CONTRIBUTIONS OF IMMOBILIZED PENICILLINASE TO THE ANALYTICAL CHEMISTRY OF PENICILLINS

By

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CONTRIBUTIONS OF IMMOBILIZED PENICILLINASE TO

THE ANALYTICAL CHEMISTRY

OF PENICILLINS

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PREFACE

This thesis is divided into six chapters for clarity and comprehension. Chapter I introduces the subject of the study, while Chapter II is a literature review of the analytical chemistry of penicillins. Chapter III describes the methods and procedures that are common to the entire study. Other methods and procedures are covered in the respective chapters. Chapters IV, V, and VI describe the immobilization, characterization and design of reactors, and the use of the best reactor configuration in a continuous-flow system for the determination of penicillins in real samples. Chapter VII draws some conclusions from this study.

I am greatly indebted to my adviser, Dr. H. A. Mottola, for his help and guidance with this study, and throughout my graduate work at Oklahoma State University. He was not only an excellent adviser, but also a good friend and counselor.

I would like to express my appreciation to the other members of my advisory committee, Dr. L. P. Varga, Dr. E. C. Nelson, Dr. L. B. McGown, and Dr. T. E. Moore.

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Thanks are also extended to my friends and colleagues, namely, Al Goodwin, Albahadily, Dennis, Monte, Ricky, Sudha, Matt, El-Gendy, Kazi, Iob, Tonia, Ramasamy, Young-ok, and Dr. Yamane, for their friendship and encouragement, and for providing a cheerful atmosphere in the laboratory.

My heartfelt gratitude is extended to my dear friend, Marissa, without her help, guidance, and friendship, I would not have accomplished this much.

Appreciation is also expressed to Mr. V. A. Ponnusamy and Dr. V. A. Palanisamy, for their help and encouragement in making my study at Oklahoma State University possible.

Finally, I wish to thank my father, Ramaswamy, and my mother, Kokilam, for their inspiration, understanding, and sacrifice that has enabled me to achieve this goal.

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CHAPTER I

INTRODUCTION

1.1 History of Penicillin

Penicillin was identified by Sir Alexander Fleming in 1929 from a mold, Penicillium (1). Florey and Chain of Oxford University successfully demonstrated the value of penicillin in treating a variety of otherwise intractable diseases. The Noble Prize was awarded in 1945 to Fleming, Florey, and Chain for the discovery of penicillin and its curative effect in various infectious diseases.

During the Second World War, a full scale cooperation was established between the United States and Great Britain to produce penicillin on a large scale (2). The Manhattan Project, leading to the development of the atomic bomb, only equalled the efforts of the Office of Scientific Research and Development during World War II to produce a synthetic penicillin (3). This goal was accomplished, however, only long after the end of the war. Initially, the yield of penicillin from penicillium in fermentation tanks was very low. The discovery of corn steep liquor as an excellent nutrient for penicillium made corn steep liquor an essential war time product (4). The magic ingredient that increased the yield several folds was then found to be phenyl acetic acid (4). Several penicillins, with differing side chains, were then prepared by adding certain compounds like phenyl acetic acid to the fermentation broth.

This formed the basis for the precursor method of penicillin synthesis.

In the late 1950's scientists from Beecham Laboratories in England developed two ways of isolating 6-amino penicillanic acid (6-APA). One method was to deprive penicillium of essential precursors and the other by removing the side chain from penicillin enzymatically using penicillin amidase. This 6-APA was then coupled with different acetyl chlorides to give a large number of semisynthetic penicillins. The total chemical synthesis of penicillin was completed by Professor John C. Sheehan of Massachusetts Institute of Technology in 1958 (5). Since then, numerous penicillins have been produced.

The purpose of synthesizing new penicillins was directed at solving three problems (6). The first problem was concerned with stabilizing the molecules against the action of staphylococcal penicillinases. Methicillin, oxacillin, and nafcillin were the successful products of these research efforts. The second problem was to expand the spectrum of the penicillin to include gram negative bacteria. Derivatives such as ampicillin and carbenicillin resulted from these efforts. The third problem, chemical stability and enhanced absorption, was solved by the development of acid stable and orally absorbable penicillin derivatives like penicillin-V and oral ampicillin.

1.2 Chemistry of Penicillins

The penicillin molecule contains a thiazolidine ring, carrying a carboxyl and two methyl groups, fused to a β-lactam ring carrying a long side chain as shown in Figure 1. The various penicillins differ only in the nature of the R_1 and R_2 groups. Some of the common penicillins are shown in Table I.

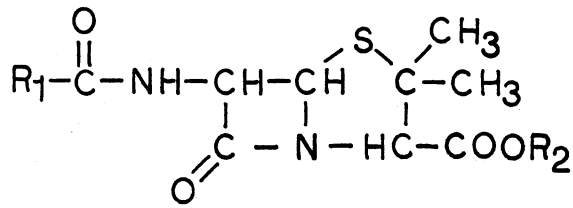
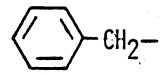
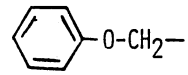
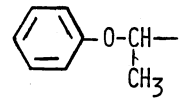
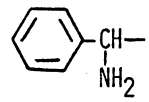
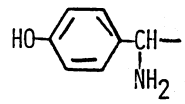
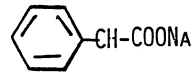
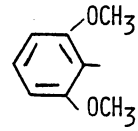
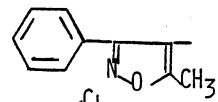
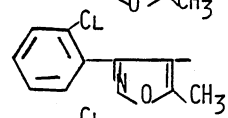
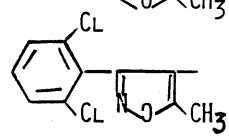
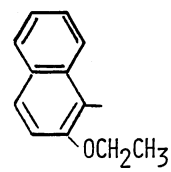


Figure 1. General Structure of a Penicillin

Some of the important reactions of penicillins are summarized in Figure 2 (7). Alkalies and the enzyme penicillinase attack the β-lactam ring of the penicillins to give a penicilloic acid. This reaction has been used in enzymatic methods of determination of penicillins. Various colorimetric and polarographic methods of determination are based on the indirect determination of the penicilloic acid. Penicilloic acid on treatment with $HgCl_2$ gives penamaldic acid, which decomposes to give a penicillamine and a penaldic acid. Penaldic acid further decarboxylates to give a penilloaldehyde. Penicilloic acid on heating or treatment with acid decarboxylates yielding a penilloic acid. In acidic solution, penicillins undergo an intramolecular rearrangement to yield penicillenic acid which is unstable and decomposes rapidly to form penillic and penicilloic acids. The common degradation products of penicillin such as penicilloic acid, penamaldic acid, and penicillamine, and penicillenic acid are electroactive. This property has been made use of in the polarographic determination of penicillins by first subjecting the penicillins to acidic or basic degradations.

TABLE I

STRUCTURES OF COMMON PENICILLINS

	R_1	NAME	R_2
1.		PENICILLIN G	K, NA, BENZATHINE, PROCAINE
2.		PENICILLIN V	H, K, BENZATHINE
3.		PHENETHICILLIN	K
4.		AMPICILLIN	H, NA
5.		AMOXYCILLIN	H
6.		CARBENICILLIN	NA
7.		METHICILLIN	NA
8.		OXACILLIN	NA
9.		CLOXACILLIN	NA
10.		DICLOXACILLIN	NA
11.		NAFICILLIN	NA

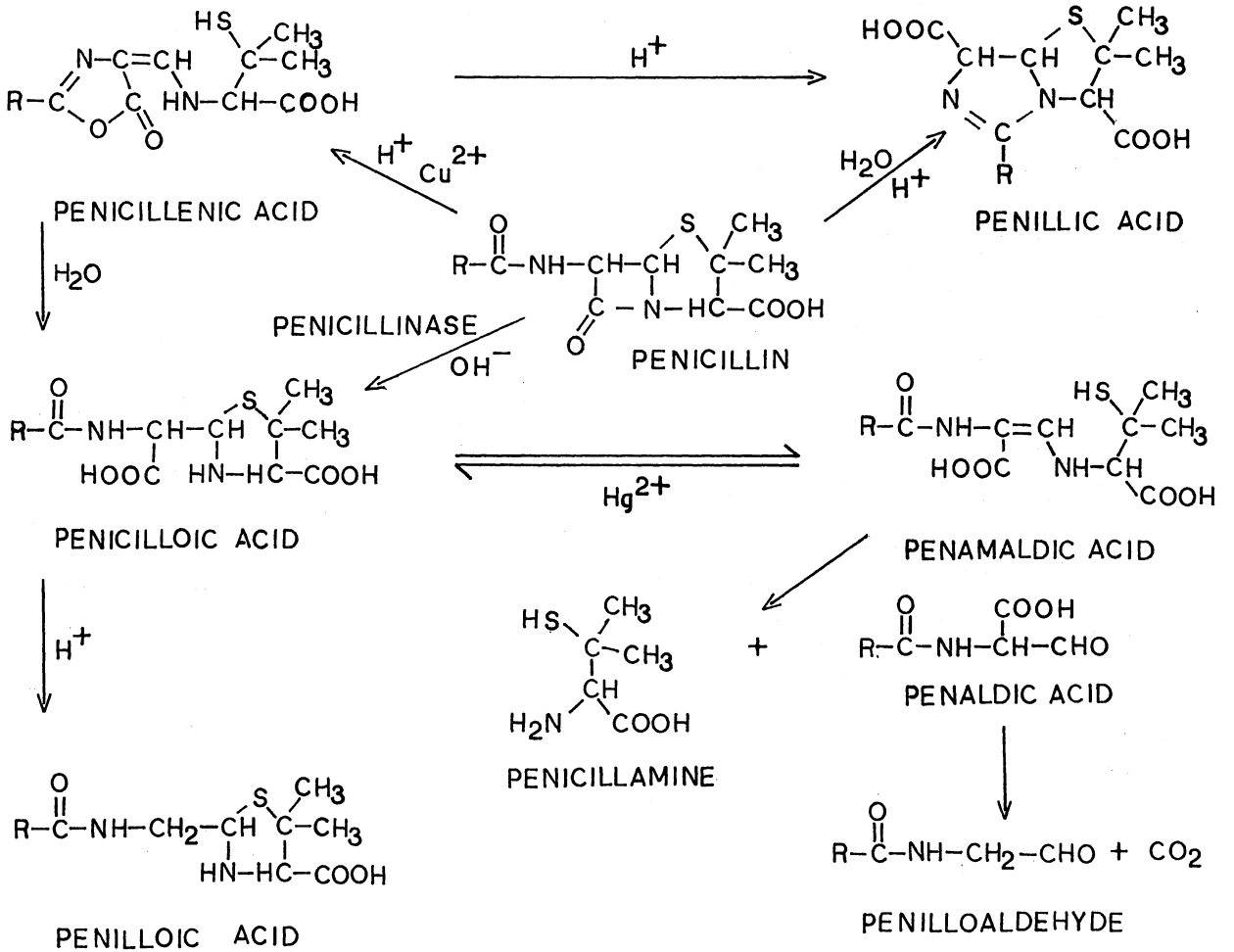


Figure 2. Common Degradation Reactions of Penicillins

Penicillins are oxidized by oxidizing agents like iodine, hypobromous acid, dibromohydantoin, and iodine monochloride to give penicillamine and penilloaldehyde as two of the oxidation products. This property has been exploited in the titrimetric methods of determination.

1.3 Automation Using Continuous-Flow

Pharmaceutical industries and clinical laboratories determine penicillins in a large number of similar samples everyday. A large sample throughput, in such laboratories, can be achieved with the help of automation. The first automation in wet chemistry, using continuous flow, was introduced by Leonard T. Skeggs in the 1950's (8), for which he was given an award in the 1982 Pittsburgh Conference. The idea of Skeggs was commercialized by the Technicon Company with their introduction of the Auto Analyzer.

In the Auto Analyzer, processes such as mixing of reagents, filtration, heating, and measurement of a property like absorbance are performed automatically and continuously. Skeggs segmented the sample solution with air bubbles, hence, the method was called segmented continuous-flow analysis. Although automation was achieved with the Auto Analyzer, the sampling rate was still very low, about 15 samples per hour.

Ruzicka and Hansen (9) introduced a simple unsegmented continuous-flow system also called Flow Injection Analysis (FIA). They showed that expensive instrumentation was not needed for automation. An inexpensive continuous-flow system could be assembled in any small laboratory, achieving sampling rates greater than 100 samples per hour. Sampling rates of 700 samples per hour have been reported (10).

1.4 Use of Enzymes in Analytical Chemistry

The use of enzymes as analytical reagents has been gaining popularity because of the high selectivity of enzymes. Enzymes are biochemical catalysts that occur "in vivo" in living systems. Enzyme reaction rates corresponding to the turnover of 100 to 10,000 substrate molecules per molecule of enzyme per second may be regarded as typical. Due to the high selectivity of enzymes, interferences from many constituents of complex samples are avoided. As a result, lengthy separations and sample preparations are unnecessary in enzymatic analyses. However, the high cost of most of the enzymes had limited the use of enzymes as analytical reagents until the development of immobilization technology.

Immobilization refers to the localization or confinement of enzymes during a process that allows them to be physically separated from the substrate and products for re-use. The tremendous surge of interest in immobilized enzymes resulted from the many advantages that immobilized enzymes exhibit in comparison with soluble ones (11, 12, 13). Some of these advantages that are of interest in analytical chemistry are:

1. Expensive enzymes like penicillinase when immobilized can be re-used several times before the activity decreases.

2. When an enzyme is immobilized, the products of the enzymatic reaction are free of enzymes and hence, separation of enzyme is unnecessary.

