical methods based on the quantitative attachment of a probe (preferably chromophoric, although other labeling may be used), subsequent detachment of the immobilized probe by

changing experimental conditions, and final measurement of the released probe.

Colorimetric determinations of aminosilanes in air (94) and on glass (95) with 1- chloro- 2,4- dinitrobenzene have been described. The method on glass is destructive since it measures the absorbance of the aminosilane-dinitrobezene product in solution. Conventional titration has been used after aminosilanes bound to glass fibers were decomposed and nitrogen trapped as NH3 (96). Coulometric titration was used to determine aminosilanes in solution (97). More recently ninhydrin was employed to determine terminal amino groups on glass (98,99). The ninhydrin reaction is, however, destructive (100).

The Merrifield synthesis of peptides generated an interest in spectrophotometrically determining amino groups on various solid supports (101-104). A spot test with ninhydrin was developed (101). Two methods used excess 2-hydroxy- 1- naphthaldehyde to react via quantitative Schiff base formation to surface amine groups. Excess reagent is removed and a soluble Schiff base is formed by reacting the resin with excess benzylamine. The spectrophotometric determination of the benzylamine-naphthaldehyde product is related to the amine content of the resin (102,103). Diisopropylethylamine was used to detach the picric acid probe from aminated polymers (104). This method is nondestructive with a reproductibility of $\pm$2% but uses potentially explosive material and importantly the bonding

and size of the probe does not mimic the actual immobilization bond (imine bond).

A study of the chemistry of enzyme reactor production and optimization should vary the reaction conditions and/or reagents and monitor both the resultant enzyme activity and the intermediate product formation. This will enable the analyst to identify those parameters important to enzyme reactor performance (high apparent activity). The immobilization process produces, in turn, terminal amines, aldehydes, and protein. Reactive aldehyde groups have been nondestructively quantitated using p - Nitrophenylhydrazine (90). Brilliant Blue G was employed to get a relative measure of amount of protein immobilized on reactors surfaces (91).

These studies were initiated when observers noted large variations in continuous flow performance in visually identical immobilized-enzyme reactors. Slight differences in surface chemical composition, chemical species distribution, surface area or surface topography of very similar reactors can void comparisons made by varying immobilization chemistry. The comparisons can be complicated further by using different reactors to study the immobilization of each functional group. These variables can be removed by using one reactor to immobilize then quantify, sequentially (1) aminosilane, (2) dialdehyde, and (3) enzyme (Figure 14). Comparisons that use this scheme assume that quantification is nondestructive such that a

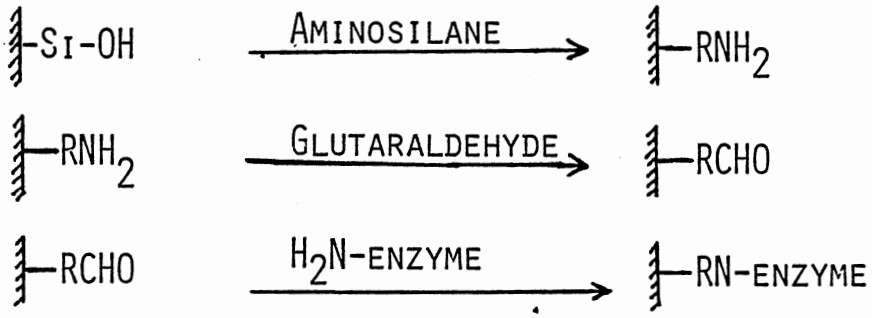


Figure 14. Terminal Groups Attached After Each Step of the Glutaraldehyde Immobilization Procedure

subsequent immobilization reaction will be exposed to the measured amount of functional groups. Extrapolations to different possible reactor configurations can be eliminated by utilization of a method with the actual reactor to be used in a flow system. This need precludes the use of a number of nondestructive spectroscopic means of determination which give good results but are not adaptable to use in the actual reactor.

Ideally, the chromophoric probe should chemically react with the "reactive" group under determination in the same fashion and under the same experimental conditions to be used in successive immobilization steps (or as close to it as possible). p-Dimethylaminocinnamaldehyde (DACA) has been chosen as the probe in the method developed and described here since it reacts with primary amines to form an α-β-unsaturated imine similar to that postulated for the glutaraldehyde/amine immobilization-reaction product (49-51,64,65). The method, being nondestructive, is of special interest for in situ determination in reactors. It involves an on/off chemistry based on the attachment of a chromophoric probe, subsequent detachment of the immobilized probe under different experimental conditions, and final spectrophotometric measurement of the released probe at 390 nm. Several aspects of incidental interest regarding covalent attachment of amino groups to silica surfaces are also discussed.

CHAPTER IV

EXPERIMENTAL DESIGN

Plastic-CPG Reactors

The apparatus, reagents, solutions, materials and procedures used in the preparation and evaluation of plastic-embedded controlled-pore glass open-tubular reactors are described in this section.

Heating Apparatus

The heating of Teflon-based reactors was performed in a custom made muffle furnace equipped with a Barber-Coleman (Rockford, IL) Model 293C temperature sensing unit. Tygon tubing was heated in a Lab-Line Imperial IV laboratory oven (Lab-Line Instruments, Inc., Melrose Park, IL).

Reagents, Solutions, and Materials

Teflon tubing of 0.8 mm i.d. and 1.6 mm o.d. (Cole-Palmer, Chicago, IL) and Tygon tubing of 1.02 mm i.d. and 1.78 o.d. (Cole-Parmer, Chicago, IL) were used for preparation of the open tubular reactors. Controlled-pore glass (CPG) of different pore size and particle size was obtained from Electro-Nucleonics Inc. (Fairfield, NJ).

All chemicals used were of AR grade except as noted. The water used for solution preparation was deionized and further purified by distillation in an all-borosilicate glass still with a quartz immersion heater (Wheaton Instruments, Millville, NJ). Penicillinase (EC 3.5.2.6) from Bacillus cereus and Glucose Oxidase (EC 1.1.3.4), Type II, from Aspergillus niger were obtained from Sigma Chemical Co. (St. Louis, MO).

Procedure for Preparing
Open-Tubular Reactors

Both, Teflon and Tygon, tubing sections were heated at one end with a flame so that the could be closed with pressure a pair of pliers. Controlled-pore glass was poured into the other end by using a small funnel. Once the pieces of tubing were tightly packed, the open end of the tubing was sealed as before. Each piece of packed tubing was then tightly wound around a 7-mm-diameter glass rod and secured with metal wire to maintain the coil shape during heating. This assembly was heated in the appropriate at the given temperature and for the length of time specified later. After heating, the coil was allowed to cool to room temperature and the unattached CPG particles removed. This was performed by either aspirating at one end (Teflon reactors) or passing nitrogen gas (tygon reactors) and shaking loose the glass particles that were not embedded

with the help of vibration from an electrical engraving tool.

Procedure for Determination of Aldehyde

The aldehyde groups immobilized on the CPG-reactor walls were determined according to the published procedure (90). (p-Nitrophenyl) hydrazine (in pH 5 buffer) was pumped into the reactor to form a Schiff-base type coupling to surface aldehyde groups. The probe was hydrolytically removed by pumping a pH 7 buffer solution through the reactor. The absorbance of the eluted hydrazine was photometrically monitored to determine the amount of reactive aldehyde groups present.

Procedure for "Whisker" Growth on Capillary Walls

A glass capillary (i.d. 0.8 mm and 4 to 6 m in length) was filled completely with concentrated HCl to wet the entire surface. Approximately 1/3 of the volume of the capillary was emptied and both ends sealed by torch. The sealed capillary was placed in an oven at 80 C overnight to leach any metal ions from the surface.

A solution of ammonium bifloride saturated in anhydrous methanol was pumped through the capillary to coat the walls. A steady nitrogen flow was used to remove residual moisture. Each end was torch sealed and the capillary place in a furnace and heated to 400-450 C overnight. This etching

step was repeated at least 3 times until the surface modification appeared even to the naked eye. Prior to the immobilization procedures a second HCl leach was performed to remove metal ions which migrated to the surface during heating.

Procedure for Immobilizing Enzymes on Plastic-CPG Reactors

The coiled reactors were connected to a pump in such a way as to allow fresh reagent solutions to flow into the reactor then to waste. In this manner, 60 mL of a 10% solution of 3-aminopropyltrimethoxysilane in 95% ethanol was introduced and pumped through the reactor for 1 hour. The reactor was then flushed with copious amounts of ethanol for at least 5 min, followed by rinsing with pH 7 buffer. A buffered (pH 7) solution of 5% glutaraldehyde was introduced into the reactor at 1 mL/min for 30 min.

The reactor was rinsed with one or two reactor volumes of buffer then a cold (4 °C) 5% enzyme solution was allowed to fill the reactor. Enzyme solution first rinsed the reactor (~2 mL) then filled it. The ends of the reactor were sealed and the reaction was allowed to proceed overnight at 4 °C. The reactor was rinsed with 1 - mL of 1.0 M KCL to remove absorbed protein. Finally, the reactor was rinsed and filled with buffer solution and stored until used.

Procedure for Flow Injection Analysis

Determination of Substrate

Plastic-embedded CPG OTR or glass capillary OTR were incorporated in the experimental setup shown in Figure 12. A Minipuls 2 peristaltic pump (Gilson Medical Electronics, Middleton, WI) was used to propel the (1 mM phosphate) buffer solutions through the system. Samples (70 µL) were intercalated into the carrier stream with the aid of a model 5041 Teflon rotary valve (Rheodyne, Inc., Cottati, CA). All wetted surfaces from the injection valve to the detector were made of Teflon. Potentiometric measurements were performed in a flow-through cell using an epoxy body combination electrode (Sensorex, Westminster, CA) connected to a model PHM 84 Research pH Meter (Radiometer, Inc., Copenhagen).

Determination of Amines on Silica Surfaces

The following sections describe the apparatus, reagents, and procedures using in developing and validating a method to determine reactive amino groups on silica surfaces.

Apparatus

A Perkin-Elmer Lambda 3840 UV/VIS linear diode array spectrophotometer operated by a Perkin-Elmer 7300 computer (Perkin-Elmer Inc., Nowalk, CT) was used for collection,

manipulation, and output of spectral and absorptiometric measurements.

Attachment of the probe was carried out using either of two inverting shakers for mixing. For room temperature reactions a Universal Oscillating shaker (Model 2095, Lab-Line Instruments, Inc., Melrose Park, IL) was used, and for reactions at elevated temperatures a Multi-Blok heater (Model 2093, Lab-Line Instruments) was mounted on a custom-made air-driven shaker.

Detachment studies were conducted by placing 30-mL glass vials containing DACA-reacted controlled-pore glass and the appropriate hydrolysis medium into a test tube rack located within the well of an ultrasonic mixer. The temperature of the hydrolysis reaction mixture was controlled ($\pm$ 0.1 °C) by water circulated from a Haake R-22 water bath (PolyScience Corp., Evanston, IL). The extent of hydrolysis as a function of time was determined by adding to the setup a Minipuls 2 peristaltic pump (Gilson Medical Electronics) to pump the hydrolysis medium through a gas dispersion tube (to filter out suspended glass from the vial) to a flow-through cell in the spectrophotometer and back into the capped vial.

Reagents and Glass Supports

All chemicals used were AR grade. The water used for solution preparation was de-ionized/distilled as described earlier. Anhydrous reagents were prepared by drying over

type 4A molecular sieves (Union Carbide Corp., Linde Division, Danbury, CT). The buffer solutions used for detachment were 0.050 M in total phosphate and 0.10 M in NaCl. Aminopropyl CPG was obtained from Electro-Nucleonics Inc. (Fairfield, NJ) and used without further treatment.

Procedure for the Attachment of Chromophoric Probe

About 50 mg of aminopropyl-CPG was placed in a 30-mL glass vial with an excess (40 mg) of DACA (Aldrich Chemical Co.) and 20 mL of reaction solvent. For the recommended procedure the solution was also 1.0×10^{-3} M in piperidine (Aldrich Chemical Co.). The piperidine was added in solution with anhydrous ethanol. The vial was then capped and mechanically shaken for 60 min. The reacted CPG was separated from the mixture by aspirated filtration, sequentially washed with four 10-mL portions of anhydrous ethanol, and finally allowed to dry under air flow.

Procedure for the Detachment of Chromophoric Probe

At least 10.0 mg of the reacted CPG was carefully weighed into each of three 30-mL glass vials and each weight was recorded since all determinations are on a per gram basis. Hydrolysis medium, preferably 95% ethanol, (25.00 mL) was pipetted into the vial and the vial placed in a thermostated ($\pm$ 0.1 °C) ultrasonic bath for 60 min.

Suspended glass particles were removed by centrifugation and the absorbance was measured at 390 nm.

Procedure for Reaction vs. Time Studies

The reaction of DACA with aminated CPG as a function of time was monitored in a variety of solvents by stopping the reaction at designated times and subjecting the reacted CPG to standard hydrolysis.

Detachment of probe as a function of time was studies in a close-loop continuous-flow system. The hydrolysis medium was circulated between the reaction vial and a flow cell located in the spectrophotometer. Absorbance measurements were made every 30 s and used to locate an absorbance plateau.

Procedure for the Determination of Limit of Detection

Un-aminated CPG was reacted with DACA and piperidine in anhydrous ethanol for one hour. The CPG was then filtered out, rinsed, dried, and weighed into portions of about 30 mg. Each portion was subjected to hydrolysis at 40.0 °C with 95% ethanol. The Limit of detection was were taken as the average of ten blank readings plus three times the standard deviation of those radings.

Solutions and Procedure for Titrations

Anhydrous titrant was prepared by adding 200 μL of 70%

perchloric acid (Mallinckrodt, Inc., Paris, KY) and 450 µL of acetic anhydride (Mallinckrodt) to about 1.00 L of glacial acetic acid (J.T. Baker, Phillipsburg, NJ). Care was taken to avoid an excess of acetic anhydride since it reacts with primary amines. The indicator solution was prepared by dissolving 0.25 g of Methyl Violet 2B (Matheson Co., East Rutherford, NJ) in 100 mL of glacial acetic acid.

Aminated CPG (20 mg) was placed in 25 ml of glacial acetic acid and 2 drops of the 0.25% (w/v) indicator solution was added. The mixture was titrated (105,106) until the last tinge of purple color disappeared (Methyl Violet endpoint).

CHAPTER V

RESULTS AND DISCUSSION

Plastic-CPG Reactor

Open tubular reactors (OTRs) were among the first type configurations used in implementing immobilized enzymes as analytical reagents in flow systems (7,9,73,74). The advantageous flow characteristics, sample throughput, low back pressure, and steady electrochemical baseline were the tradeoff for the low surface area per unit of this type reactor. This section details the results of a project to improve the ease of preparation and the use of OTRs by the innovative use of high surface area glass particles and plastic tubing.

Nylon tubing was first used to fabricate immobilized enzyme OTRs. The limited ability to increase the surface area, and adsorption or organics have precluded its widespread use in unsegmented flow systems. Glass capillary surface areas have been successfully increased 2 or 3 orders of magnitude by the procedure depicted in Figure 15 (80). The surfaces of treated and untreated inner walls of glass capillaries are illustrated here. The process of making reactors by drawing capillaries then etching those capillaries is prone to failure at several points. Upon

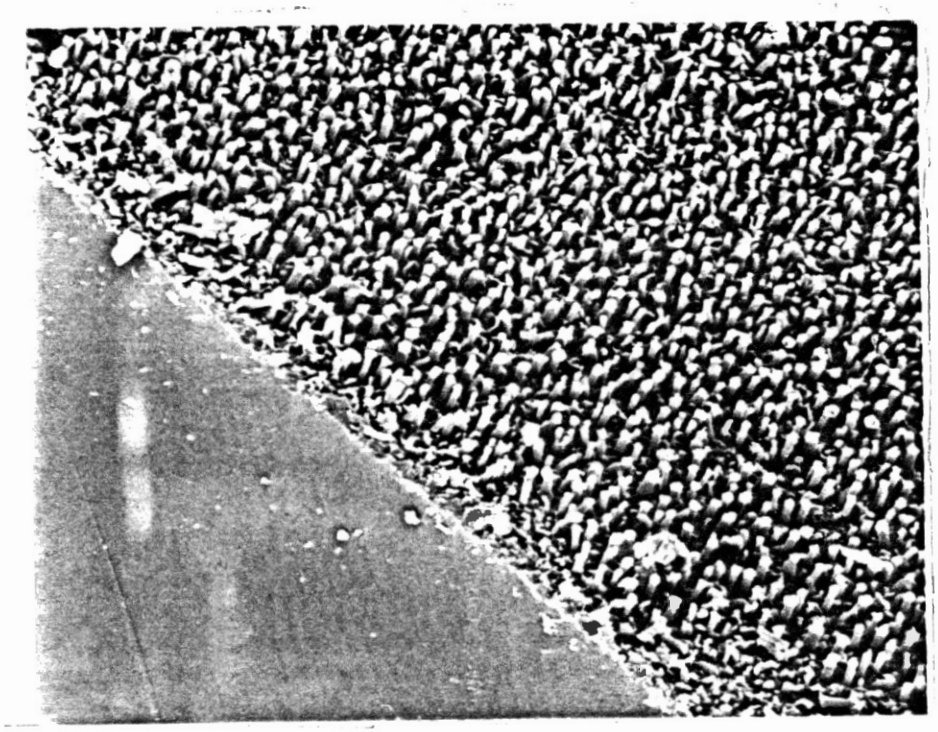


Figure 15. The Effect of the Whisker Growth Process on Glass, Untreated (left) and Treated (x 10000)

applying heat in either the HCl leaching or HF etching steps (Table II), the sealed capillary is subject to destructive explosion. The experimentalist only option is start with another capillary from the first step.

In practice, the etching solution, saturated ammonium bifloride wets the inner walls of the capillaries. The methanol solvent is removed by evaporation under nitrogen flow. In the leaching steps a capillary is filled to 2/3 its length with concentrated HCl, sealed and then heated. Heating glass to temperatures as high as 400 °C causes metals in the glass framework to migrate to the surface. These metal ions, which can be enzyme inhibitors, must be removed prior to immobilization procedures.

Whisker growth proceed, best when the glass surface is very clean, in particular the surface should be free of metals and water. Temperature gradients in the heating oven lead to uneven or nonuniform surface modification. Minute variation in temperature in parts of the glass surface, together with localized metal and water content, causes the development of zones of great surface area and zones of almost no modification.

In addition to the nonuniformity within the reactor, the procedure is not very reproducible. An etched glass capillary produced on day 2 is likely to be very different from a capillary etched on day 1. This problem is addressed by resubjecting the treated surface to the etching procedure for a second, third, or even fourth treatment. The

TABLE II

COMPARISON OF PROCEDCURES TO PRODUCE GLASS CAPILLARY AND PLASTIC-CPG OTR'S

ETCHED GLASS OTR		PLASTIC-CPG OTR	
- DRAW CAPILLARY	4 H	- PACK TUBING with CPG	30 MIN
- HCl LEACH @ 80° C	16 H	- HEAT *	10 MIN
--COAT WITH NH_4HF_2	1 H	- REMOVE LOOSE CPG	15 MIN
- HEAT @ 400° C	4-16 H		
- HCl LEACH @ 80° C	16 H		
	2-3 DAYS		1 H
		* TEFLON 10 MIN @ 400° C TYGON 5 MIN @ 190°C	

expectation is that zonal variations will even out as the number of etching steps increase producing a more uniform surface. Likewise, a group of capillaries that undergoes multiple etchings would be very similar to each other (reproducible). Another group subjected to a single treatment would demonstrate nonreproducible fabrication.

Table II lists an optimistic assessment of the time required to etch a glass capillary surface. The table was constructed using a "best case" scenario with all procedures working to perfection. In reality a capillary can be prepared for the immobilization process in about 5 days if no failures occur. The 3 day preparation of high surface area glass capillaries should be compared with the 1 - 2 h procedure to produce plastic-CPG hybrid OTRs. Tubing is simply filled with CPG, heated to just above the tubing melting point, then the excess CPG is removed (Table II). The procedures used in the construction of the plastic-CPG OTRs are milder and do not require toxic chemicals or corrosive steps.