As shown in Figure 2, the enzyme penicillinase catalyzes the hydrolysis of penicillin to penicilloic acid. Thus, penicillinase can be used for the determination of penicillins by potentiometrically measuring the amount of acid released. The use of immobilized penicillinase in a continuous-flow system would combine the selectivity of

penicillinase with the speed of continuous-flow analysis. This would enable the determination of penicillins in complex media like fermentation broths and pharmaceutical preparations of penicillins.

Penicillinase electrodes have been constructed by the use of physical immobilization techniques (14, 15, 16). These electrodes cannot be used in continuous-flow systems. The lifetime of the immobilized enzyme is only a few weeks. The reaction and detection steps in an enzyme electrode cannot be controlled and optimized independently.

Penicillinase has been immobilized on controlled-pore glass and used in a packed reactor configuration in continuous-flow system (17). This system suffered from serious back pressure problems due to the use of a packed column. The sampling rate was also very low, 15 samples per hour, not typical of a continuous-flow system.

The purposes of this study were:

1. to develop better immobilization methods to increase the lifetime of the immobilized penicillinase;

2. to use improved reactor designs, with minimum back pressure associated with it;

3. to make use of the new reactor design in a continuous-flow system for the determination of penicillins in real samples like penicillin broths; and

4. to design a new flow-through pH detector for use in the continuous-flow system.

CHAPTER II

A LITERATURE REVIEW OF THE ANALYTICAL CHEMISTRY OF PENICILLINS

The determination of penicillins is necessary for several reasons: a) monitoring the production of penicillins in fermentation tanks; b) quality control of various pharmaceutical preparations; c) studying the pharmacologic efficiency of penicillins in biological fluids; and d) detecting trace levels of penicillin residues in dairy products. The presence of antibiotics in milk causes failure of culture organisms in the production of cheese. Allergic reactions and loss of sensitivity of the general population to antibiotics are some of the other problems associated with the consumption of dairy products with antibiotic residues. Thus, the Food and Drug Administration sets limits on the penicillin content of milk.

The problems usually encountered in the determination of penicillins in various samples can be summarized as follows:

1. Interference from additives: Penicillin tablets contain several additives like gelatin, starch, talc, ascorbic acid, and sodium bicarbonate which would interfere in some of the chemical and physical methods of determination.

2. Interference from proteins: Biological fluids (blood serum and urine), fermentation broths, and milk contain a variety of proteins that could interfere in the chemical and physical methods of

determination. If spectrophotometric detection is used, the proteins tend to coat the cuvette walls.

3. Detection limits: In addition to the problem of proteinaceous materials in biological fluids, penicillins are present in such low levels (ng levels) that most of the chemical, physical, and enzymatic methods are not able to detect them.

4. Time factor: In the case of biological fluids, results are often needed immediately (within a few hours), so that further treatments could be given to the patients. This is the case with milk, where on the spot decisions have to be made to see if a certain batch of milk is suitable for human consumption. In this type of situation, procedures requiring elaborate and lengthy determinations are not suitable.

Numerous methods can be found in the literature for the determination of penicillins and they have been reviewed (18, 19, 20). None of the reviews cover enzymatic methods using immobilized enzymes nor do they include biological methods of determination.

To cover all the work done to date would be beyond the scope of this review and has been avoided. This review is concerned with some of the important chemical, physical, and biological methods of determinations published during the past twenty years.

2.1 Titrimetric Methods

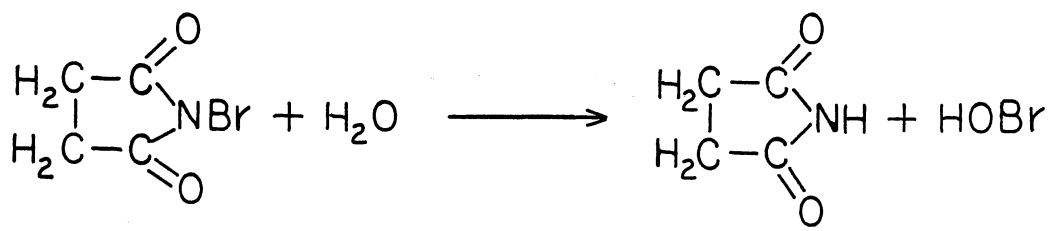
Most of the titrimetric methods are based on the titration of a known weight of sample with a standard solution of an oxidizing agent. The end point is usually signalled by starch in the case of iodometric titrations and by the use of redox indicators in other redox reactions.

2.1.(a) Iodometric Method

Penicillin is inert to iodine in neutral aqueous solutions while penicilloic acid consumes about six to nine equivalents of iodine depending on the reaction conditions (21). This property was used by Alicino (21) to determine penicillins indirectly. He inactivated the penicillins with alkali or enzyme (penicillinase) to penicilloic acid, which was then treated with a known excess of iodine solution. The unreacted iodine was back titrated with standard thiosulfate using starch as the indicator. A blank was run by treating the intact penicillins in a similar manner. The mechanism of the degradation of penicilloic acid by iodine is unknown, but some of the degradation products of phenoxy methyl penicilloic acid have been identified to be phenoxy acetic acid, phenoxy acetamide, glyoxal, carbon dioxide, and ammonia (22).

2.1.(b) N-Bromosuccinimide Method

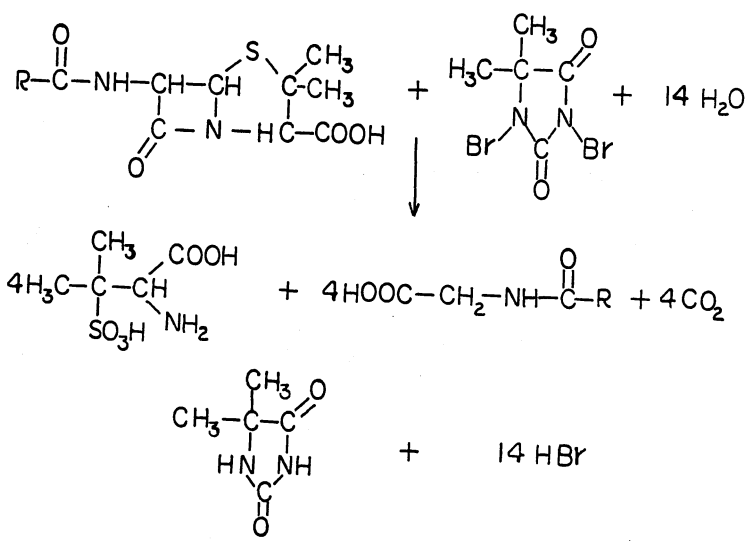
Alicino (23) also proposed a determination based on the use of N-Bromosuccinimide as an oxidizing agent. This method required no prior inactivation of the penicillins with alkali or enzyme. Penicillins were treated with an excess of N-Bromosuccinimide (NBS). NBS on hydrolysis yielded hypobromous acid.



Hypobromous acid formed in the first step attacks the ring system of penicillins to give various degradation products, which have not been identified. The excess HOBr is determined by the addition of solid KI and titrating the liberated iodine with standard thiosulfate solution. This method could also be used for the determination of penicillamine and penicillanic acid. Therefore, this method is not suitable for the determination of penicillins in the presence of these compounds as impurities.

2.1.(c) Dibromohydantoin Method

Dibromohydantoin (DBH) (1,3-Dibromo, 5,5-dimethyl hydantoin) (24) is an oxidizing agent which could oxidize penicillamine and penilloaldehyde to the corresponding sulfonic and carboxylic acids respectively. This method is based on the degradation of penicillins in strong acid medium into penicillamine and penilloaldehyde, followed by oxidation of these compounds with DBH. The overall reaction can be written as follows.



The equivalence point was detected potentiometrically or with the use of methyl red as indicator. A spectrophotometric titration was also performed which, the authors claimed without justification, increased the sensitivity of the method. Six penicillins, penicillamine, and 6-aminopenicillanic acid (6-APA) could be determined by this method. Therefore, penicillins cannot be determined accurately in the presence of these impurities.

2.1.(d) Potassium Iodate Method

Potassium iodate is a powerful oxidizing agent, but the course of the reaction is governed by the conditions under which it is employed (25). The reaction between potassium iodate and reducing agents such as iodide ion in solutions of moderate acidity (0.1-2.0 M HCl) stop at the stage when the iodate is reduced to iodine.

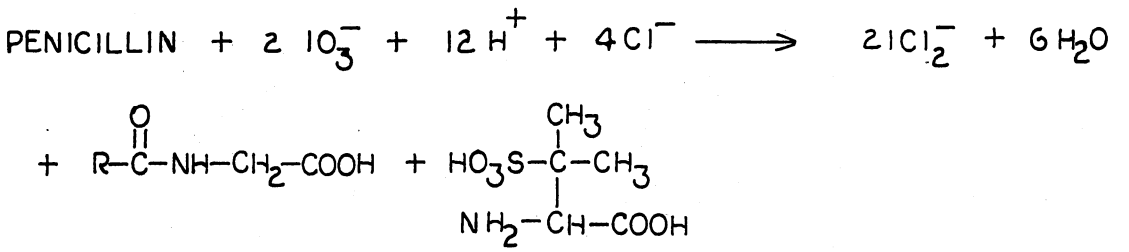
$$IO_3^- + 5I^- + 6H^+ \longrightarrow 3I_2 + 3H_2O$$

In stronger acidic solutions (3-6 M HCl), reduction occurs to iodine monochloride, which forms a stable complex with chloride ion. The overall reaction maybe written as follows:

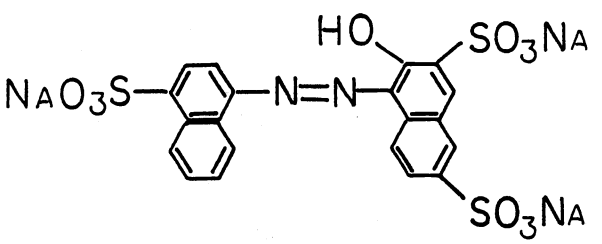
$$IO_3^- + 6H^+ + 2Cl^- + 4e \longrightarrow ICl_2^- + 3H_2O$$

Ibrahim (26) oxidized the penicillins with iodine monochloride in highly acidic medium and titrated the liberated iodine with potassium iodate until the disapperance of the iodine color from the chloroform layer. The experimentally observed stoichiometry of one mole of penicillin to two moles of iodate was stated to be consistent with the formation of the appropriate sulfoxide derivative of the penicillin (27).

However, Grime (28) later proved that iodate not only oxidizes the iodine produced by the reduction of iodine monochloride (in the non-aqueous layer) but further oxidized the sulfoxide produced in this reaction to a sulfonic acid. The overall reaction is given below.

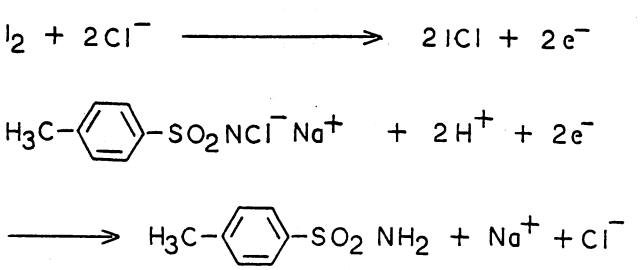


Grime has also titrated the penicillins in an entirely aqueous medium using a dye, amaranth, as indicator. The red color of the indicator was decolorized by the first excess of iodate.



Amaranth

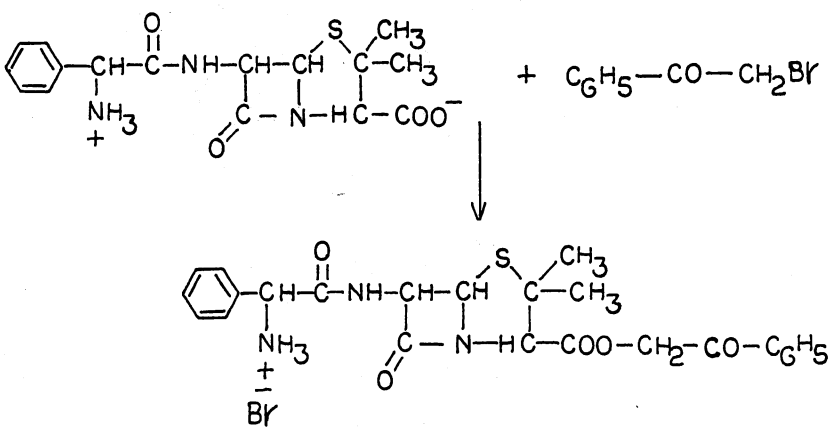
Nayak (29) modified the method of Grime by titrating the liberated iodine with chloramine-T, (N-chloro p-toluene sulfonamide) to a chloroform extraction end point.



Titration of penicillins in an entirely aqueous medium using amaranth as indicator is superior to other methods using a chloroform extraction end point. The chloroform extraction end point technique, unlike the aqueous phase titration, requires vigorous shaking during titration.