Effect of Temperature and Heating Time

The effect of temperature on surface coverage was more dramatic in the case of Teflon-based reactors. Figure 16 shows samples heated at 325 °C and 350 °C for the same length of time. The Teflon used was of the TFE type which has a reported melting point of 327 °C (10). Figure 16A is the electron micrograph at the higher magnification and

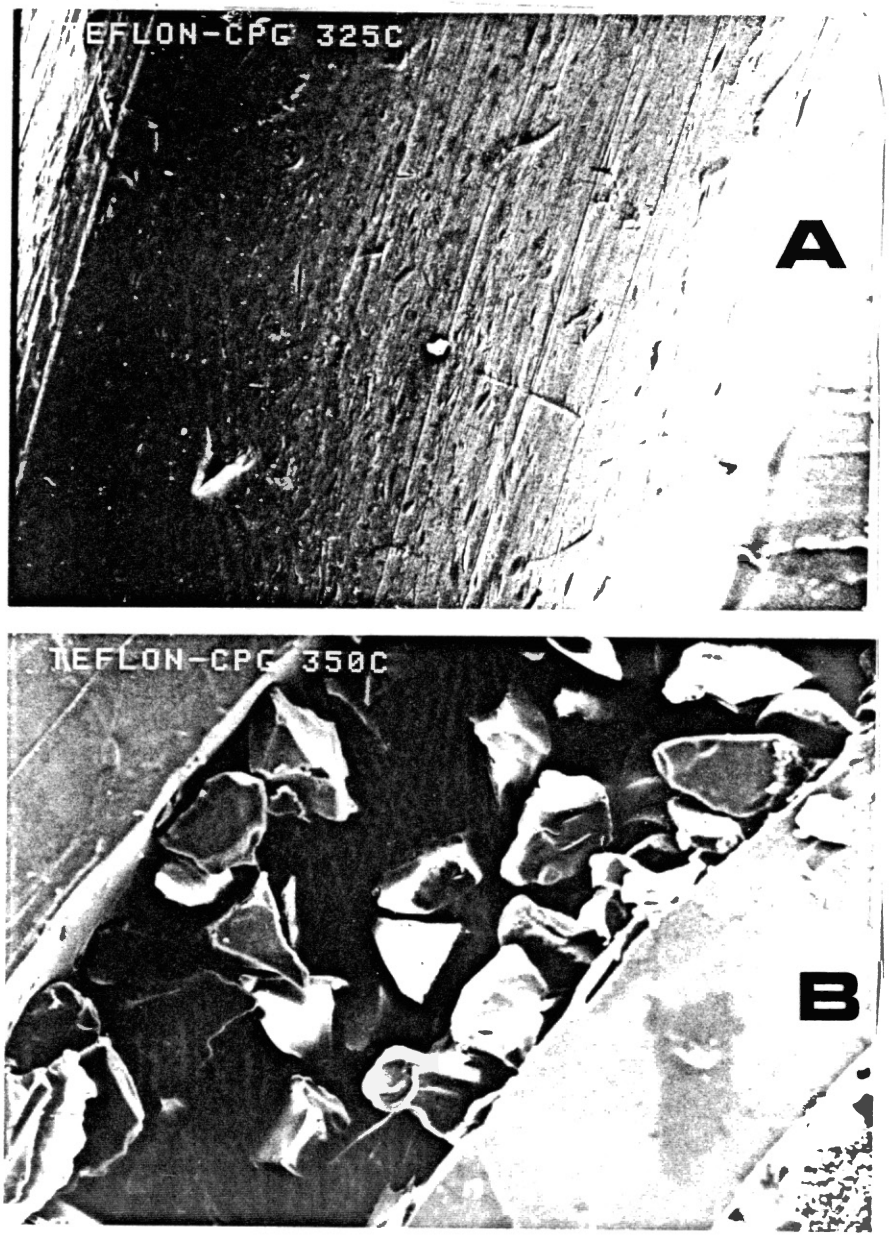


Figure 16. Scanning Electron Micrograph of Reactors Made by Heating Teflon 10 min. at (A) 325 °C (x108) (B) 350 °C (x68)

demonstrates no adherence of CPG particles at temperatures below the Teflon melting point. At temperatures slightly above the melting point the plastic tubing becomes soft enough for CPG particles to be embedded in the tubing walls (Figure 16B). The tubing was found to have a narrow heating exposure tolerance (combination of temperature and time) for this application. Temperatures higher than 350 °C could not be withstood long enough to give effective particle coverage on the tubing. The Teflon would decompose or become unusably brittle. The same effect could be observed if the heating times were extended. Figure 16B illustrates heating Teflon at 350 °C for 10 minutes gives good results.

Tygon exhibited a different behavior (Figure 17 and 18). The Tygon used was observed to melt around 150 °C. At temperatures lower than the melting point only smaller chips can be observed (using magnifications of over 1000) to stick to the walls via static charges (Figure 17A). As the temperature is increased. gradually larger and larger particles are embedded in the surface. Tygon must have a broad melting point range as compared to the Teflon behavior of an abrupt change in embedding character from just 2 °C under the melting point. Tygon tubing can be covered effectively by glass chips at temperatures of 175 °C to 200 °C (Figure 17B and Figure 18A,B) and exposures of 2 to 3 minutes. Greater exposure or temperatures causes decoloration, plasticizer release, and tubing decomposition.

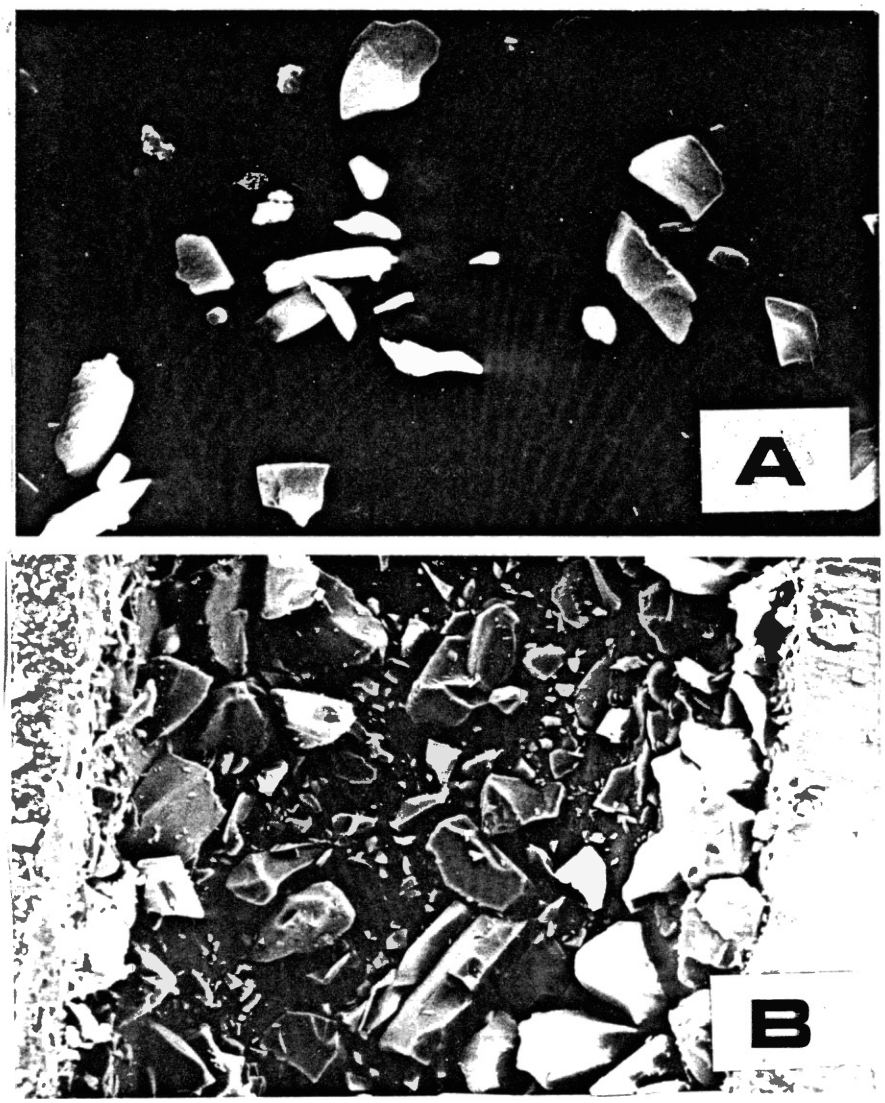


Figure 17. Scanning Electron Micrograph of Reactors Made by heating Tygon (A) 5 min. at 125 °C (x1430) (B) 5 min. at 175 °C (x68)

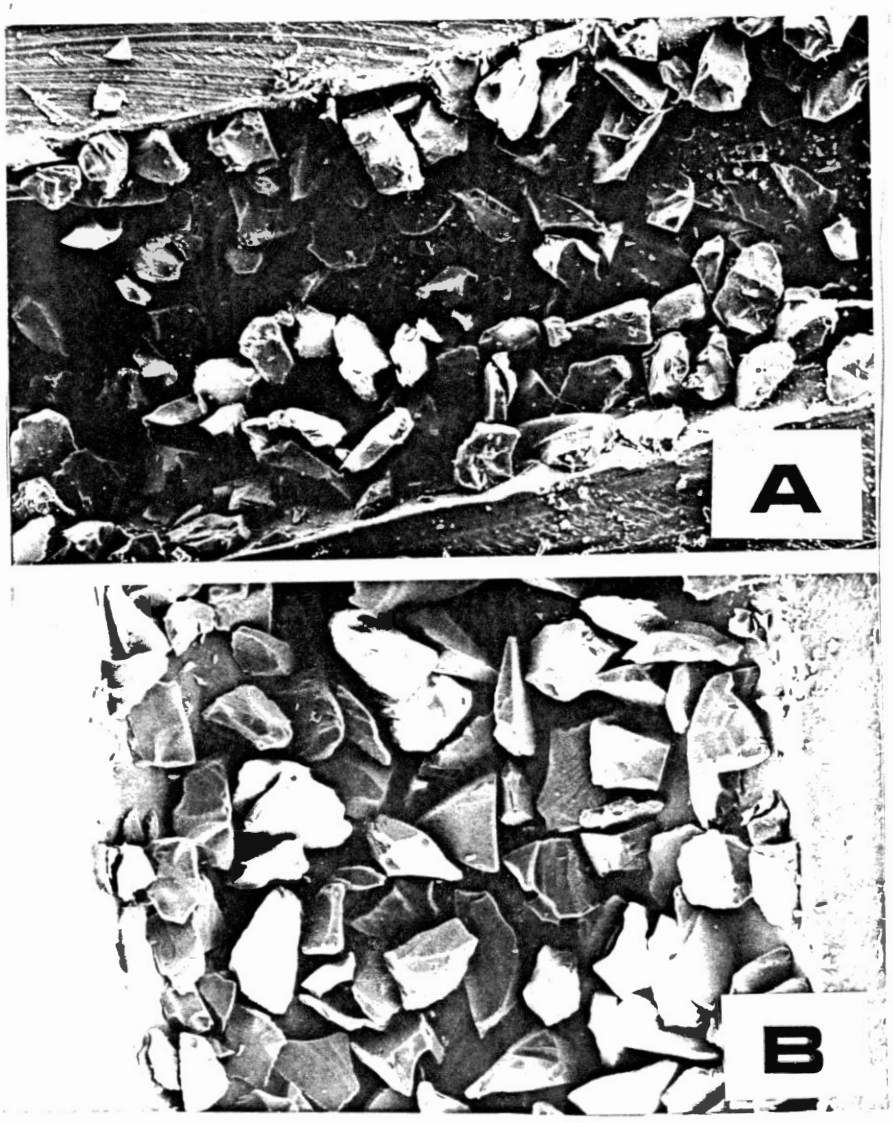


Figure 18. Scanning Electron Micrograph of Reactors Made by Heating Tygon (A) 2 min. at 180°C (x42) (B) 5 min. at 200°C (x57)