2.1.(e) ω-bromo Acetophenone Method

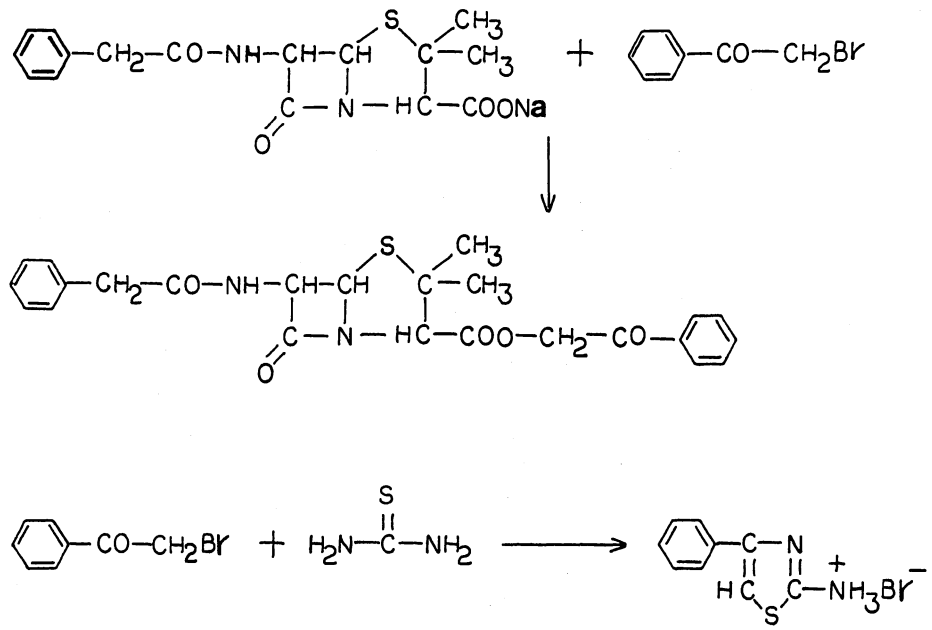
Recently, ampicillins (30) were determined by converting them into phenacyl ampicillinate hydrobromide with ω-bromo acetophenone and titrating the hydrobromide with standard sodium carbonate to a thymol blue end point (green color).



Although this method was applied only to the determination of ampicillin, this method could, in principle, be applied to any penicillin with an NH_2 group in the side chain.

Penicillins without an amino group in the side chain can be determined by treating the penicillins with an excess of ω-bromo acetophenone (31). The unreacted ω-bromo acetophenone can be determined by reaction with thiourea and titrating the 4-phenyl-2-amino thiazole

hydrobromide with alkali to a phenolphthalein end point.



2.1.(f) Desulphurization Method

The thiazole nucleus of penicillin breaks down on treatment with KOH at 250-280°C to yield one mole of sulfide ion (32). In the presence of plumbite ion (prepared by mixing lead nitrate and potassium hydroxide to give $K_2Pb(OH)_6$) the sulfide ion is precipitated as lead sulphide. Penicillins have been determined by desulphurization with excess plumbite, and back titrating the lead with EDTA at a pH of 4.5 with the use of a lead ion selective electrode. This method is applicable to any penicillin. In the case of a mixture it gives the total amount of penicillins present. The main drawback is that this method does not differentiate penicillin degradation products like penicilloic acid from penicillin.

2.1.(g) Titration with Hg(II)

Karlberg (33) used the reaction of penicilloic acid with Hg(II) to determine penicillins. He hydrolyzed the penicillins with enzyme or alkali (Figure 2) followed by titrating the penicilloate with Hg(II) potentiometrically. Penicillin does not react with Hg(II), so a blank run without hydrolyzing the penicillins gives the amount of penicilloate initially present. The main advantage of this method is that it does not need any penicillin standards. The advantages and limitations of the various titrimetric methods are summarized in Table II.

2.2 Colorimetric Methods

The determination of penicillins by colorimetric methods is based on: (a) direct formation of a colored complex by treatment of the penicillins with suitable reagents; (b) degradation of penicillins with acid, alkali, or enzyme followed by treating the resulting degradation products like penicilloic acid, penamaldic acid, penicillenic acid or penicillamine with suitable reagents that would produce a chromophore; or (c) degradation of penicillins with a known excess of oxidizing agents like iodine and determining colorimetrically the unreacted oxidizing agent. Both equilibrium and kinetic measurement approaches have been used in connection with colorimetric procedures.

2.2.(a) Equilibrium Methods

In equilibrium methods, the system is allowed to attain equilibrium before absorbances are read. Often, operations like mixing various reagents, extractions, and measurement of absorbances are done manually.

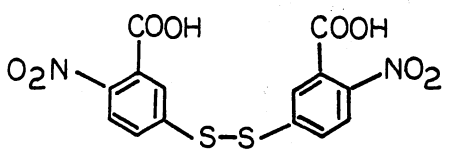
TABLE II

OBSERVATIONS ON THE TITRIMETRIC METHODS

Penicillins Determined (see Table I)	Method of Determination and Reference	Advantages	Limitations	Time Required for Determination
1	Iodometric (21)		Iodine consuming impurities cause low results. Stoichiometry fluctuates between 8 and 9 depending on the reaction conditions.	≃1 hour
1,2,4,8 & 9	N-Bromosuccinimide (23)	Prior inactivation of penicillin unnecessary as in the iodometric method.	NBS is light and air sensitive. Penicillamine and penicillenic acid interfere.	≃ 2 hours
1,4,5,6,9	Dibromohydantoin (24)	Fast	Penicillamine and 6-APA interfere.	0.25 hours
1,2,4	Potassium iodate (26)	Simple	Procaine interferes.	0.50 hours
1,4,9	Potassium iodate (28)	Direct titration applicable to penicillin with unsaturated side chain.	Not applicable to tablets. (They contain oxidizable additives)	0.25 hours
1,2,4,9	Potassium iodate (29)	Simple, fast		0.25 hours
4	ω- bromoacetophenone (30)		Slow	2.25 hours
1	ω- bromoacetophenone (31)	Simple	Temperature has to be maintained below 15°C.	0.75 hours
1,2,4,8	Desulfurization (32)	Simple and fast	Does not differentiate between penicillin and penicilloic acid.	0.25 hours
1	Mercury(II) (33)	Fast; no penicillin standards needed.		0.25 hours

This approach is quite slow and is inconvenient for the routine analysis of a large number of samples. On the other hand, kinetic approaches, as will be seen later, are suitable for the routine determination of a large number of samples.

The Dithionitrobenzoic Acid Method. Penicillins were first degraded into penicillamine by treatment with alkali followed by acid. The determination of penicillamine with DTNB (34) is based on the cleavage of the S-S bond in DTNB by the aliphatic thiol (penicillamine) in weakly alkaline medium to form an intensely yellow colored anion of 5 thio-2-nitrobenzoic acid, which displays a maximum absorption at 412nm.

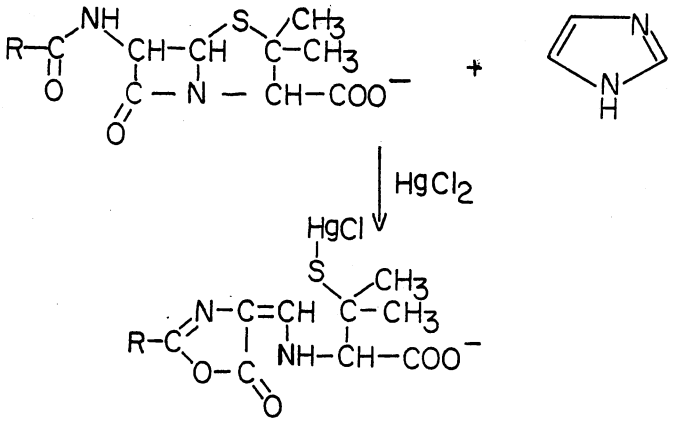


This method was used for the determination of penicillins in urine after extraction with chloroform or n-butanol. This method can determine penicillin in urine at concentrations as low as 300 mg L^{-1}.

The p-N-Methylamino Phenol Sulfate (Metol) Method. Penicillamine, a degradation product of penicillin, reacts with chromium (VI) and metol to give a colored complex with an absorption maximum of 490nm (35). Although the mechanism of the reaction is unknown, the primary amine group in penicillamine is believed to form a ternary complex with metol and chromium (VI). Penicillins G and V in tablets were determined by converting them to penicillamine by acidic hydrolysis followed by treatment with metol and chromium (VI). Common additives in tablets like dextrose, sucrose, starch, nitrate, sulphate, stearate, phosphate, and

gum acacia did not interfere. However, citrate was found to interfere.

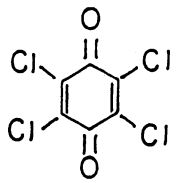
The Imidazole Method. Penicillenic acid mercuric mercaptide is formed in quantitative yield when penicillins are heated with imidazole and mercuric chloride at a pH of 6.8 (36).



The mercaptide has absorption maxima ranging from 324-345nm depending on the R-group. The reaction is slow, taking about 30 minutes to attain equilibrium. This method has been employed for the determination of penicillins in pharmaceutical preparations.

The Ammonium Vanadate Method. Ibrahim (37) used ammonium vanadate to determine several penicillins. The method was based on the reduction of vanadium (V) to vanadium (IV) by the acid degradation products of penicillin. The penicillins were boiled with ammonium vanadate and concentrated H_2SO_4 for ten minutes, cooled, diluted, and the absorbance of the resulting blue colored vanadium (IV) was measured at 750nm.

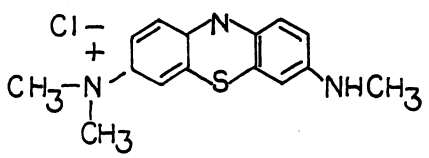
The Chloranil Method. p-Chloranil can act as a π acceptor due to the strong electron withdrawing effect of the four chlorine atoms.



p-Chloranil

Chloranil has been used to determine penicillin G by the formation of charge transfer complexes with penicillin G as the n-donor and chloranil as the π-acceptor (38). In the ground state, the two molecules are held together by Van der Waals forces. When this complex is irradiated with light at 560nm, an electron is completely transferred to the acceptor resulting in a purple color. The charge transfer complex was formed by heating penicillin G with chloranil for fifteen minutes in 80 percent dioxane.

The Azure-B Method. Azure-B forms ion pairs with various ions (39).



Azure-B

Penicilllins G and V form ion pairs with azure-B with an absorption maximum at 634nm. Penicillins in aqueous solutions were shaken with azure-B and chloroform. The absorbance of the ion-pair in chloroform was measured at 634nm. The calibration curve was observed to be linear in the range of 6 to 95ppm of penicillin G. This method enjoys virtual freedom from interference.

2.2.(b) Kinetic Methods

In kinetic methods of analysis, measurements are made before the attainment of equilibrium. Simultaneous determination of closely related species can be performed using the differential reaction rates. Continuous-flow sample processing is kinetic in nature (40), because the system is attaining equilibrium by a chemical or a physical process (that is, dispersion in the intercalated sample plug) or both. The simplest continuous-flow manifold is illustrated in Figure 3.

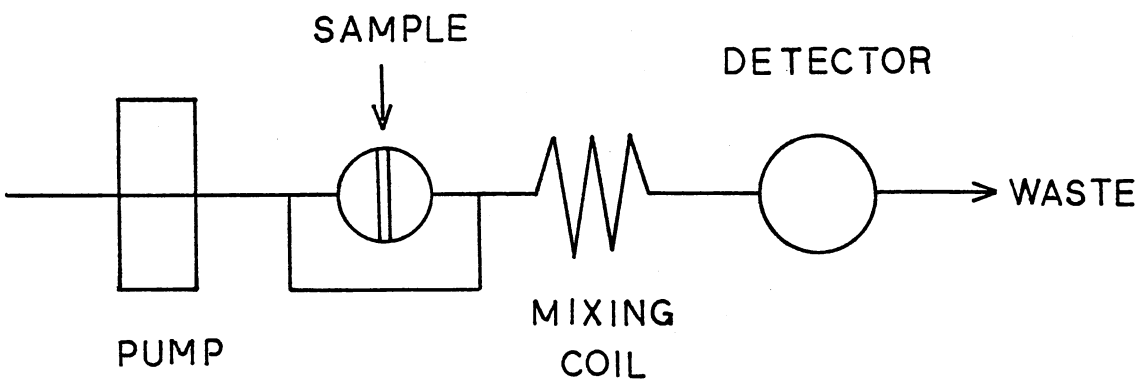


Figure 3. A Simple Continuous-Flow Analysis Manifold

In this type of system, called an "unsegmented continuous-flow system," a sample (liquid or gas) is intercalated into a continuously flowing carrier stream propelled by means of a pump. The intercalated sample plug moves towards the detector during which, time dispersion into and out of the sample plug occurs. The mixing coil provides controlled mixing between the sample and the carrier stream after which a product

or reactant is detected. When gaseous samples are used, a debubbler is introduced before the sample enters the detector (41). In segmented continuous-flow analysis, air bubbles are introduced into the stream to control dispersion in the sample plug.

The Iodometric Methods. The manual titrimetric method of Alicino (21) described earlier, was modified and automated by Ferrari (42) to determine penicillin in fermentation media by making use of an Auto Analyzer. Sample, reagents, and air were pumped with the aid of a proportioning pump. The penicillin solution was segmented with air bubbles. The sample solution was merged with a stream of penicillinase and passed through a mixing coil, where mixing of penicillin and penicillinase takes place. The mixed sample and penicillinase passed through a five minute delay coil where degradation of penicillin to penicilloic acid is completed. The penicilloic acid was dialyzed into a phosphate buffer stream, with the proteinaceous materials being sent to waste. This dialyzed stream was then merged with iodine solution, passed through a mixing coil and a delay coil to effect iodination of penicilloic acid. The solution was debubbled and the decrease in absorbance of the iodine solution was monitored at 420nm.

The decrease in absorbance of the iodine solution is proportional to the concentration of penicillin. The signal was recorded on a strip chart recorder, while the signal height was used for quantification. A blank can be run by substituting distilled water for penicillinase. This was the first completely automated method for the determination of penicillins.

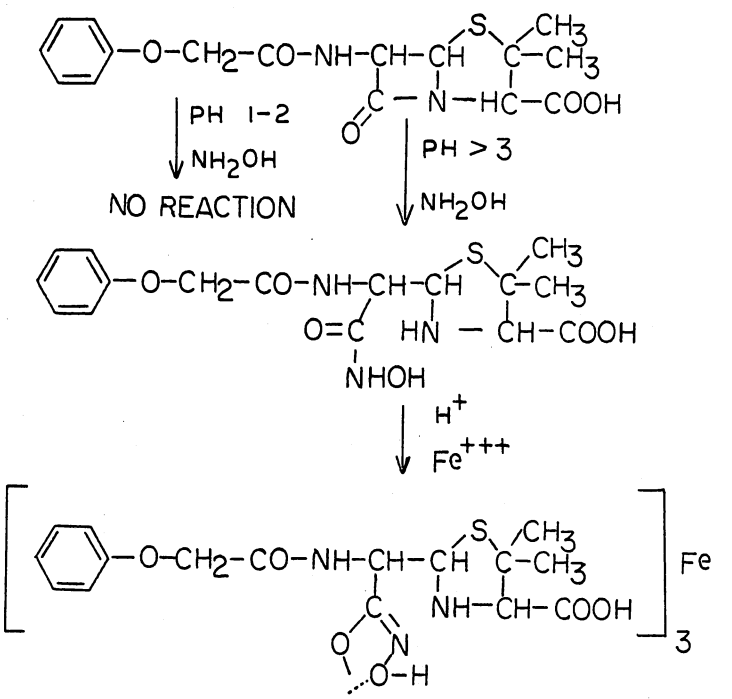
Goodall (43) modified the method developed by Ferrari. In the

modified method, enzymatic inactivation was done manually, and the unreacted iodine was monitored as its blue complex with starch at 525nm. The use of starch extended the limit of determination to <1 μg/ml which was not possible in the previous method.

After manual degradation of penicillin to penicilloic acid, the penicilloate was determined by first merging it with iodine solution followed by treatment with starch-potassium iodide solution. Dialysis was eliminated and an allowance was made for the iodine absorbing species in the broth by injecting a sample of penicillin in broth without penicillinase treatment. Phenoxymethyl penicillin was determined in the concentration range of 2500 to 5000 units/ml at a rate of 18 samples per hour by use of this method.

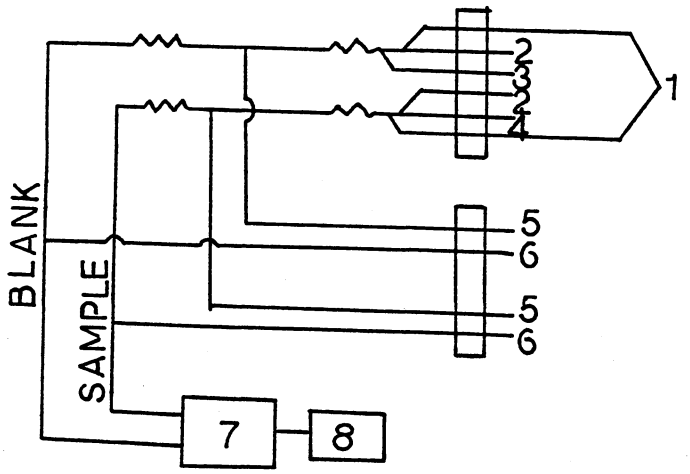
The main drawbacks of the iodometric methods are: (a) the stoichiometry of the iodine uptake by penicilloic acid is not constant, varying from 8 to 9 depending on the reaction conditions; (b) penicillins with an unsaturated side chain take up iodine and cause high results; (c) iodine is absorbed by many organic compounds; and (d) iodine is absorbed by Tygon and silicone tubings, which are generally used in the continuous flow manifold.

The Hydroxylamine Method. This method was based on the formation of a hydroxamate complex when penicillins are treated with hydroxylamine and ferric ions (44). The complex has an absorption maximum at 480nm. The reactions taking place can be represented as follows:



An automated colorimetric method based on the above reaction was presented by Niedermayer (45). Sample processing was done with the help of an Auto Analyzer. The online dialysis step in the Auto Analyzer was eliminated because of a curvilinear response. Since penicilloic acid is inert to hydroxylamine, a blank was run after inactivating the penicillins by merging it with penicillinase in the Auto Analyzer. This resulted in adsorption of the enzyme on walls of the tubings. An extensive cleaning was necessary before injection of penicillin samples. This problem could have been avoided by the use of immobilized enzymes.

In order to eliminate the time consuming step of cleaning up the system after using penicillinase, a dual and differential colorimetric system (46) was developed to run sample and the blank simultaneously. In this method, sample and blank were treated simultaneously by using separate lines for sample and blank as illustrated in Figure 4.



(1) Penicillin; (2) Air; (3) Penicillinase; (4) Water; (5) NH_2OH; (6) Fe^{+++}; (7) Dual Flow Cell Colorimeter; (8) Recorder

Figure 4. Differential Colorimeter System

The signal compensated for the blank, using a dual flow cell colorimeter, was recorded. Later, penicillinase was completely eliminated by utilizing a "reagent inversion blanking technique" (44). This technique was also a differential method like the previous one but, no enzyme was used in the blank line. Instead, the pH of the blank was reduced to a pH of < 2 so that no reaction took place between penicillin, hydroxylamine, and Fe^{+++}. A "solid prep" sampler was introduced in the manifold which eliminated the need for manual sampling of solid samples. An extensive study was done to adapt this method to all available liquid (47) and solid (48) formulations.

The Differential Rate Method. The different rates of degradation exhibited by some penicillins were used for the determination of the components in binary mixtures (49). The logarithmic extrapolation

method was used to follow the acid catalyzed hydrolysis of penicillins in a binary mixture by monitoring the unreacted penicillins as a function of time using the hydroxylamine reaction described earlier.

Acid catalyzed hydrolysis of binary mixtures can be considered as a system of two competing pseudo-first-order irreversible reactions of the type.

$$A \xrightarrow{K_A} \text{Products}$$

$$B \xrightarrow{K_B} \text{Products}$$

The rate equations $\frac{-d_A}{d_t} = K_A \cdot A$ and $\frac{-d_B}{d_t} = K_B \cdot B$ can be integrated to

$$[A]_t = [A]_o \cdot e^{-K_A t}$$

$$[B]_t = [B]_o \cdot e^{-K_B t}$$

The sum of the concentrations of A and B at any time can be represented by

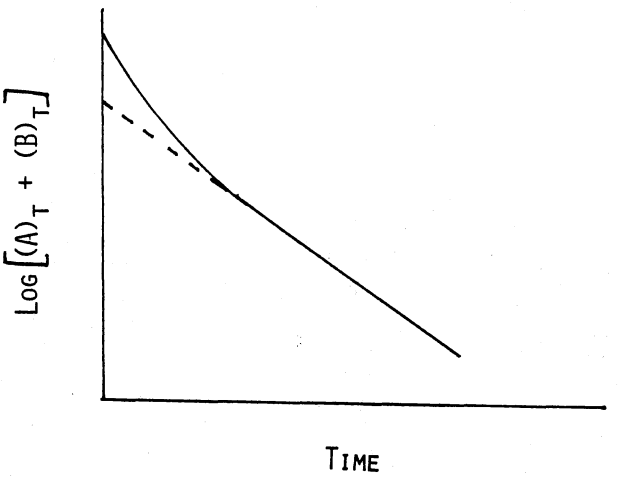
$$[A]_t + [B]_t = [A]_o \cdot e^{-K_A t} + [B]_o \cdot e^{-K_B t}$$

$$\log ([A]_t + [B]_t) = \log ([A]_o \cdot e^{-K_A t} + [B]_o \cdot e^{-K_B t}) \qquad (2.2\text{-}1)$$

When the faster reacting component A has almost completely reacted the above equation reduces to

$$\log [B]_t = \log [B]_o - \frac{K_B t}{2.303} \qquad (2.2\text{-}2)$$

Thus, by plotting $\log([A]_t + [B]_t)$ versus time, a nonlinear portion and a linear portion corresponding to equations (2.2-1) and (2.2-2) respectively can be obtained as illustrated in Figure 5.



$[A]_t$ - Absorbance of faster reacting component

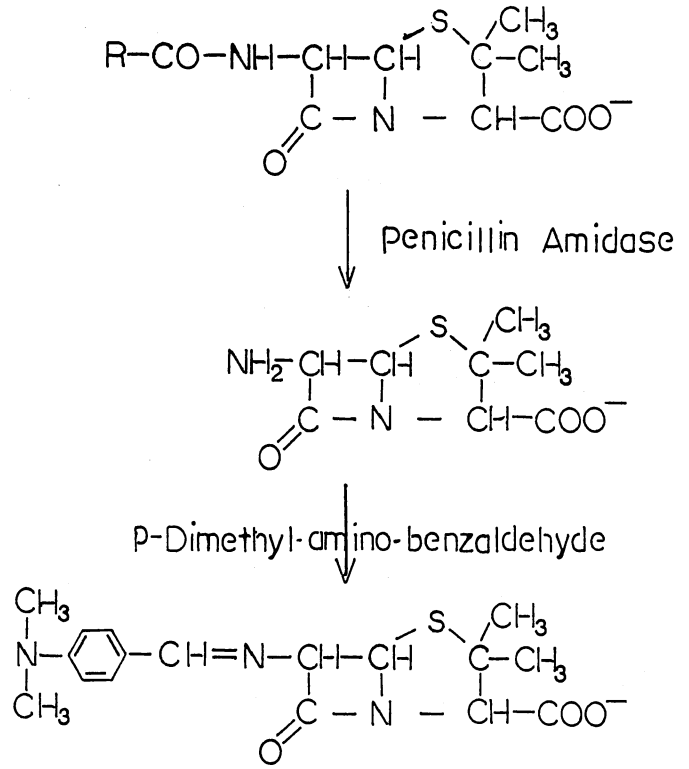
$[B]_t$ - Absorbance of slower reacting component

Figure 5. Differential Hydrolysis Plot

The initial concentration of the slower reacting component $[B]_o$ is extracted from the intercept. The authors did not say how they obtained the total initial concentration. The total initial concentration of the faster reacting component $[A]_o$ can be found by difference. This is the only method reported for penicillins, that would determine individual amounts of penicillin in a binary mixture without resorting to separation. The limitations of this method are: (a) K_A/K_B needs to be large (at least >5) so that the faster reacting component goes to almost completion before the slower reacting component can be monitored; and (b) B_o/A_o needs to be large.

The p-Dimethylamino Benzaldehyde Method. This method is based on the relatively specific action of an enzyme on the amide group of

penicillin G to form 6-aminopenicillanic acid (6-APA). Evans (50) determined penicillin G by converting it to 6-APA using penicillin amidase followed by a schiff-base coupling with p-dimethylamino benzaldehyde.



The method was completely automated using an Auto Analyzer. The absorbance of the complex was monitored at 415nm and penicillin G was determined in the concentration range of 250-2500 µg/ml.

Determination of penicillins using colorimetric methods are summarized in Table III.

TABLE III

SUMMARY OF THE COLORIMETRIC METHODS

Penicillins Determined (Table I) & Type of Sample	Error ±%	Precision ±%	Speed of Process	Advantages	Limitations	Reference
EQUILIBRIUM METHODS:						
1,2, & 4 in urine		2.7	2.5 hours per sample	More accurate than microbiological methods.	Slow	34
1 & 2 in formulations	0.4-1.0	1.0-1.8	2.5 hours per sample	Simple	Slow	35
1 & 2 in formulations	1.0	0.5	0.75 hours per sample	Simple		36
1,2,4,7 & 9 in formulations	0.6-2.4	0.9-2.1	0.5 hours per sample	Simple and Fast	Other drugs with basic centers interfere	37
1 in formulations	1.0	1.51	0.5 hours per sample	Simple and no interference		38
1 & 2 in formulations	0.6-2.7	0.6-1.6		No interference	Slow	39
KINETIC METHODS:						
1 in fermentation media	3	3		First automated method	Rate of dialysis not linear with concentration of penicillin.	42
2 in fermentation media		2-2.5	18 samples per hour	Simple; no dialysis unit required; sensitive; 1 μg/ml of penicillin determined.		43
1 in fermentation media	3		10 samples per hour	Simultaneous determination of sample and blank.		45,46
2 in formulations				Completely automated with a solid sampler		44
1,2 & 4 in formulations				Above method modified to accomodate tablets, liquid formulations and capsules		47,48
1 in formulations		3.4-5.6	40 samples per hours	Large sample throughout		50
1,4 & 9 in formulations				Useful in analyzing binary mixtures	K_A/K_B should be large	49

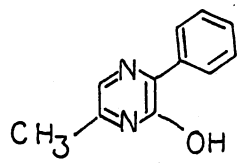
2.2.(c) Comparison Between Equilibrium and Kinetic Methods

Reported equilibrium methods have better accuracy and precision than kinetic ones as can be seen from Table III. Equilibrium methods, however, are usually very slow, some taking as long as two and a half hours per sample. On the otherhand, some kinetic based methods can analyze as many as 40 samples per hour. So, when there are a large number of similar type of samples to be analyzed, continuous flow analyzers would be the method of choice. Although, commercially marketed Auto Analyzers are quite expensive, a simple continuous flow analyzer can be "home-made" quite inexpensively to do the same function. Using some kinetic based differential rate methods binary mixtures can be analyzed without resorting to separation.