Comparative Performance of Reactors Containing Immobilized Enzymes

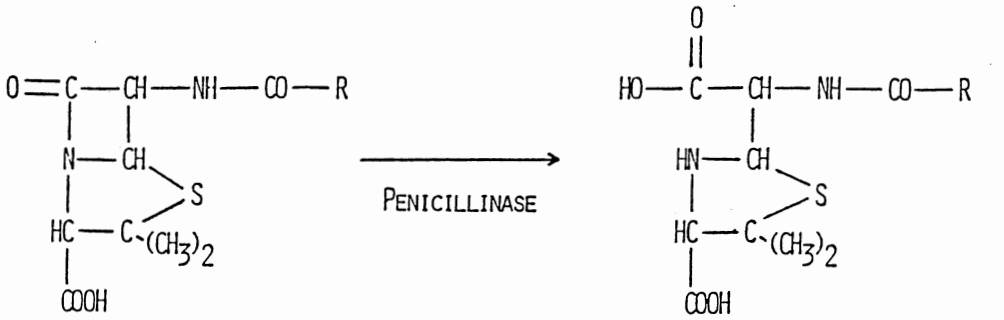
The hybrid reactors compare favorably to other OTRs, specifically etched glass capillaries (Table III). Reactors were constructed then glutaraldehyde attachment was performed. The increased loading capacity of the plastic-CPG type reactor was demonstrated by using a recently developed method for determining reactive aldehyde groups on reactor surfaces (90). Using this (p-nitrophenyl)hydrazine method aldehyde groups attached to plastic embedded CPG (75 Å, 80/120 mesh) were determined. As indicated in Table III, a 3750-fold increase in aldehydes immobilized per meter was observed for plastic-CPG reactors compared to a etched glass capillary reactor. There was more uniform coverage with CPG embedded reactors than with borosilicate glass reactors. The larger uncertainty for Tygon depicted in Table III is caused in large part by adsorption of the chromophoric probe used to measure aldehydes onto the Tygon itself. Teflon did not adsorb the probe.

Additional comparative and characterization studies were performed by preparing immobilized Glucose Oxidase and immobilized Penicillinase reactors. The substrate conversions yielded by these two types of enzyme reactors are illustrated in Figure 19. The product concentrations in the FIA stream were followed by monitoring the change in hydrogen ion activity potentiometrically. Figure 20 shows how the FIA peak shape changes when using different pore

TABLE III

COMPARISON OF OTR PERFORMANCE

	ALDEHYDE (μMOL/M)	SIGNAL/ LENGTH	SENSITIVITY
TEFLON (75 Å)	45 ± 0.04	-	-
TYGON (75Å)	43 ± 0.12	15	1.5
TYGON (3000Å)	-	18	3
ETCHED GLASS CAPILLARY	0.012 ± 0.004	1	1



GIVEN PENICILLIN

CORRESPONDING PENICILLOIC ACID

D GLUCOSE

D GLUCONIC ACID

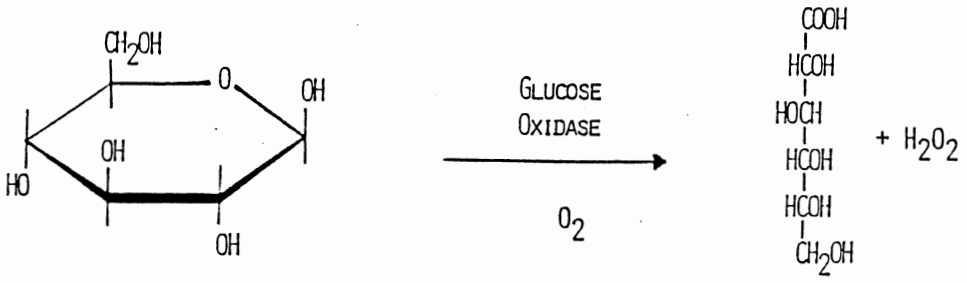


Figure 19. Substrate Conversions Catalyzed by Enzyme Reactors Utilized in Comparison Study

GLUCOSE OXIDASE REACTORS

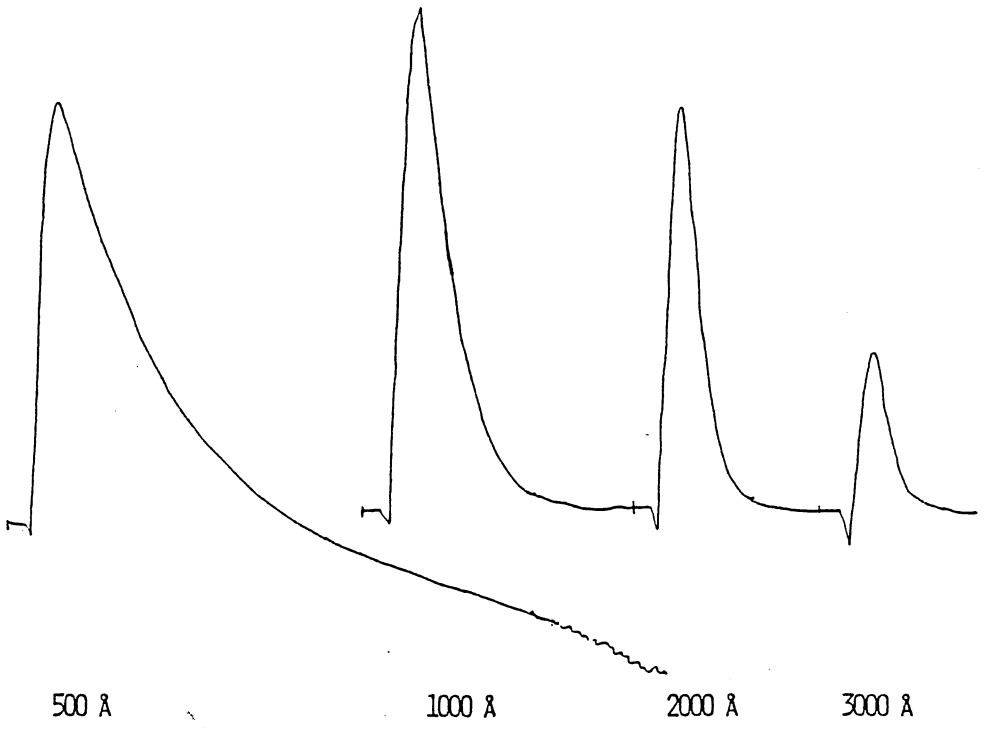


Figure 20. The Effect of CPG Pore Size on FIA Signal Profile

sizes of available CPG. Undoubtably there is an optimum CPG pore size for each enzyme and perhaps each application. For Glucose Oxidase reactors, good sensitivity (large peak height) and good throughput (short return to baseline) can be achieved using 2000 Å CPG enbedded in plastic. Reactors made with 500 A pore CPG exhibited unacceptable dispersion. Product molecules entered the pores and slowly diffused back into the carrier stream. Peaks did not return to baseline after 30 minutes.

Figure 21 illustrates how FIA conditions can be varied to improve the performance of the reactor system. At a flow rate of 4 mL/min there is very little opportunity for molecules to become trapped in the inner caverns of the CPG. This higher flow rate improves the FIA peak shape (and sample throughput) of this reactor but at the expense of additional reagent use and sensitivity. In practice, the judicious choice of the appropriate pore-sized CPG makes more sense.

The plastic-CPG reactors compare favorably with etched glass capillary reactors. Table III shows that plastic-CPG penicillinase reactors were as much as 3 times more sensitive (as measured by the slope of the calibration curve) than reactors made of etched borosilicate. The greater local enzyme activity indicates that these reactors can be much shorter than previously used OTRs. The plastic-CPG reactors were about 1/10 as long as the etched glass capillary reactors. The normalization of signal height by

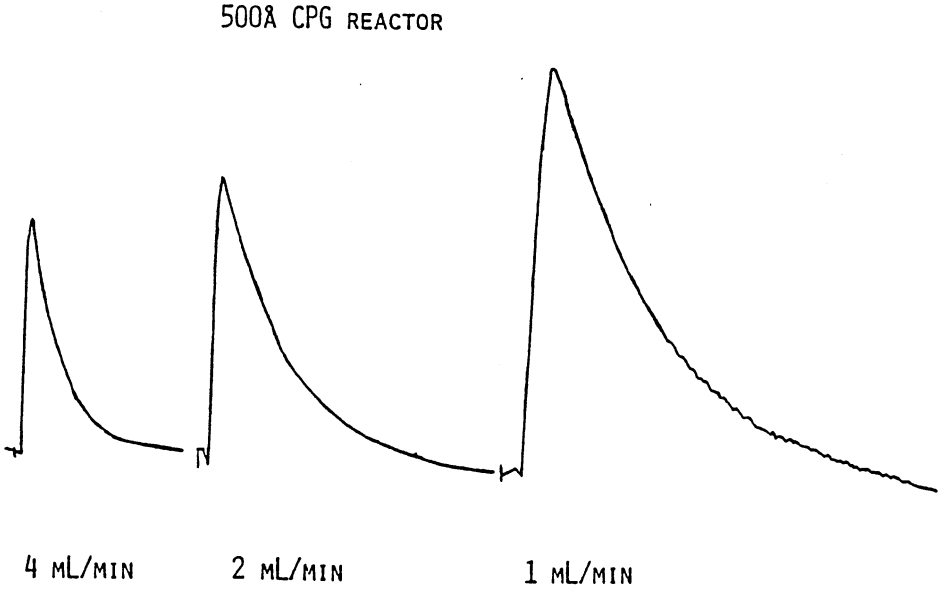
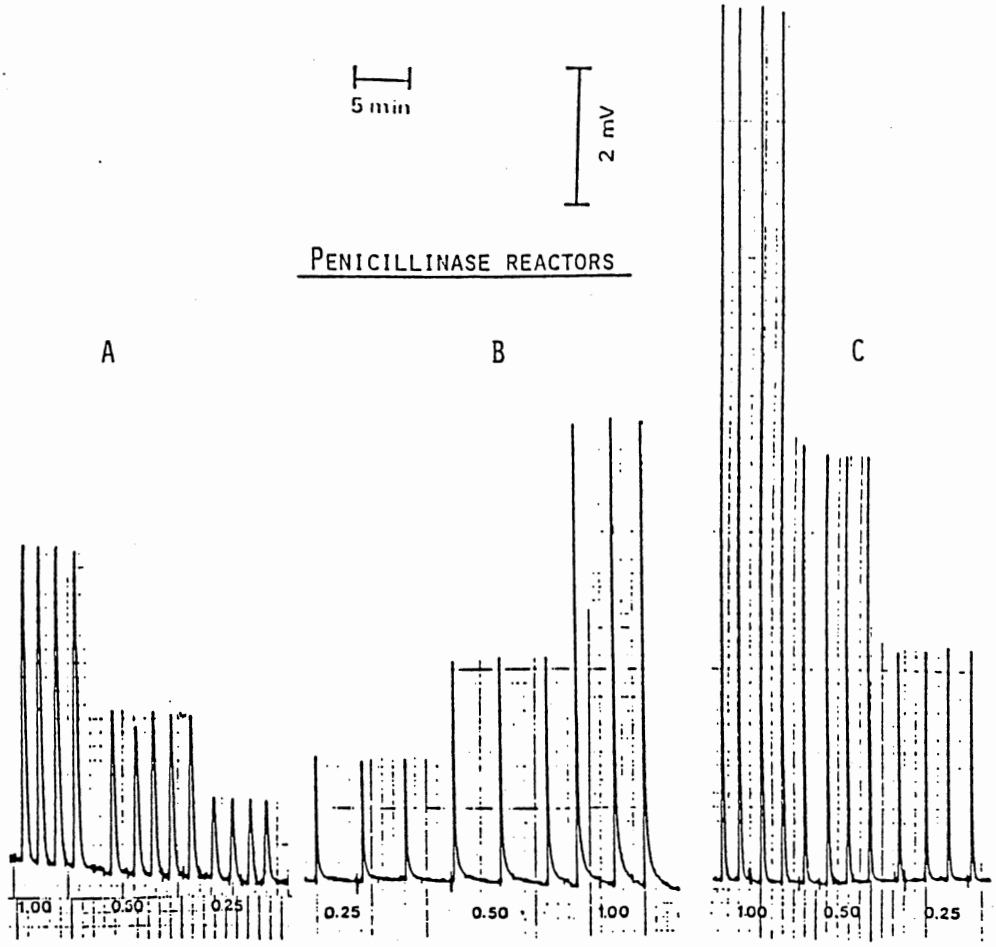