2.3 Polarographic Methods

Polarographic methods of determination are based on the measurement of the current developed at a dropping mercury electrode when diffusion is the rate limiting step in the electrochemical process. Intact penicillin is polarographically inactive. Therefore, in all the polarographic methods of determination of penicillins, it was initially degraded with acid or alkali into an electroactive product.

Penicillins with an α-amino group on hydrolysis with acid yield penicillamine and 2-hydroxy, 3-phenyl, 6-methyl pyrazine (51, 52), both of which are electroactive, the former giving an anodic peak and the latter giving a cathodic peak.



2-Hydroxy, 3-Phenyl, 6-Methyl Pyrazine

2-Hydroxy, 3-phenyl, 6-methyl pyrazine is believed to be formed by cyclization of the α-amino group and carbonyl group of the β-lactam ring followed by a disproportionation reaction (51). The reduction peak due to 2-hydroxy, 3-phenyl, 6-methyl pyrazine was utilized for the determination of amoxycillin (53, 54), epicillin (55), and ampicillin (56). The results are summarized in Table IV.

Penicillins that do not contain an α-amino group in the side chain could also be converted into an electroactive product by acidic hydrolysis of the C-S bond in the thiazolidine ring of penicillin to give RSH (57) which is electroactive. Although the mechanism of the reaction nor the products are known, it is quite likely that the electroactive product is penicillenic acid. Penicillamine (RSH) gives an anodic wave (58) forming a complex with mercury as shown below.

$$RSH + Hg \longrightarrow RS \cdot Hg + H^+ + e^-$$

So, it is likely, that this type of reaction is taking place with penicillenic acid. Penicillin V, penicillin G, carbenicillin, cloxacillin, and flucloxacillin could be determined in the range of 0.01-1.0mg mL^{-1} with a standard deviation of 0.02 at 1.0mg mL^{-1}.

Penicilloic acid gives an anodic wave at -0.25V versus SCE (59). The major reaction product at the electrode was found to be mercury penamaldate complex. Ulf Forsman (60) described a flow injection method for the determination of the penicilloic acid content of several

TABLE IV

SUMMARY OF THE POLAROGRAPHIC DETERMINATION OF PENICILLINS WITH AN α-AMINO GROUP

Sample Type	Linear Dynamic Range	Recovery %	Precision rsd %	Advantages	Reference
Amoxycillin in (i) capsules and (ii) urine		94.7-99.0		No interference from from constituents of	53
Amoxycillin in blood plasma	0.1-5.0 mg/ml	100	< 10	biological fluids	54
Epicillin in capsules	1×10^{-2} - 1×10^{-5} M	98.5	0.7		55
Ampicillin in capsules	1×10^{-2} - 1×10^{-5} M	100.85	1.09		56

penicillins. The penicilloate was detected polarographically at a dropping mercury flow through detector. The current response was higher for a droptime of one second, due to a higher surface area.

The calibration plot was linear in the range of 2×10^{-6} M to 1×10^{-4} M with a relative standard deviation of 0.2 percent. As many as 120 samples per hour could be analyzed by this method.

For the determination of penicillins in a mixture, Forsman also suggested that penicillins could be separated on a liquid chromatograph, hydrolyzed by post column addition of alkali and the penicilloic acid detected with a polarographic detector. Benner (61) used polarography to determine penicillins to regulate the amount of drug in the serum during drug theraphy. He used acidic as well as basic degradation procedures to determine nine penicillins. In the case of basic degradation, the polarographic peak appeared at 0.65V versus SCE. The acidic degradation resulted in a peak at 1.20V versus SCE. Although Benner did not identify the degradation products, knowing that acidic degradation gives penicillenic acid and basic degradation yields penicilloic acid, it could be assumed that the peak at 0.65V versus SCE is due to penicilloate and that at 1.20V versus SCE is due to penicillenic acid. Since serum interfered with the polarographic peak for all drugs, it was filtered with an ultrafiltration cell. Penicillins in serum could be determined at the µg level using this method.

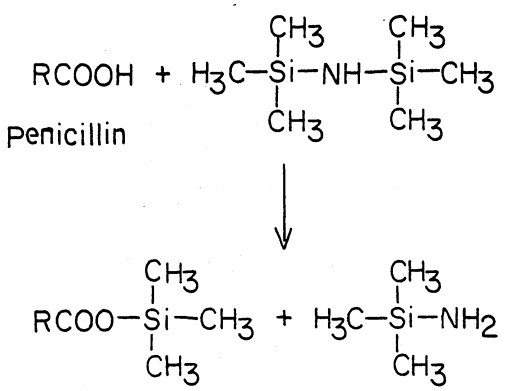
2.4 Chromatographic Methods

The word chromatography (color writing) comes from Tswett, a Russian botanist, who first employed the technique for the separation of plant pigments. Since then, chromatography has undergone several

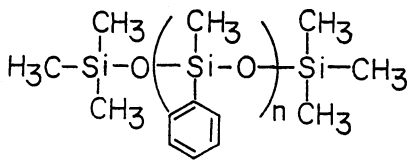
changes resulting in the use of a variety of stationary phases (liquid or solid) and mobile phases (gas or liquid). Penicillins have been separated and determined by: (a) gas-liquid chromatography; and (b) liquid-liquid chromatography.

2.4.(a) Gas-Liquid Chromatography

Gas-liquid chromatography acccomplishes separation of penicillins by partitioning them between a mobile gas phase and a thin layer of non-volatile liquid on an inert solid support. Although gas chromatography is limited to volatile materials, non-volatile materials can be derivatized to decrease their boiling point. Hishta (62) silylated the penicillins with hexamethyldisilazane. He separated the penicillins along with 5-α cholestane (internal standard) on an OV-17 column.



The structure of the stationary phase used by Hishta is shown below.



Some of the forces responsible for the separation of penicillins on this stationary phase are: (a) hydrogen bonding forces; and (b) dispersion forces.

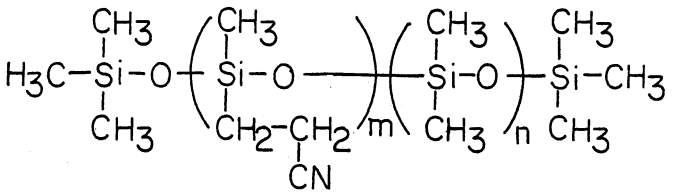
Hydrogen bonding is possible between the imine hydrogen of the penicillins and the π electrons of the phenyl ring on the stationary phase. Hydrogen bonding can also occur between the imine hydrogen and the ether groups on the stationary phase provided there is no steric hindrance.

Dispersion forces exist among all molecules. They result from the attraction of individual dipoles for the instantaneous and varying dipoles among molecules. Dispersion interactions increase with the sizes and unsaturation of solute molecules. The silyl esters of penicillins, therefore, get separated into bands based on their differing size - polarizabilities and hydrogen bonding. The various bands were detected with a flame ionization detector and recorded as peaks on a recorder.

Known weight ratios of the penicillin and internal standard were prepared and chromatographed. Area ratios (component/standard) were plotted against weight ratios (component/standard) to obtain a calibration plot. Penicillin in a test sample along with a known amount of internal standard was injected and the area ratios calculated. Using the calibration plot, the weight of penicillin in the test sample was calculated.

Roy (63) used pyrolysis gas-liquid chromatography (PGLC) to determine penicillins. PGLC can provide a reproducible succession of peaks from a single penicillin which, in effect, represents a finger print of the material. Separation of the pyrolyzed fragments of penicillins were carried out on an XE-60 column, which is a

cyanoethylmethyl silicone whose structure is shown as follows.



This strong dipolar cyano group would interact selectively depending on the dipole moment and polarizability of penicillins being separated on the column. Even a fragment of penicillin with no permanent dipole moment may have a dipole moment induced by the dipolar stationary phase (Dipole-Induced Dipole). Based on the differing polarizabilities, the various fragments of penicillin get separated on this column. Thus, the more polarizable a fragment is, the longer it is retained in the column.

Apart from the induction forces, hydrogen bonding can also take place between the cyano group of the stationary phase and the fragments. The separated fragments were detected using a flame ionization detector. Quantitative analysis of the penicillins were carried out based on the peak height of the most intense peak in the pyrogram. Calibration curves for penicillins were linear in the range of 10 ng to 100 μg. Fourteen penicillins have been determined using this method.

Disadvantages of PGLC can be summarized as follows: (a) This method cannot differentiate between penicillins that have close resembling "R" groups. For example, penicillin G and carbenicillin give similar pyrograms; (b) This method is not suitable for a mixture of penicillins; and (c) This method does not have an internal standard. Minor fluctuations in experimental conditions could affect the accuracy and precision of

the results. An advantage of PGLC is that no derivatization, unlike in GLC, is necessary.

The advantages of gas chromatographic determination of penicillins are: (a) separation and determination of a multicomponent mixture is possible; (b) results are obtained rapidly; (c) only small samples are required with minimum sample preparation; (d) the method is applicable to widely divergent sample types; and (e) good accuracy and precision are obtainable.

2.4.(b) Liquid-Liquid Chromatography

The different migration rates of the various penicillins as they move through a chromatographic column result in their separation. Relatively nonpolar compounds like penicillins cannot be successfully separated in normal liquid-liquid chromatographic systems where the stationary phase is more polar than the mobile phase. However, penicillins have been separated on reverse-phase systems where the normally polar stationary phase and nonpolar mobile phase were interchanged. A good separation and determination of five penicillins were effected by the use of high efficiency small particle (10μm) reverse phase C_{18} Lichrosorb columns (octadecyl silane) (64). A mobile phase of 35 percent methanol in 0.01 M sodium hydrogen phosphate was used. Pure compounds, complex mixtures of antibiotics in a variety of dosage forms and fermentation broths could be analyzed by this technique. This system could also be used for preparative applications because of the small particles and totally porous nature of the stationary phase. Westerlund (65) determined ampicillin in biological material by reverse phase liquid chromatography and post column derivatization.

The inherent light absorbance of the penicillins are difficult to use for bioanalytical purposes since these compounds have a sufficiently high molar absorptivity only in the wavelength range of 220-240nm, where many endogenous compounds also absorb light. The biological fluid after protein precipitation with trichloroacetic acid was injected into a Lichrosorb RP8 column (octyl silane) and eluted with 30 percent methanol in pH 8 phosphate buffer. The penicillins after separation were segmented with air bubbles, to control dispersion, derivatized with imidazole to form the mercuric mercaptide and the absorbance of the complex was monitored at 310 nm.

Horvath and others (66) attributed the retention of solutes on octadecyl silica to a reversible association of the solutes with the hydrocarbonaceous liquid of the stationary phase.

$$S + L \rightleftharpoons SL$$

S = Substrate L = Ligand

It is also assumed that no ionic or hydrogen bonding occurs between solute and the stationary phase. The equilibrium constant $K_{assoc.}$ is given as

$$K_{assoc.} = \frac{[SL]}{[S][L]} . \qquad (2.4\text{-}1)$$

The dissociation of a monoprotic acid, HA, like penicillin in the mobile phase is governed by

$$HA \rightleftharpoons H^+ + A^- .$$

The equilibrium constant in the mobile phase is given as

$$K_{a_m} = \frac{[H^+]_m[A^-]_m}{[HA]_m} . \qquad (2.4\text{-}2)$$

Equation (2.4-1) can be modified by substituting HA for S

$$K_{LHA} = \frac{[LHA]_s}{[HA]_m[L]_s} \cdot \qquad (2.4\text{-}3)$$

The interaction between the anion and the ligand results in the formation of the complex LA^- according to the following equilibrium.

$$A^- + L \rightleftharpoons LA^-$$

$$K_{LA^-} = \frac{[LA^-]_s}{[A^-]_m[L]_s} \qquad (2.4\text{-}4)$$

The magnitude of solute retention is expressed by the capacity factor, k.

$$k = \frac{V_s}{V_m} \bullet \frac{[LHA]_s + [LA^-]_s}{[HA]_m + [A^-]_m} \qquad (2.4\text{-}5)$$

Substituting (2.4-2), (2.4-3), and (2.4-4) in (2.4-5) results in

$$k = \frac{V_s}{V_m} \bullet \frac{k_{LHA}[L]_s + k_{LA^-}[L]_s \bullet \frac{Ka_m}{[H^+]_m}}{1 + \frac{Ka_m}{[H^+]_m}} \cdot \qquad (2.4\text{-}6)$$

From the equation (2.4-6) it is evident that pH plays an important role in the capacity factor and hence, the separation of penicillins in reversed phase systems. The optimum pH was found to be 8 for amphoteric penicillins like ampicillin. The retention profile for amphoteric penicillins increased at low as well as high pH values. This was explained as due to the interaction of penicillins with stationary phase both in the uncharged form (HP) and as ion pairs (HP H^+X^- and M^+P^-, where X and M are buffer components).