Figure 21. The Effect of Flow Rate on FIA Signal Profile

reactor length (Table III) shows these CPG to be roughly 20 times as good as borosilicate glass reactors. This is visually depicted in Figure 22 where typical signals obtained with each type reactor are illustrated.

Summary

The comparative studies presented here show the advantaged of the plastic-CPG construction for OTRs to be used as immobilized enzyme reactors in unsegmented flow systems. plastic-OTR reactors can be contrasted to etched borosilicate glass capillary reactors on several points. 1) The reactors are quickly and more easily prepared. The production of these reactors do not require special equipment such as capillary drawing machine. A CPG-embedded-on-plastic reactor can be made in minutes whereas the elaborate procedures of whisker growth requires days. 2) The loading of immobilized aldehyde is 3750 times as high. 3) These reactors in FIA systems were roughly 20 times better than the glass counterparts when the signal height to reactor length ratio was considered. 4) When sensitivity was measured as the slope of the calibration curve, the plastic-CPG reactors were as much as 3 times more sensitive than glass reactors ten times longer. 5) The increased surface area affords the opportunity to achieve high local enzyme activity. The resultant use of shorter reactors to achieve identical substrate conversion should decrease the dispersion due to length of travel. 6) The



A. 3.6 M GLASS CAPILLARY (NH_4HF_2 ETCHED).

B. 0.35 M CPG-TYGON (75Å CPG, 80/120 MESH).

C. 0.55 M CPG-TYGON (3000Å CPG, 200/400 MESH).

Figure 22. Comparison of Typical FIA Signal Profiles Generated by Immobilized Enzyme Reactors.

increase in surface area is not very dependent on operator skill. The process of whisker growth is nearly an art, the results obtained in one lab may differ significantly from those obtained by another. With plastic-CPG Reactors, the increase in surface area is inherent in the material to be embedded in the walls of the plastic. Figure 22 suggest the plastic tubing surface area enhancement is greater than the 2 to 3 orders of magnitude often quoted for whisker growth on glass (9). 7) Uniform coverage is obtained. There are undoubtedly temperature variations in the heating of CPG laden tubing. The rate of whisker growth, however, is kinetically controlled and therefore much more affected by temperature gradients. Once the melting point threshold is crossed CPG chips are embedded in the soft plastic independent of a 2 or 3 degree temperature fluctuations. 8) The simplicity of simplicity of fabrication, independence from operator skill, temperature variations, and tubing preparation are factors which make this procedure vary reproducible. Capillaries which have undergone "whisker growth" etching may appear quite different from each other. 9) The plastic reactors produced are easier to handle and connect to flow systems than glass tubing. 10) A CPG-plastic reactor can be made without procedures which use corrosive chemicals such as refluxing concentrated HCl and etching with HF (NH_4HF_2 at 450 °C).

The simplicity of fabrication along with the fast preparation should appeal to those interested in producing

immobilized enzyme reactors for use in continuous flow systems. This type hybrid reactor could conceivable be initiated with other materials as well. The inclusion of beads to form a SBSR is another area to be explored. Based on comparative studies reported above the plastic-embedded-CPG reactor is the more attractive choice for use as FIA immobilized enzyme reactors.

Glyoxal Studies

Studies were performed to compare the coupling effectiveness of two dialdehydes (glyoxal and glutaraldehyde). Plastic-CPG immobilized-enzyme reactors were prepared using each of the dialdehydes. A visual comparison of the resulting FIA peaks did not show significant difference between glyoxal coupled reactors and glutaraldehyde coupled reactors.

Additional studies were performed on batch CPG to measure the amount of reactive aldehyde immobilized on the glass chips. The appropriate aminosilane (10% in ethanol) was shaken with 1 g of CPG for 5 minutes, then a 5% solution of aldehyde was shaken with about 0.25 g of the aminated CPG for 1 hour. The amount of aldehyde immobilized was determined according to published procedures (90).

These studies, in the batch mode were undertaken to see if the length and stiffness of the coupling agent would affect the number of reactive aldehyde groups available for immobilizing enzymes. When a molecule of glutaraldehyde

TABLE IV

COMPARISON OF GLUTARALDEHYDE AND GLYOXAL AS IMMOBILIZATION CROSS LINKERS

SILANE	DIALDEHYDE	REACTIVE ALDEHYDES (μmol-CHO/g CPG) 1st	2nd	X ± S
amino propyl trimethoxy silane	glutaraldehyde	14.86	18.62	17.74 ± 2.66
amino propyl trimethoxy silane	glyoxal	14.96	15.49	15.23 ± 0.37
amino phenyl trimethoxy silane	glutaraldehyde	0.30		
amino phenyl trimethoxy silane	glyoxal	1.38		
amino phenyl triethoxy silane	glutaraldehyde	0.42		

reacts with a surface-amino group at one end, the other aldehyde group is free to rotate and perhaps react with other surface amino groups. This has the effect of reducing the amount of aldehyde groups available (reactive aldehydes). Glyoxal (a two carbon dialdehyde) was thought less likely to participate in this type of "double" coupling. Glyoxal coupled to a still aminosilane (i.e. aminophenylsilane) was not expected to couple to surface amino groups at all.

The results in Table IV do not show a statistically significant difference in the amount of reactive aldehyde immobilized on CPG using glyoxal or glutaraldehyde. The smaller numbers seen with the aminophenyl silanes is attributed to low efficiency of the silanization reaction usually performed in refluxing toluene instead of room temperature ethanol.

Determination of Reactive Amines on Silica Surfaces

The reversible addition of a chemical probe to an immobilized chemical species with the subsequent quantitative removal and measurement of the released probe is referred to herein as "on/off chemistry" (107). Formations of Schiff bases (imination) are good candidates for use in this procedure. Product formation is easily reversed by hydrolysis of the imine and a wide range of conditions can be selected to favor the forward reaction.

DACA has been proposed as a colorimetric reagent for detecting amines (108) and was selected for the work reported here over I_2 and chloranil, two other potential probes tested, because it provided better behavior for the type of application sought.

Iodine is released slowly during the exposure of samples to air; this result in very poor reproducibility. chloranil, on the other hand, is attached practically irreversibly; neither concentrated solutions of the bases morpholine of piperdine, or HCl, or NaOH were effective in removing chloranil. Iodine was studied because it has been used to enhance the UV detectability of N, N-dimethylbenzylamine in liquid chromatography (109), and chloranil because it has been proposed for the spectrophotometric determination of surface-bound amines (110). The probe of choice, DACA, has a relatively large molar absorptivity of 3.2×10^5 cm^{-1} M^{-1} and a very distinct peak maximum at 390 nm (Figure 23).

Effect of solvent on Probe Attachment

Organic solvents which can facilitate the removal of water produced in the coupling reaction should favor more product formation at equilibrium. The use of molecular sieves is not feasible in a method developed for use within small diameter continuous flow reactors. Acetic anhydride reacts with primary amines. Since there is 'no such thing as a completely water free silica surface', choosing a

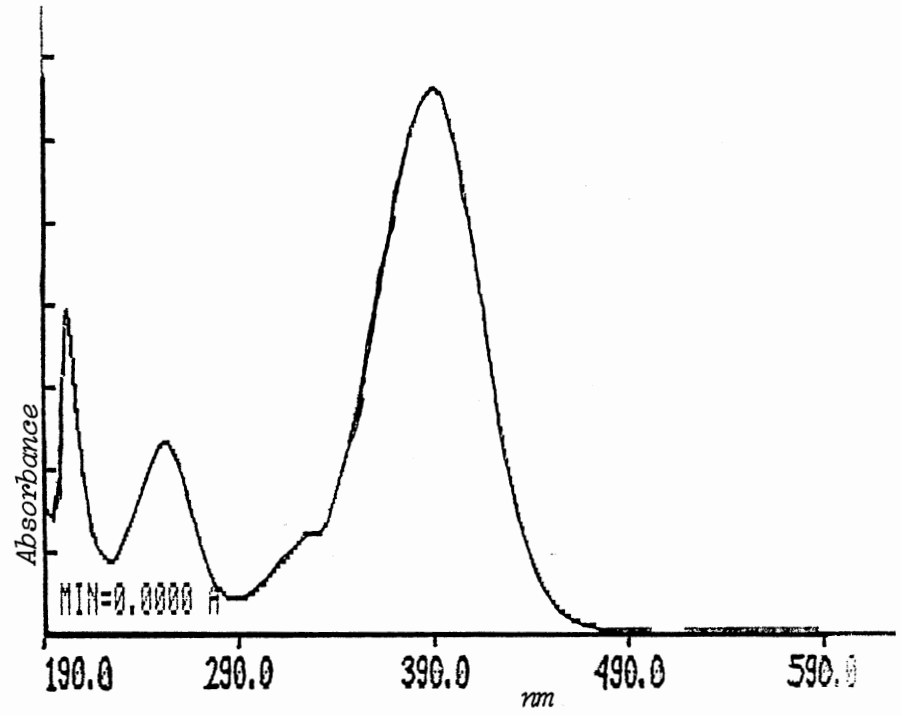


Figure 23. UV-Visible Spectrum of DACA

TABLE V

EFFECT OF REACTION SOLVENT ON PROBE ATTACHMENT

SOLVENT	DACA ATTACHED (μmole/g CPG)
(A. Using CPG B, 60 min contact time)	
Anhydrous Methanol	44.7 ± 1.0
Ethanol	32.7 ± 0.4
Anhydrous Ethanol	39.1 ± 0.6
Propanol	40.7 ± 0.3
0.25 N ethanolic HCl	0.19 ± 0.02
Anhydrous Acetone	4.39 ± 0.75
(B. Using CPG E, 150 min contact time)	
pH 5.0 buffer	3.96 ± 0.19
distill H_2O	9.56 ± 0.08
95% ethanol	176 ± 6

solvent which can solubilized water and dilute the local surface concentration of water is a reasonable alternative.