As expected in a reverse phase system, the presence of polar substituents such as $-NH_2$, -OH, and $-COO^-$ on the penicillins decrease the retention times. By the injection of 100µl of plasma, ampicillin, and mecillanam could be quantitatively determined at 100ng/ml with a

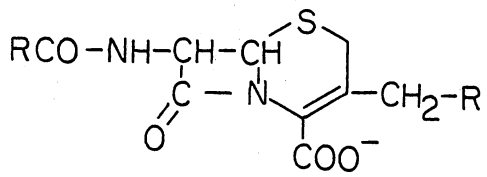
relative standard deviation of eight percent. This method is applicable to the determination of ampicillin in plasma, whole blood, urine, and lymph.

Rudrick (67) used a μ-Bondapak (5 μm) C-18 column (octadecyl silane) to separate and determine penicillinase resistant penicillins in serum using a 28 percent acetonitrile in 0.2M pH 5.6 ammonium acetate as the mobile phase. The absorbance of the penicillins were monitored at 254 nm. Serum proteins were precipitated with acetonitrile before injection into the column. Methicillin, oxacillin, cloxacillin, dicloxacillin, and naficillin in serum could be determined at concentrations as low as 0.5 μg/mL. Although this technique does not use any post column derivatization, the minimum concentration that can be determined is 0.5 μg/mL which is higher than that of the previous method (0.1 μg/mL) which incorporates a post column derivatization. The extra column band broadening due to the post column derivatization is probably offset by the use of high efficiency small particle (5 μm) columns. The Van Deemter equation predicts that the smaller the particle size in the column, the smaller is the band broadening.

2.5 Enzymatic Methods

Enzymes are high molecular weight biochemicals which catalyze numerous reactions. Enzymes are selective in their mode of action. In 1940, Abraham and Chain (68) reported that an extract of Escherichia coli destroys the antibacterial activity of penicillin G. They proved that an enzyme was responsible for the antibacterial activity which they named as penicillinase. Later, many other bacteria were found to contain or secrete similar enzymes for which the more general term

β-lactamase has been adopted. β-Lactamase denotes an enzyme which catalyzes the hydrolysis of the amide bond in the β-lactam ring of penicillins (see Figure 1) or cephalosporins.



Cephalosporin

The term penicillinase has been retained to denote β-lactamase preparations which predominantly catalyze the hydrolysis of penicillins. Enzymatic determinations of penicillins are based on monitoring the amount of penicilloate produced either directly or indirectly. In the direct methods, the increase in hydrogen ion concentration upon inactivation of the penicillins was used. In the indirect methods, the penicilloates are further broken down to various degradation products. These degradation products are then treated with suitable reagents to form colored complexes which are determined spectrophotometrically. These indirect enzymatic methods have already been covered under colorimetric methods of determinations.

All of these procedures use native enzymes in solution. In solution, native penicillinase is stable for several hours at room temperature, but a concentrated solution (10 mg/mL) of penicillinase will be denatured in 15 minutes (69). They can also be denatured by vigorous agitation. Many of the difficulties discussed above can be negated, or at least minimized by the technique of enzyme immobilization (70). Once immobilized, an enzyme is often stable for weeks or even months, making

it possible, to reuse the enzyme several times. Reuse of expensive enzymes results in decreasing the cost per determination.

Determination of penicillins using immobilized penicillinase can be done via the use of (a) enzyme electrodes, or (b) enzyme reactors.

2.5.(a) Enzyme Electrodes

An enzyme electrode may be considered as the combination of any type of electrochemical sensor and a small layer of enzyme which is used to measure the concentration of a substrate (71). There are two important electrode designs by which penicillins have been determined: (a) containment of enzyme in a semi-permeable membrane; and (b) adsorption of enzyme onto a fritted glass disc.

Containment of Enzyme in a Semi-permeable Membrane. Nilsson and co-workers (14) designed an enzyme electrode by entrapping the enzyme in a thin liquid layer around a pH electrode with the help of a semi-permeable membrance as shown in Figure 5.

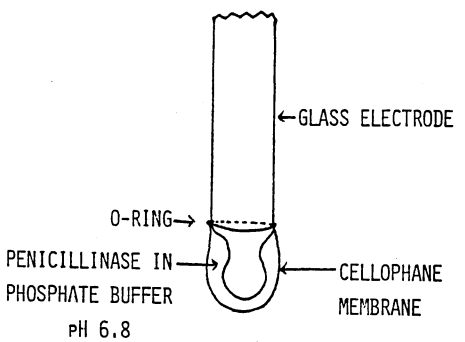


Figure 5. Entrapment of Enzyme With A Semi-Permeable Membrane

This electrode and a saturated calomel reference electrode were dipped in stirred penicillin solutions and the change in pH was noted. The electrode was equilibrated by dipping it in a stirred pH 6.8 buffer before testing another sample. The calibration plot was linear in the range of 10^{-2}M to 10^{-3}M. The electrode performed in an acceptable manner for two to three weeks after which replacement of the enzyme was necessary. Although this kind of electrode with enzyme in the solution form is the least stable, the ease with which this enzyme electrode can be constructed makes it useful.

Enfors (15) designed an autoclavable enzyme electrode for the determination of penicillin in fermentation broths. This electrode was designed so that the electrode could be dipped directly into fermentation tanks under aseptic conditions. The design of the electrode is shown in Figure 6.

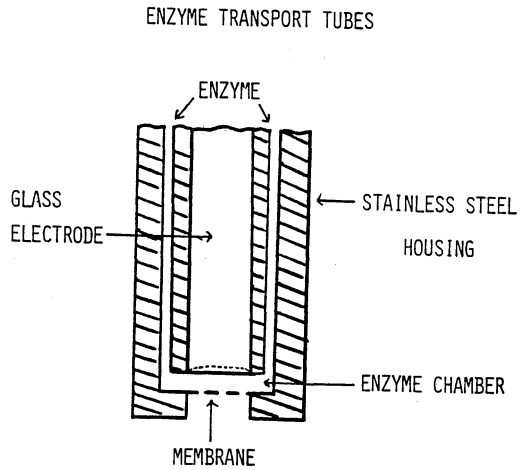


Figure 6. Autoclavable Enzyme Electrode

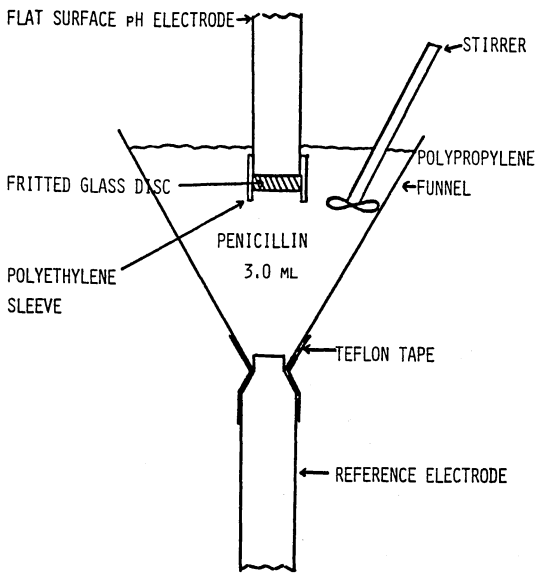
A flat surface pH electrode was mounted in a stainless steel housing. A semi-permeable membrane formed one of the walls of the enzyme chamber, the other wall being the sensing surface of the pH electrode. The enzyme chamber was connected to the upper part of the housing by stainless steel tubes. The stainless steel probe with the pH electrode was sterilized to prevent contamination of the fermenter, as any contamination would reduce the enzymatic activity.

Penicillinase was charged into the enzyme chamber through stainless steel tubes following sterilization of the probe. The enzyme electrode was inserted into fermentation broths and the change in pH was monitored. The change in pH was proportional to the concentration of penicillin in the range of 0-50mM. The response time of the electrode was about one minute. However, this could be decreased by decreasing the chamber thickness at the expense of reducing the linear dynamic range.

Adsorption of Penicillinase onto a Fritted Glass Disc. Cullen (16) immobilized penicillinase by adsorption of the enzyme onto a fritted glass disc which was affixed to the end of a flat surface pH glass electrode. The electrode assembly is diagrammatically represented in Figure 7.

Calibration curves for different penicillins were constructed by pipetting 3mL of the penicillin solutions adjusted to a pH of 6.900 ± 0.005 into the funnel and measuring the change in potential. One mole of penicilloic acid is released per mole of penicillin upon enzymatic inactivation. Therefore, the Nernst equation can be modified to read

$$E = E^{o} + 2.3 \frac{RT}{F} \log (\text{penicillin}).$$



Source: Cullen, L.F.; Rusling, J.F.; Schleifer, A.; Papariello, G.J. Anal. Chem., 46, 1955 (1974).

Figure 7. Glass Disc Penicillin Selective Enzyme Electrode Assembly

The equation predicts a slope of 59 mV/decade change in penicillin concentration when log (penicillin) is plotted against the observed potential. The experimentally observed slopes were in the order of 56-58 mV in close agreement with the predicted value. This electrode showed a linear relationship in the concentration range of 10^{-5} to 3 x 10^{-3}M. The electrode was used for six weeks after which replacement of the fritted disc was necessary. During this period, as many as 300 measurements could be made with the same electrode. Penicilloic acid, if present as an impurity, does not interfere in the determination of penicillins.

The advantages of using enzyme electrodes discussed this far can

be summarized as follows:

(a) Pretreatment of samples were not necessary. No incubation to develop color and fluorescence are needed.

(b) Electrodes are as easy to calibrate and use as glass pH electrodes.

(c) The electrodes are quite fast. They reach a steady state within 30 seconds to two minutes.

(d) Since a given electrode can be reused many times, the cost per determination is quite small.

The main drawback of enzyme electrodes is that reaction and detection steps cannot be controlled and optimized independently. This was overcome by using immobilized enzyme reactors in continuous flow analysis. The detector was placed downstream from the reactor.

2.5.(b) Enzyme Reactors

Enzyme reactors contain immobilized enzymes bound to the wall of the reactor (open tubular reactor) or bound to the packing material (packed reactor) by strong chemical forces. Reactors are used online in continuous flow analysis. Physical methods of immobilization like entrapment and adsorption are not quite suitable for use in reactors as enzymes tend to be washed away. The substrate is introduced into the reactor as a plug. The enzyme in the reactor catalyzes the conversion of substrate to product, which is monitored with a suitable detector at the end of the reactor.

Covalent Binding Method. An immobilized penicillinase based flowing stream analyzer for measurement of penicillin in fermentation

broths was reported by Rusling (17). A covalently bound penicillinase glass derivative was synthesized, packed in a microcolumn and used in a continuous flow system for the determination of penicillins. The reactions leading to the penicillinase glass derivative are shown in Figure 8.

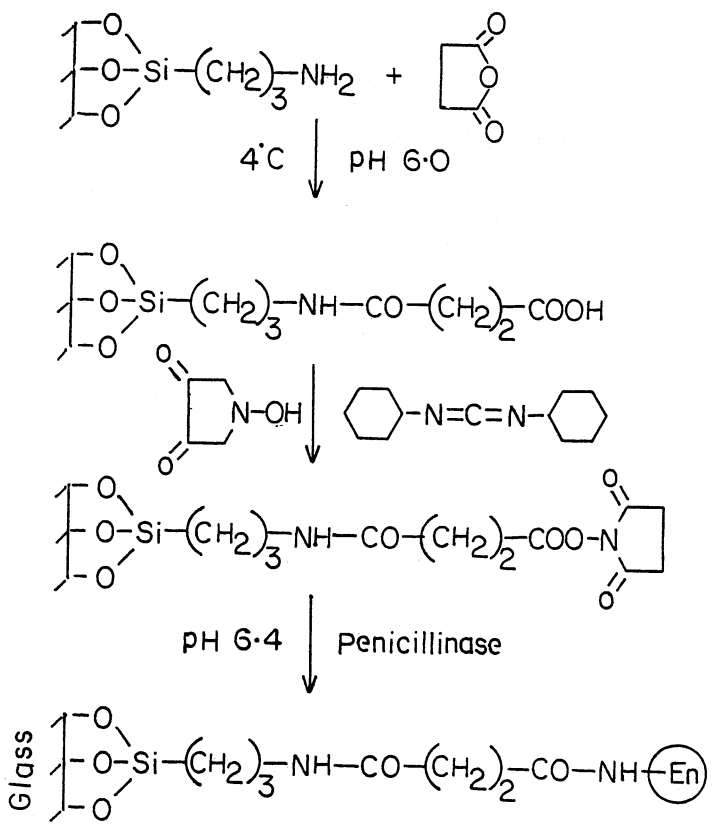


Figure 8. Reactions Leading to the Penicillinase Glass Derivative

A flow through potentiometric detection system was constructed to monitor the change in hydrogen ion concentration in the continuous flowing stream as a result of enzymatic hydrolysis of penicillins. The penicillin and the carrier solution were made up in a 4×10^{-4}M K_2HPO_4 pH 7.00. The potentiometric response of the system, E_p, can be related to the substrate concentration, S, as follows.

$$E_p = 59.2 \cdot \frac{V_m \, \Delta t \, S}{K_m \, \beta}$$

where V_m = maximum velocity of reaction
Δt = sample residence time in column
K_m = Michaelis constant
β = buffer capacity

The above equation predicts that if V_m/K_m and β remain constant or change slowly in compensating directions, the system response will be a linear function of penicillin concentration. This equation holds good at low concentrations. On the other hand, at high penicillin concentrations V_m/K_m is lowered because of a drop in the enzymatic activity due to a decrease in pH. This is reflected as lower E_p values than predicted by the equation. The equation also predicts that a decrease in buffer capacity would increase sensitivity of the method. A plot of the change in potential versus the concentration of penicillin is linear in the range of 0.1 to 0.8 mg/mL. An optional online dialysis system was studied which improved the accuracy of the method.

Whenever protons are consumed or released in any reaction, it is more sensitive to measure the change in potential rather than measuring the change in pH (72). Consider a buffered solution in which protons are released. Let v = volume of reaction mixture in liters
h_o = number of moles of H^+ in the initial reaction mixture so that $pH_o = -\log \frac{h_o}{v}$

n = number of moles of H^+ produced during the reaction
a = number of moles of buffer acid in the initial reaction mixture
s = number of moles of buffer salt in the initial reaction mixture
c = total amount of buffer in the system (c=a+s)
K = dissociation constant of the buffer acid
A = $pK - pH_o$

$$pH_o = pK + \log s/a$$

$$pH_t = pK + \log \frac{(s-n)}{(a+n)} \qquad (2.5\text{-}1)$$

where pH_o is the initial pH and pH_t is the pH t_{min} after the start of the reaction.

$$K = \frac{(h_o/v \cdot s/v)}{(a/v)}$$

$$= h_o s/av \quad \text{and}$$

$$c = a + s$$

therefore, $$s = Kcv/(h_o + Kv) \qquad (2.5\text{-}2)$$

$$a = h_o c/(h_o + Kv) \qquad (2.5\text{-}3)$$

$$A = pK - pH_o$$

therefore, $$Kv = 10^{-A} \cdot h_o. \qquad (2.5\text{-}4)$$

Substitution of (2.5-4) in (2.5-2) and (2.5-3) gives

$$s = c/(10^A + 1) \qquad (2.5\text{-}5)$$

$$a = c/(10^{-A} + 1). \qquad (2.5\text{-}6)$$

Substitution of (2.5-5) and (2.5-6) in (2.5-1) gives

$$\Delta pH = \log \frac{c-n(10^A + 1)}{c+n(10^{-A} + 1)} \qquad (2.5\text{-}7)$$

but $$\Delta E = \left[\frac{RT}{F} \ln 10\right] \cdot \Delta pH$$

$$= 6.15 \cdot \Delta pH \text{ at } 37^oC \qquad (2.5\text{-}8)$$

From equation (2.5-7) and (2.5-8), it is evident that sensitivity is greater when change in potential is monitored rather than when

monitoring the change in pH.

Antigen-Antibody Complex Technique. Antibody molecules appear in the blood serum and certain cells of vertebrates in response to the introduction of a protein or some other macromolecule foreign to that species (called antigen) (73). The antibody moelcules generated in this manner can combine with the antigen which elicited their formation to form an "Antigen-Antibody Complex." This reaction is called the immune response.

Matiasson (74) immobilized penicillinase on sepharose by using the antigen-antibody interaction. Anti-human serum albumin (anti-HSA) was immobilized on BrCN activated sepharose. The coupling of the protein to the activated sepharose occurs through the amine function of the protein. The antibody containing sepharose was packed in a heat insulated column with flow upwards. A thermistor was immersed in the top of the bed.

Penicillinase was coupled to HSA using the bifunctional coupling agent glutaraldehyde. The glutaraldehyde couples amino groups of the penicillinase to the amine groups of HSA.

$$\text{Enzyme} + \text{OHC-(CH}_2\text{)}_3\text{-CHO} + \text{HSA} \xrightarrow[4^{\circ}\text{C } 17\text{hrs.}]{\text{pH } 7.0} \text{Enzyme-N=CH-(CH}_2\text{)-CH=N-HSA}$$

Immobilization of penicillinase on the sepharose was achieved by passing the Enzyme-HSA through the column containing sepharose-anti-HSA. Penicillin solutions in 0.1 M phosphate buffer pH 7.0 were injected into the column and the heat signals caused by enzymatic conversion of pulses of substrate were registered by the thermistor and recorded.

When the activity declines or another enzyme activity was needed,

the column was washed with 0.2 M glycine-HCl, pH 2.2. By these washes, the antigen-antibody interaction is split and the antibody column is ready for reloading with the new enzyme. The entire process of washing and immobilization took only 8-10 minutes.

Determination of penicillins using immobilized penicillinase are summarized in Table V.

2.6 Radioimmunoassay

Radioimmunoassay combines the sensitivity of radiochemistry with the specificty of immunology. In immunoassay an analyte is determined by the exploitation of an antigen-antibody binding reaction in which the analyte itself is the antigen. The principle of antigen-antibody binding reaction can be illustrated in Figure 9.

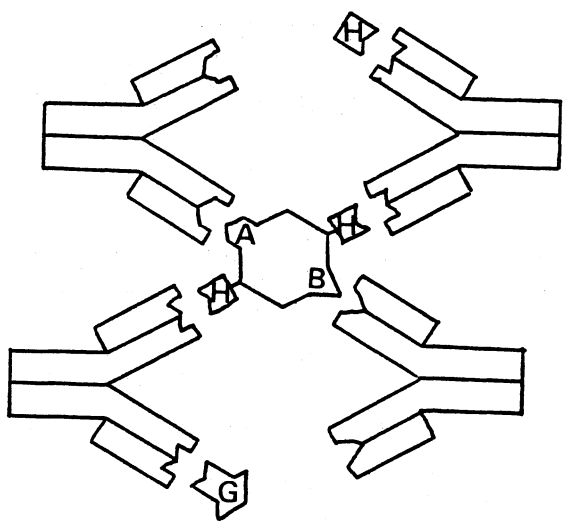


Figure 9. Schematic Representation of Antigen-Antibody Complex

TABLE V

OBSERVATIONS ON THE DETERMINATION OF PENICILLINS
USING IMMOBILIZED PENICILLINASE

Sample Type (Refer to Table I)	Method of Immobilization	Linear Dynamic Range	Stability	Observations	Reference
1	Entrapment in a cellophane membrane	10^{-2}M - 10^{-3}M	3 weeks	Enzyme electrode preparation very simple.	14
1 in fermentation broths	Entrapment	0-50 mM		Electrode can be autoclaved, making it possible to dip it directly into fermentation tanks	15
1,2,4, & 12	Adsorption on a fritted glass disc	3.5 - 1100 µg/ml	4 weeks	Replacement of fritted disc done easily	16
1 in fermentation media	Covalent binding	0.1 - 0.8 mg/ml		More sample throughput achieved with a continuous flow system. Due to compaction in column repacking was necessary once every 3 days.	17
1 in biological fluids	Antigen-antibody interaction	10^{-4} - 10^{-2}M	4 weeks	Reversible immobilization used which permits change from one enzyme assay to another within a few minutes	72

The Y shaped molecules shown in the figure are antibody (Immunoglobulin G) molecules. G and H are "haptens" (small molecular weight antigens that cannot by themselves induce the immune response but can do so when bound to large antigens). A and B are antigenic determinants (sites) to which two hapten groups are covalently attached. This antigen (with two hapten groups) when inoculated into the blood stream of a vertebrate induces the formation of the Y shaped antibody molecules with specific sites for A, B, and H. Although antigen H cannot induce the immune response by itself, it can bind with the antibody against H (see Figure 9). A hapten G with a structure similar to hapten H can also bind with the antibody against H, and block the antigenic determinants.

The initial reaction vessel contains antibody solution, radiolabelled antigen, and unlabelled antigen (analyte). Upon incubation, the antibody (Ab) will form an antigen-antibody immuno complex (Ag^*-Ab). In the absence of unlabelled antigen, a certain fraction of the radio labelled antigen (Ag^*) is bound as (Ag^*-Ab). But when increasing amounts of unlabelled antigen (Ag) are added, the limited binding sites of the antibody are progressively saturated and the antibody can bind less of the radio labelled antigen.

Following incubation, the bound antigens are separated from the unbound antigens by precipatation and the radioactivity of either or both the phases (precipitate or filtrate) was measured to determine the percent bound labelled antigen. A calibration curve was prepared by using antigen standards of known concentration by plotting either the percent bound labelled antigen or the ratio of percent bound to free (B/F) as a function of the unlabelled antigen concentration. From this the

unknown concentration of antigen can be calculated. Usually the tyrosine residues of antigens are labelled with ^{131}I or ^{125}I by iodination of the phenyl ring. Iodine is the tracer of choice since it is a gamma emitter. It is easy to count gamma radiation when compared to low energy beta emitters like ^{3}H and ^{14}C where liquid scintillation counting has to be used.

Wall and co-workers (75) used radioimmunoassay to determine penicillins in serum, urine and milk at concentrations as low as 10ng/ml. Antibodies for penicilloyl-BGG (Bovine Gamma Globulin) conjugate were raised in rabbits. Anti-BGG antibodies were removed from the penicilloyl-BGG antibody with BSA-BGG immunoadsorbent which would bind with BGG antibodies, where BSA is Bovine Serum Albumin. The tracer was obtained by coupling the penicilloyl groups to BSA previously labelled with ^{125}I. The incubation of the tracer, unlabelled penicilloyl and antibody was carried out overnight at room temperature. The labelled antibody (penicilloyl-iodinated BSA) complex was precipitated with anti-rabbit sheep serum. The precipitate was separated by centrifugation and the radioactivity of the precipitate was measured with a gamma scintillation spectrometer. A standard curve was constructed with penicilloyl ε-amino caproate of known concentration.

Penicillins in serum, milk, and urine were determined by first converting them to penicilloyl ε-amino caproate by incubating them with ε-amino caproic acid, followed by incubation with tracer and antibody. Free penicillin and penicilloic acid do not bind with the antibody. Penicilloic acid does not react with the antibody, probably because the antibody was produced with penicilloyl-BGG conjugate and the penicilloyl antibodies are not accessible to free penicilloic acid. This property

was exploited to determine intact penicillins in presence of penicilloic acid.

2.7 Conclusions

Penicillins are present in a wide variety of samples in varying concentrations from a few ng/mL (in biological fluids) to a few mg/mL (in pharmaceutical preparations). There is no method available that can be applied successfully to all kinds of samples. On the otherhand, based on the type of sample, concentration range, and problems encountered, suitable methods of determination can be selected.

Automated colorimetric methods (48, 49) using hydroxylamine are ideal for pharmaceutical preparations. Additives commonly found in formulations do not interfere. Being automated, a large number of samples can be analyzed everyday.

Enzymatic methods using immobilized penicillinase are comparable or even better because the catalyst (enzyme) is reused several times, which reduces the cost per determination. No method using immobilized penicillinase has been completely automated for the determination of penicillins in pharmaceutical preparations. If done, the methods would have a great potential as it would decrease the cost per determination. At the same time, semi-synthetic penicillins, which are resistant to penicillinases cannot be determined using enzymatic methods.

Titrimetric methods are simple and do not need expensive instrumentation. So, titrimetric methods can be used in small laboratories where penicillin samples are analyzed occasionally.

Due to the complex nature of fermentation media, penicillins are not easily amenable to chemical methods of determination. Methods

using immobilized penicillinase in continuous-flow can be used with minimum sample preparation. On the other hand, it is quite convenient to dip autoclavable enzyme electrodes into fermentation broths and get the result immediately by referring to a calibration plot.

Penicillins are present in ng levels in biological fluids and sometimes in the milk of dairy cows, that it is practically impossible to determine them by colorimetric or enzymatic methods. Radioimmunoassay would be the method of choice because of its specificity and senesitivity. Proteins and other macromolecules do not interfere and hence need not be separated.

When components of a mixture of penicillins have to be determined, chromatography is convenient. But, chromatographic methods cannot determine concentration levels lower than 0.1 ng/mL. Therefore, radioimmunoassay has to be used for concentrations of penicillins lower than 0.1 ng/mL.

CHAPTER III

METHODS AND PROCEDURES

3.1 Apparatus

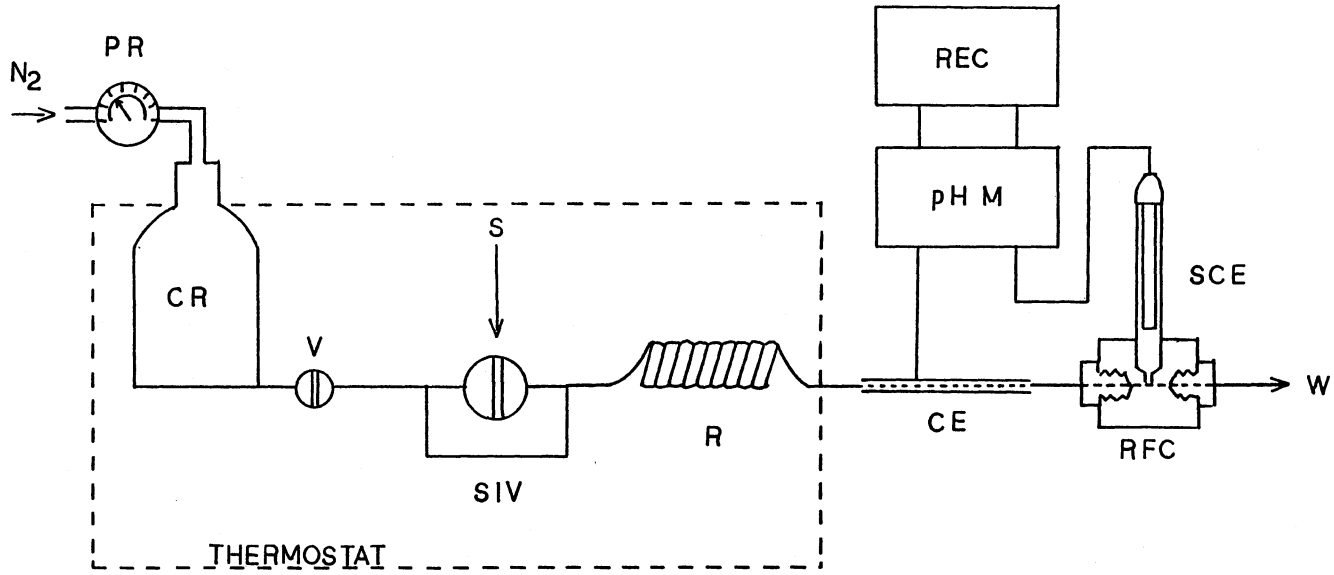
The experimental set-up used for the continuous flow system is shown in Figure 10.