Low molecular weight, comparatively polar alcohols were tested as solvents (Table V). Low carbon-content alcohols may participate in hydrogen bonding with surface amines and water produced during the reaction. Hydrogen bonding may effectively shield water molecules from imines formed at the surface and facilitate transfer from the local environment. The orientation of amines for a more efficient attach by aldehydes has been postulated as another benefit of hydrogen bonding. Methanol is expected to participate more in hydrogen bonding than ethanol, this is evident by comparing the results from reactions in anhydrous ethanol and anhydrous methanol. A comparison of the results obtained when using anhydrous ethanol and reagent ethanol indicates the maximum forward reaction is obtained in 'dry' solvents.

Less successful results were obtained with ethanolic HCl and acetone. Ethanolic HCl (0.25N) has been used as a solvent for chromogenic aldehydes used in imine formation reactions to determine amino groups on surfaces (111). The DACA reaction solution and unwashed reacted CPG did displayed a more intense color than others observed. This was a solvation effect since the amount of probe that reacted and was subsequently detached is poor (Table V). Water is very miscible with acetone, however ketones can react with primary amines and the results, listed in Table V, are predictable.

Water as a reaction solvent was discarded since the equilibrium constant for imination in aqueous media does not favor product formation (60); the results for absolute ethanol and anhydrous absolute ethanol (stored over molecular sieves) in Table V are lower when water is present in the solvent. In another experiment (Table VB) 18 times as much probe was attached in 95% ethanol as in deionized/distilled water under otherwise identical conditions. In pH 5.00 phosphate buffer, conditions were even worse since comparatively 44 times as much probe was attached when 95% ethanol was used as solvent.

Effect of Acid or Base Promotion

Imine formating can be either acid catalyzed or base catalyzed, in general (112), and piperdine has been proposed as a basic promoter of imine formation (113). Base-promoted imine formation can be considered to occur as follows:

$$R\text{-}NH_3^+ + B: \rightleftharpoons R\text{-}NH_2 + BH^+ \qquad (7)$$

$$R\text{-}NH_2 + O{=}CH\text{-}R' \longrightarrow R\text{-}N(H)\text{-}C(OH)(H)\text{-}R' \qquad (8)$$

$$R\text{-}N(H)\text{-}C(OH)(H)\text{-}R' + B: \longrightarrow R\text{-}N{=}CH\text{-}R' + BH^+ + HO^- \qquad (9)$$

Piperdine as a basic promoter, (B:), first shifts the amine protonation equilibrium (reaction 7) to the unprotonated primary amine; then it removes a proton from the carbinolamine intermediate during the dehydration step (reaction 9).

Table VI shows results in presence and absence of piperdine and of acetic acid as potential promoters of imine formation. Acetic acid as a potential promoter shows no effect at all except as relatively high concentrations when a detrimental rather than a beneficial action is observed. Piperdine, however, shows a definite promoting effect; results after only 1 h in anhydrous ethanol in the presence of piperdine, for instance, are comparable to those obtained in its absence but after overnight contact time. Hence the recommended procedure calls for piperdine in anhydrous absolute ethanol, room temperature, and 60 min contact time.

The effect of acid concentration was further studied by varying the concentration of glacial acetic acid in anhydrous ethanol eight orders of magnitude. The amount of DACA detached using standard conditions was recorded in Table VII. The effect of acetic acid concentration is minimum at low acid concentration and long reaction times. When reaction times are extended (4.5 hrs) the results of reactions with and without acetic acid are not significantly different. High acid concentration may protonate surface amines and initially decrease the reaction rate.

TABLE VI

EFFECT OF DIFFERENT SOLVENTS AND PIPERIDINE OR ACETIC ACID ADDED AS POTENTIAL PROMOTERS OF THE ATTACHMENT OF DACA TO AMINOSILANE-DPG

SOLVENT	10 POTENTIAL PROMOTER ADDED		PIPERDINE ADDED (1.0 x 10_{-3} M)		ACETIC ACID ADDED	
	60 min[a]	19 h[a]	60 min[a]	19 h[a]	5.7×10^{-2} M 60 min[a]	5.7×10^{-4} M 60 min[a]
Ethanol (absolute)	32.7 ± 0.4	50.0 ± 0.3				
Anhydrous absolute[b]	39.1 ± 0.6	53.9 ± 0.9	51.8 ± 0.5	61.2 ± 0.6	29.5 ± 0.8	39.0 ± 0.2
Anhydrous methanol	44.7 ± 1.0	61.6 ± 1.1	58.4 ± 1.7	63.9 ± 1.0		

[a] Contact time.
[b] Absolute ethanol dried over molecular sieves.

All results are average of three determinations on the same sample of aminosilane-CPG and reported in µmol -NH_2/ g CPG. Temperature: 25.0 ± 0.2 °C. DACA concentration: 0.01 M.

Effect of Reaction Time on Probe Attachment

Figure 24 illustrates reaction vs time profiles of probe attachment in various solvents. Piperdine promoted reactions reach a plateau after about one hour of reaction. Beyond this point substantial increases in reaction times are justified by increased probe attachment. By way of example, the reaction using 1.0×10^{-3} M piperdine in anhydrous ethanol attached 51.8 $\pm$ 0.5 µmol/g, increasing the reaction time 450% to 4.5 h only produces an 11% increase in the amount of probe attached (57.5 $\pm$ 0.6 moles/g). True comparative quantities should be obtained under reaction conditions which are typically used to couple glutaraldehyde. Reaction times of one hour are common. The piperdine promoted reaction reaches a yield plateau after 60 min contact time and longer times are not justified.

Effect of Temperature on Probe Attachment

As expected increasing the reaction temperature 15 °C to 40 $\pm$ 1 °C speeds the reaction. Table VIII details the results of increasing the reaction temperature with two different CPG's. A one hour room temperature reaction produces a result that is only 85% of that obtained when the reaction temperature is 40 °C. The results of an overnight (19 hr) reaction is still less than that of a one hour heated reaction. Table VIII shows for the heated reaction, attachment of probe increases steadily with time, however,

TABLE VII

EFFECT OF ACETIC ACID CONCENTRATION
ON PROBE ATTACHMENT

ACETIC ACID CONCENTRATION (5.7x)	μmoles OF DACA ATTACHED	
	1 h REACTION	4.5 h REACTION
10^{-2}	29.5 ± 0.8	47.4 ± 1.1
10^{-4}	39.0 ± 0.2	48.4 ± 0.3
10^{-6}	40.5 ± 0.4	49.8 ± 0.8
10^{-8}	39.3 ± 0.7	46.3 ± 2.2
0	39.1 ± 0.6	48.1 ± 0.6

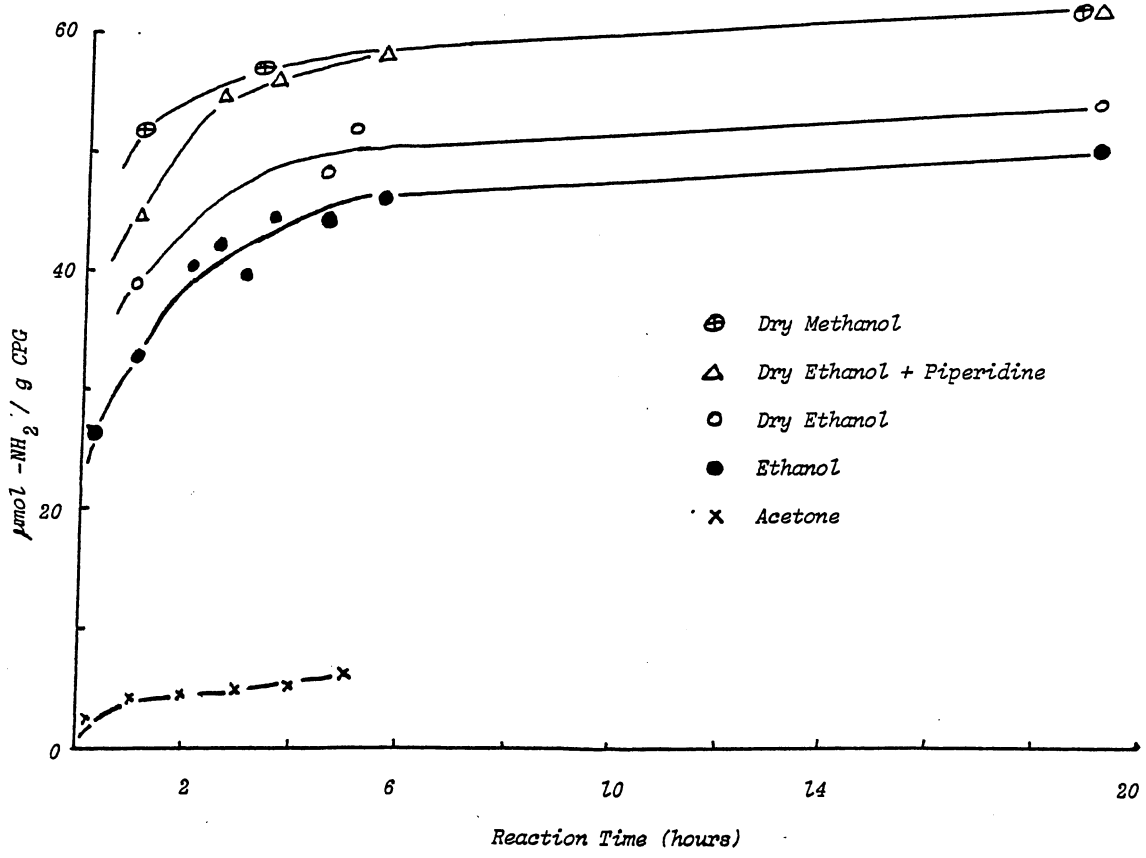
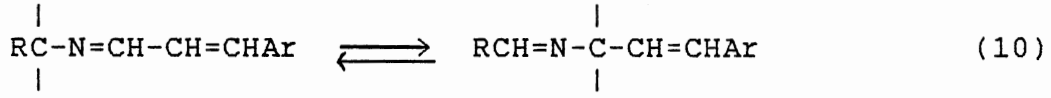


Figure 24. Reaction vs Time Profiles for Attaching DACA in Various Solvents

doubling the reaction time form 2 hrs to 4 hrs did not produce a significant change in the results.

Detachment of the Chromophoric Probe

The conjugated system of double bonds present in DACA is energetically unfavorable to tautomerization (reaction 10), this insures recovery of the original amine and aldehyde when the probe is detached by amine bond rupture.



(10)

The hydrolysis of the imine is spontaneous and rapid in the presence of water (60,112,114). To drive this reaction to completion in a reasonable length of time, however, the temperature had to be elevated to 40.0 °C and efficient mixing was required.

Effect of Time and Temperature on Detachment of Probe

The detachment step was performed immediately after attachment was completed since a decrease in the amount of probe recovered was observed after extended storage time of DACA-reacted-CPG. Over a period of ten days, for instance, the amount of DACA detached from portions of the same DACA-reacted-CPG decreased from 176 $\pm$ 6 to 142 $\pm$ 3 µmol/g.

A closed loop flow system was used to follow the detachment of the probe from reacted CPG. Plots of the

TABLE VIII

EFFECT OF REACITON TEMPERATURE
ON PROBE ATTACHMENT

Reaction time (h)	Sample CPG B	CPG D
(ROOM TEMPERATURE)		
1.0	58.4 ± 1.7	123 ± 2
19.0	63.9 ± 1.0	
(40 ± 1 °C Reactions)		
1.0	68.3 ± 2.6	151. ± 5
2.0	72.6 ± 0.8	154 ± 6
4.0	73.6 ± 0.8	158 ± 2

Attachment in 1.0 x 10^{-3}M piperioine/ethanol.
detachment in 95% ethanol, 40.0 °C, 1 h.

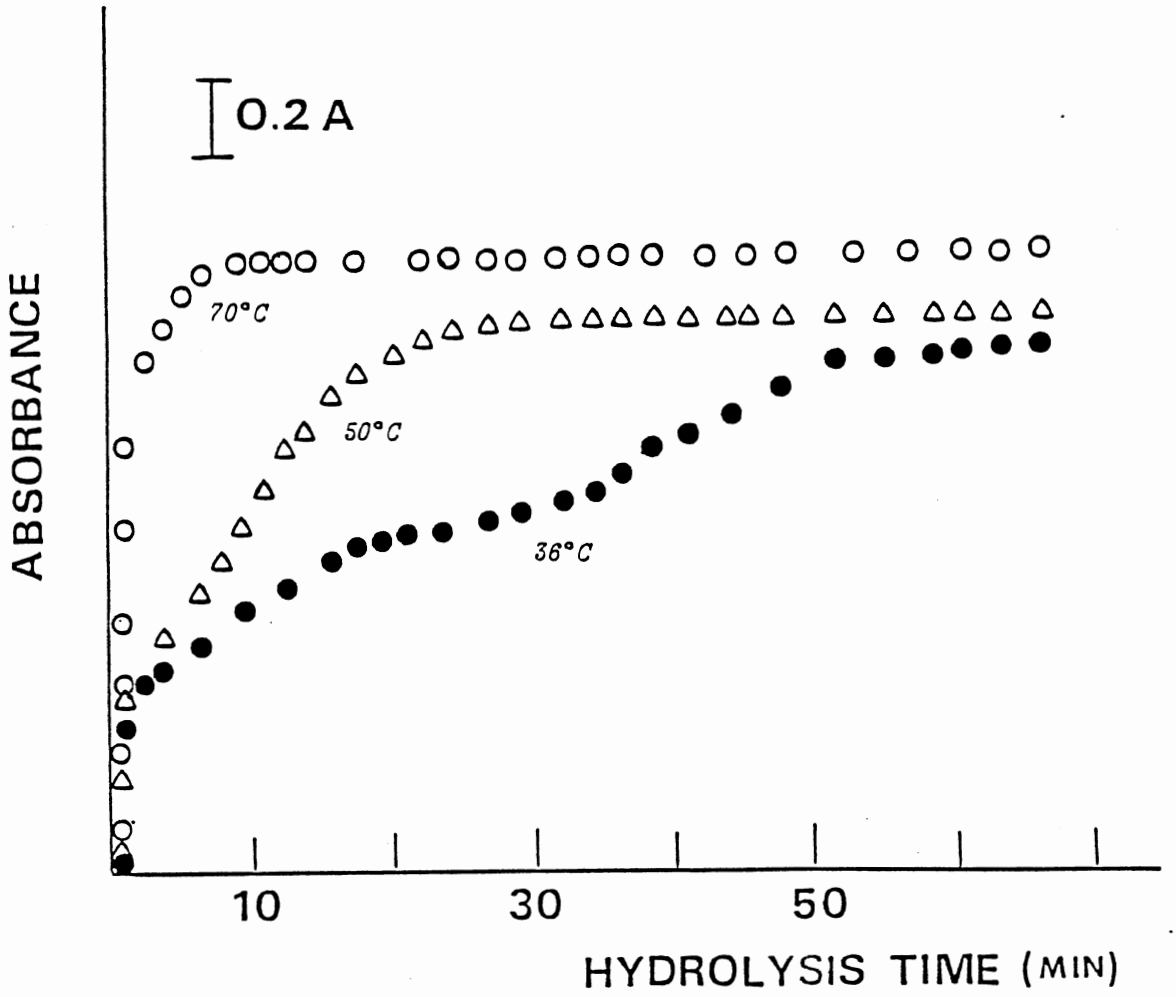


Figure 25. DACA Detachment vs Time Profiles for Different Hydrolysis Temperatures

absorbance of DACA versus time in Figure 25 show that as the temperature of hydrolysis is increased, the time needed to reach the reaction equilibrium (detachment vs time plateau) decreases. Less than 10 min is required for complete detachment at 70 °C, 30 min at 50 °C, and about 60 min at 36 °C. The same amount of probe (μmole/g) was detached at elevated temperatures in the range 36-70 °C. Room temperature detachment produced about 90% of the plateau value after several hours.

Effect of pH on Detachment of Probe

Figure 26 shows the detachment of the probe as a function of pH of the hydrolysis medium. Aminopropyl CPG and DACA were allowed to react for 5 h in anhydrous ethanol; the modified CPG was then exposed to the hydrolysis medium for 30 min at 70.0 °C. The wide plateau observed in Figure 26 indicated that temperature plays a more important role than the hydrogen ion concentration (at pH 4-7) in the detachment process. The recommended conditions for detachment of probe are 60 min at 40.0 °C in 95% ethanol. Detachment in aqueous buffers proved to create destructive conditions (e.g. irreproducible results and loss of amino groups in repeated determinations on one portion of CPG). For a one-time determination where sample destruction is unimportant, aqueous hydrolysis works well. Table IX illustrates good agreement among determinations made on four different samples using aqueous hydrolysis.

Blank Value and Limit of Detection

Un-aminated CPG showed no visual evidence of adsorption of the probe when exposed to DACA under reaction conditions. No peak at 390 nm was observed in the spectrum of the hydrolysis medium when un-aminated CPG was used. The average of ten blank determinations was 0.079 $\pm$ 0.014 μmol/g; thus a limit of detection (for the typical amount of CPG used) of 0.12 μmol/g was calculated as the average of the blank measurements plus three times the corresponding standard deviation.

Reproducibility and Repetitivity

The resproducibility of this general procedure is illustrated in Tables IX - XI. The results in Table IX show that four different portions of a sample can be subjected to identical attachment/detachment procedures with good success. The hydrolysis step was in aqueous buffers and Table XII emphasises the point that aqueous hydrolysis at elevated temperatures is destructive in successive determinations. Table X repeats Table IX, but uses the nondestructive procedure. Again, four different portions of CPG were used. Table XI uses on portion of CPG and repeats the nondestructive procedure four times.

Four different portions of the same CPG sample were separately subjected to the identical (attachment and detachment) procedures. The amount of reactive amines

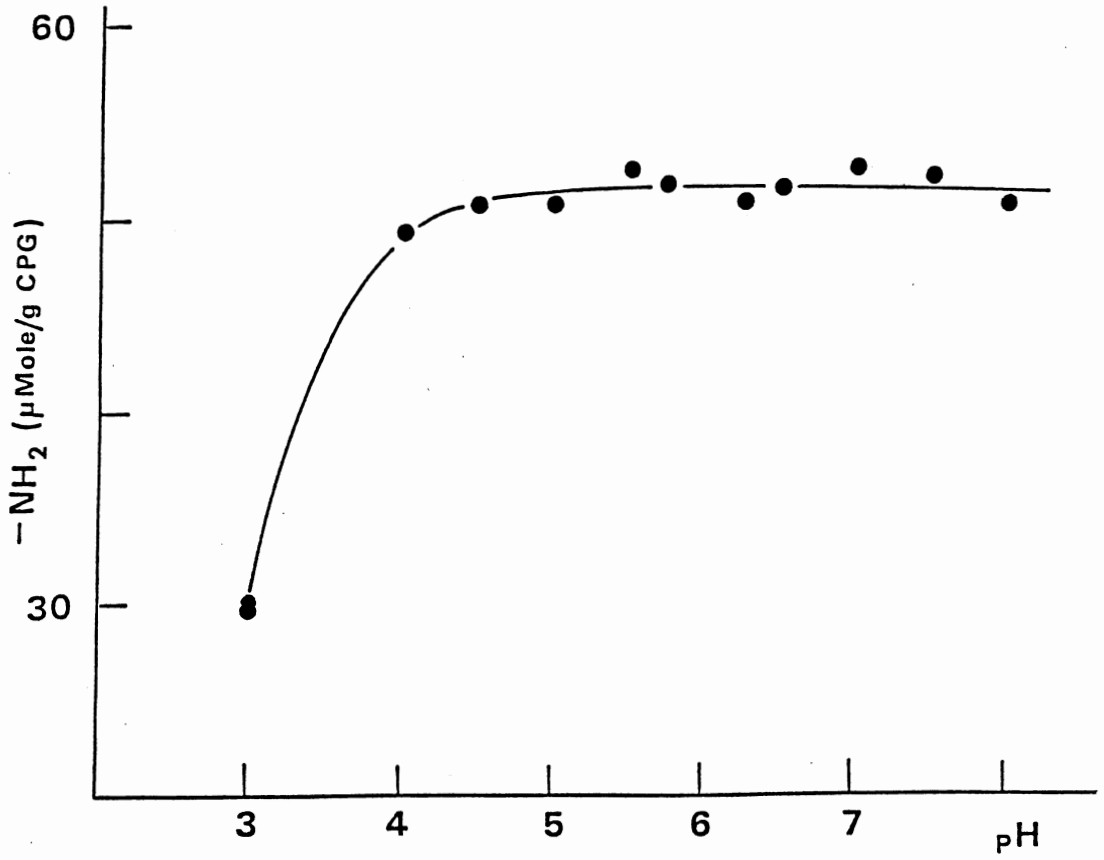


Figure 26. DACA Recovered vs pH of the Hydrolysis Buffer

TABLE IX

RESULTS OF DESTRUCTIVE DETERMINATION OF AMINO GROUPS ON FOUR DIFFERENT PORTIONS OF THE SAME CPG

Attachment: 5 h in anhydrous ethanol.

Detachment: 30 min in pH 7.50 buffer, 70 °C

Portion	μmol NH_2/g CPG
1	52.2 ± 2.7
2	52.1 ± 0.6
3	51.5 ± 0.5
4	51.9 ± 0.6
overall	51.9 ± 0.3 (0.6%)

determined (Table X) illustrates that this general methodology ("On-off" chemistry) has acceptable uncertainty (>1%). Table IX and X suggest reaction conditions influence the results and must be selected carefully. Studies of repetitive determinations on the same portion of a CPG sample were performed under conditions that were found to be nondestructive (Table XI). Silica frameworks have a great propensity to hold water. The uncertainty of successive determinations is somewhat higher due to the difficulty of quantitatively removing water from silica by solvent rinsing, reproducing the same surface chemical environment for the subsequent anhydrous reaction.

The nonrepetitive nature of detachment using aqueous buffers is detailed in Table XII. Sequential determinations made on the same portion of aminated CPG show continually decreasing amounts of amino groups available to the probe. The apparent loss of reactive amino groups is more pronounced at higher temperatures. Detachment in alkaline aqueous buffers and elevated temperatures favor the hydrolytic scission of the siloxane bond, detaching the organosilane from the glass backbone (115). In the essentially organic matrix of 95% ethanol and under milder conditions (40.0 °C), the imine is preferentially cleaved.

The enhanced attachment of probe in heated reaction media has been discussed. Repetitive determinations using heated attachment reactions on the same portion of CPG failed to prove nondestructive (Table XIII). When the

TABLE X

RESULTS OF NONDESTRUCTIVE DETERMINATION OF AMINO GROUPS ON FOUR DIFFERENT PORTIONS OF THE SAME CPG

Attachment: 1 h in ethanol/piperidine medium.

Detachment: 1 h, 40 °C, 95% ethanol.

Portion	μmol NH_2/g CPG
1	63.7 ± 0.9
2	65.5 ± 2.7
3	63.2 ± 0.8
4	64.1 ± 0.4
	over all 64.1 ± 1.0 (1.6%)

TABLE XI

RESULTS OF SUCCESSIVE DETERMINATIONS OF AMINO GROUPS ON THE SAME PORTION OF CPG.

(Attachment: 1h in ethanol/piperidine medium. Detachment: 1h 40 °C, 95% ethanol.)

DETERMINATION	μmol NH_2/g CPG
1st	54.2 ± 2.1
2nd	55.4 ± 1.5
3rd	59.3 ± 1.9
4th	53.5 ± 0.7
	overall 55.6 ± 2.6 (4.7%)

Average for each determination is the result of 3 replicates.

TABLE XII

RESULT OF SUCCESSIVE DETERMINATIONS OF AMINO GROUPS ON ONE PORTION OF CPG USING AQUEOUS HYDROLYSIS MEDIUM

	μMOL NH_2/G CPG	
Determination	70.0 °C Detachment	40.0 °C Detachment
1st	52.1 ± 0.6	51.9 ± 0.6
2nd	24.6 ± 0.1	35.2 ± 2.2
3rd	19.5 ± 0.7	28.5 ± 2.8
4th	8.28 ± 0.50	

method was optimized to enhance the recovery of probe from reacted CPG, conditions used were destructive. This points to a potential source of failure if simplex optimization were attempted. The overall goal to achieve a repetitive method should have been present in the response parameters and/or the optimization experimental design.

Comparison with Results Obtained by Nonaqueous Titration

Perchloric acid titration of amines in glacial acetic acid is well documented in the literature (105,106) and has been applied for quantitative information on amino groups immobilized on CPG (116). Table XIV compares results obtained by nonaqueous titration, an optimized form of DACA attachment/detachment (which, unfortunately, is destructive to the amino groups being determined), and the DACA attachment/detachment procedure that is nondestructive. The variation in results observed points to differences in relative accessibility of surface amino groups on different CPG samples.

A trend can be seen between reported surface area and the results obtained when DACA is used as a probe. The number of reactive amino groups increases with increase in surface area and doubling the surface area roughly doubles the amount of amino groups determined. The fact that the acid-base titration in glacial acetic acid yields higher results hints at a dependence on probe size. Uneven surface

TABLE XIII

RESULTS OF SUCCESSIVE DETERMINATIONS OF ONE PORTION OF CPG USING THE HEATED ATTACHMENT REACTION.

Determination	μmol -NH_2/g CPG
1st	77.3 ± 1.5
2nd	71.0 ± 1.6
3rd	56.2 ± 4.5

CPG A, attachment @ 40 °C in piperidine/ethanol, 4h.
Detachment 1 h, 95% ethanol at 40.0 °C.

TABLE XIV

COMPARISON OF AMINO GROUPS CONTENT DETERMINATION BY "ON/OFF" METHODS AND NONAQUEOUS TITRATIONS

	CPG sample			Nonaqueous Titration *(a)*	Optimized On-Off Method *(b)*	Repetitive On-Off method *(c)*
	Particle Size (mesh)	Pore Size Å	Surface Area m^2/g	µmol/g	µmol/g	µmol/g
A)	80/120	1273	24	91.3 ± 1.9	76.5 ±2.2	65.2 ± 0.7
B)	200/400	1273	24	70.4 ± 0.9	72.0 ± 1.3	58.7 ± 1.4
C)	80/120	530	43	268 ± 5	123 ± 2	89.9 ± 4.2
D)	80/120	547	44.5	298 ± 4	159 ± 4	123 ± 2
E)	80/120	156	91	314 ± 3	289 ± 3	266 ± 4

(a) - Nonaqueous titrations: results reported as the average of triplicate perchloric acid titrations as described in text.

(b) - Optimized On-Off method: attachment 4 h, 40 ± 1 °C in piperidine/ethanol media; detachment 1 h, 40.0 °C, 95% ethanol.

(c) - Repetitive On-Off method: attachment 1 h, 25 °C in piperidine/ethanol media; detachment 1 h, 40.0 °C, 95% ethanol.

distribution of amino groups can produce areas with clusters and areas with relatively wide separation between amino groups. The proximity of adjacent such groups may hinder the molecules of DACA from binding all amino groups on the surface. By this logic, the results form nonaqueous titration will always be equal to or larger than those obtained with larger probes. This observation is relevant to the availability of reactive amino groups for glutaraldehyde attachment since glutaraldehyde is not a small entity. The DACA aldehyde group, which mimics the reactive ends of glutaraldehyde, makes a better probe to map reactive amino groups since it has only one way to bond. the presence of a double bond and the benzene ring gives DACA a stiffness which insures the absence of intramolecular interactions (hydrogen bonding, cyclization, etc.).

The ratio of the results obtained using a larger probe, DACA, to the results obtained from anhydrous titration can be used as a measure of accessibility of immobilized amino groups to this particular probe. Table XIV shows that CPG B and CPG E appear to have very accessible surface amino groups. The variation in accessible or reactive groups with different samples of CPG can be illustrated by comparing two samples of CPG from the same manufacturing lot; CPG B and CPG A have the same surface area (24 m^2/g) and pore size (1300 Å), but CPG B has smaller particles and a greater percentage of the amines are reactive to DACA under the conditions used. The larger particles of CPG A may have a

number of narrow recesses, bottlenecks, or tortuous paths where amino groups are not easily reached by this probe. A pattern by which to generalize aminated CPG characteristics has not emerged because of the heterogeneous nature and the lot-to-lot variation of this material. For instance, CPG E, which has approximately 1/10 the pore size of CPG A, has a larger percentage (DACA results/titration results) of its amino groups available to react with the DACA probe. CPG C and CPG D, which have similar surface area (m^2/g) and pore size, give results with DACA probe that show less than 50% of the amino groups that were determined by titration.

These observations underscore the fact that the amount of amino groups immobilized on CPG surfaces may not be the amount available for subsequent chemical reactions. The microscopic physical characteristics of CPG as well as the surface distribution of immobilized groups may preclude a particular probe. The measurement of total amino groups by spectroscopic methods may no take into account these physical and/or chemical factors. The measurement of reactive groups by "on/off" chemistry, however, is dependent on amino group accessibility, probe size, type of bonding, contact time, and reaction conditions.

Duplicate reactions of aminosilanes with CPG (under vacuum to degas pores) may deposit amines in locales that can be reached only by the smallest chemical probes, allowed ample time to diffuse. The repetitive results in Table IX may be a more accurate assessment of amine

availability. These quantities should be viewed as the reactive amount for a one hour room temperature reaction in anhydrous solvent. Reactions in anhydrous alcohols proceed to a greater degree of completion than those run in aqueous solvents (Table V). Thus since glutaraldehyde coupling reactions are normally conducted in aqueous buffers, those coupling reactions may not make efficient use of the available amines on the CPG surface. Studies have shown that the amounts of immobilized aldehyde groups on CPG surfaces is about 3 μmol/g (90,91) which is similar to the amount of amine groups found by the DACA probe in aqueous reactions (Table Vb).

Comparative studies need only to yield reproducible relative results to permit conclusions about the chemistry being studied. The method described here for the batch determination of immobilized amino groups on CPG articles is nondestructive, repeatable, and readily adaptable to determinations within actual reactors to be used in flow systems (10,90,91).

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