A two litre glass bottle was used as the carrier reservoir. The flow rate of the carrier was adjusted by varying the pressure of N_2 in the reservoir with a pressure regulator. A stopcock was used as an on/off valve.

Penicillin samples were intercalated using a sample intercalation valve (76) which was built in-house. The injected sample then passes through the immobilized enzyme reactor. The decrease in pH of the sample plug, due to enzymatic hydrolysis, was sensed with a Radiometer G299A glass capillary electrode and a Radiometer K401 saturated calomel reference electrode.

A flow through cell for the reference electrode, as shown in Figure 11, was constructed for use in the flow system. The signals from the Radiometer PHM 84 RESEARCH pH METER were recorded using a Houston Instrument Superscribe Series 4900 strip chart recorder. A Brinkman IC-2 constant temperature circulator was used to thermostat the carrier reservoir, sample intercalation valve, and the enzyme reactor.

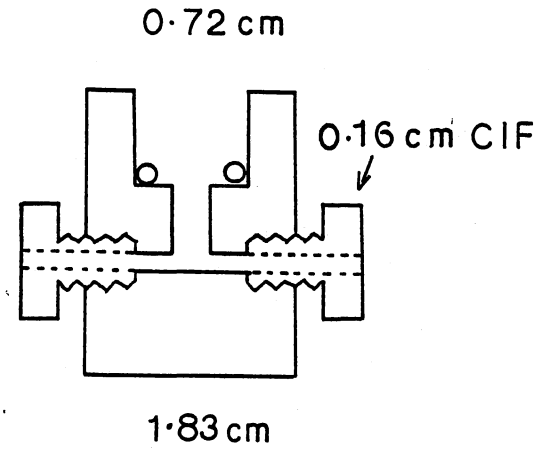
An inexpensive flow through cell for a Sensorex 450C (Stanton,



PR:	Pressure Regulator	CE:	Capillary pH Glass Electrode
CR:	Carrier Reservoir	pH M:	pH Meter
V:	On/Off Valve	REC:	Recorder
SIV:	Sample Intercalation Valve	SCE:	Saturated Calomel Electrode
R:	Enzyme Reactor	RFC:	Reference Flow Cell

Figure 10. Diagram of the Continuous Flow System for the Determination of Penicillins

California) flat surface pH electrode, as shown in Figure 12, was constructed from plexiglass. The cell volume was approximately 50 µl. The cell was fitted with 1/16" Cheminert fittings.



CIF: Cheminert Fitting

Figure 11. Flow Cell for the Reference Electrode.

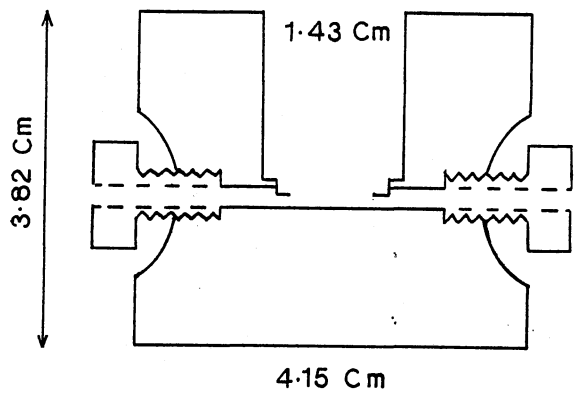


Figure 12. Flow Cell for the Flat Surface pH Electrode

In the silylation studies, enzyme activity on silica was indirectly measured by monitoring the penicillinase catalyzed hydrolysis of penicillins. The initial rate was measured with a Radiometer GK2401C combination pH electrode.

3.2 Reagents and Solutions

Water

Aqueous solutions of reagents or buffers were always prepared in deionized, distilled water.

Stock Phosphate Buffer (0.100 M)

13.6090 Grams of Fisher potassium phosphate (certified A. C. S.) were dissolved and diluted to 1 liter with distilled water.

Phosphate Buffers (1.00×10^{-2} M to 1.00×10^{-4} M)

Phosphate buffers of varying concentrations and pH were prepared by diluting the 0.100 M stock phosphate buffer and adjusting the ionic strength to 0.10 with 1.00 M KCl. The pH was adjusted with a dilute NaOH solution.

Stock Penicillin G (1.00×10^{-3} M)

0.0356 Grams of Penicillin G, Na (Sigma, St. Louis, Missori) was dissolved in the appropriate phosphate buffer and diluted to 100 mL in the same buffer. Stock solutions were prepared fresh everyday.

Penicillin G (5.00×10^{-5} M to 5.00×10^{-4} M)

Standard solutions of penicillin G for the working curve were prepared by diluting the stock penicillin G solution with the appropriate phosphate buffer.

Stock Penicillin V (1.00×10^{-3} M)

0.0388 Grams of Penicillin V, K (Sigma, St. Louis, Missouri) were dissolved in the appropriate buffer and diluted to 100 mL.

Penicillin V (5.00×10^{-5} M to 5.00×10^{-4} M)

The stock penicillin V solution was diluted with phosphate buffer to make the standard solutions for the working curve.

Glutaraldehyde (2.5%)

10 mL of 25% Glutaraldehyde, purified Grade I (Sigma, St. Louis, Missouri) was diluted to 100 mL with 0.05 M phosphate buffer, pH 7.0.

Penicillinase (Calbiochem-Behring)

From Bacillus cereus 569/Hg; E.C. No. 3.5.2.6, M.W. 32,000. Activity was 0.16 I.U./mg dry weight.

Definition of I.U. for penicillinase: one I.U. is defined as the amount of enzyme which will catalyze the conversion of 1.0 micromole of penicillin per minute at 30°C, pH 7.0.

- p-(Aminophenyl)trimethoxysilane (Petrarch, Bristol, Pennsylvania)
- 3-(Aminopropyl)triethoxysilane (Petrarch, Bristol, Pennsylvania)
- N-2-Aminoethyl-3-aminopropyltrimethoxysilane (Dow Corning,

Midland, Michigan)

- (Aminoethylaminomethyl)phenethyltrimethoxysilane (Petrarch, Bristol, Pennsylvania)
- Hydrocholoric acid ("Baker Analyzed" Reagent Grade)
- Ammonium Bifluoride (Fisher-technical Grade)
- Methanol (Fisher, Certified A.C.S.)
- Dichloromethane (Fisher, Certified A.C.S.)
- Acetone (Fisher, Certified, A.C.S.)
- Glass beads, 1.0 mm (Propper, Long Island, New York)
- Sodium Hydroxide (Malinckrodt, Reagent)
- Porasil F, 75-125μ (Waters Associates, Milford, Massachusetts)

CHAPTER IV

STUDIES ON THE IMMOBILIZATION OF THE ENZYME PENICILLINASE

4.1 Introduction

According to the literature reviewed, the enzyme penicillinase has been immobilized by a variety of methods, most of them physical. A great majority of the work on immobilized penicillinase has been performed in the area of enzyme electrodes (14, 15, 16). The enzyme electrode is a combination of an immobilized enzyme and an electrochemical sensor (77).

The three main designs of penicillinase electrodes are: (a) containment in semi-permeable membranes; (b) entrapment in polymeric gels; and (c) adsorption onto fritted glass discs. Although containment of the enzyme by membrane was very simple, the stability of the immobilized enzyme was short (three weeks). Entrapment in polymeric gels was carried out by polymerizing a mixture of monomer and enzyme on the surface of a pH glass electrode (16). This electrode was not only sensitive to penicillin but also to monovalent cations. Enzyme electrode prepared by adsorption of the penicillinase to a fritted glass disc seems to be the best electrode configuration available. Immobilization by adsorption has the advantages of being simple resulting in high initial yields (78). It is also easy to recharge the enzyme

electrode with fresh enzyme, when the activity drops below a desired level (79).

Enzyme electrodes combine the selectivity of immobilized enzymes and the sensitivity of electrochemical detection. The main advantage of the enzyme electrodes is the simplicity with which determinations can be performed without resorting to lengthy sample preparations (78, 80)

The main drawback of enzyme electrodes is the inability to use them under flow conditions. The problem is due to the slow diffusion of substrate into the membrane and the release of products out of the membrane, when the enzyme is physically immobilized in a membrane. However, when enzymes are immobilized by adsorption there is the problem of slow desorption of the enzyme with continued decrease of activity (78).

Rusling and co-workers designed a continuous-flow system, with penicillinase covalently bound to controlled-pore glass, and used it in a packed reactor configuration (17), but this system had problems of back pressure and low sampling rates. This study is concerned with the development of better immobilization techniques and the design of a better reactor configuration.

4.2 Studies on the Immobilization of Penicillinase

4.2.(a) Choice of Carrier for Immobilization

The choice of carrier used for the immobilization of an enzyme is important, since its interaction with the enzyme may have an influence

on the stability and kinetics of the enzyme (81). The other factors that must be considered are the capacity of the carrier to bind the protein, the ease of derivatization, cost and availability. Glass was chosen because it is easily available, can be derivatized, and is inexpensive. Porasil (Waters Associates, Milford, Massachusetts) was selected for the silylation studies because: (a) it has a known surface area available for silylation; and (b) it is inexpensive.

4.2.(b) Choice of Coupling Agent

The use of glutaraldehyde is one of the simplest, most gentle, and rapid of the covalent coupling techniques available for enzyme immobilization (82). The coupling reaction can be carried out in aqueous solution within a rather wide range of pH values (5 to 9). The stability of penicillinase also falls in between pH 6 and 8. The glutaraldehyde is believed to couple with amino groups on the surface of glass to the ε-amino group of Lysine in the enzyme (81).

4.2.(c) Silylation Studies

Amino silanes are generally used to introduce amino groups on the surface of glass (83). Amino silanes have the general formula $X_3Si\text{-}RNH_2$ (where X is a hydrolyzable group). Most commercial silanes are available as methoxy or ethoxysilanes. These groups merely serve as intermediates for the formation of silanol groups for bonding to the glass surface. Reaction of the silanes to a carrier involves four steps (84) as shown in Figure 13.

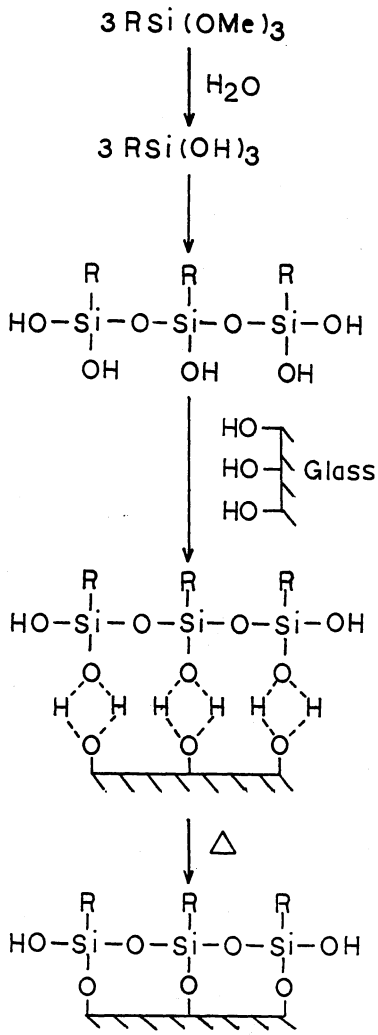
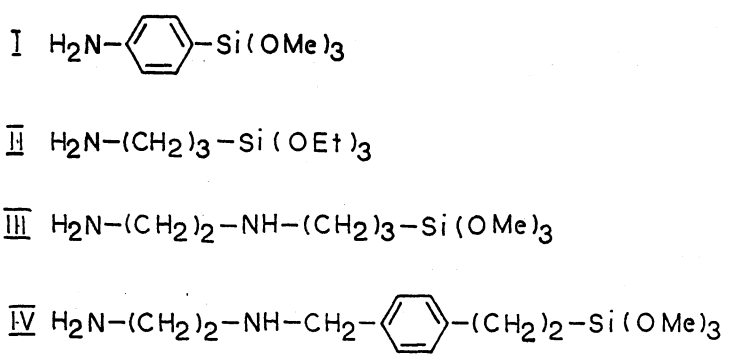


Figure 13. Steps Involved in the Silylation Process

First, hydrolysis of the three labile groups occurs. Then, condensation to oligomers follows. The oligomers then hydrogen bond with the -OH groups on the surface of glass. These hydrogen bonds are then converted to covalent bonds, in a step called curing, by heating to 80°C.

Silylation from toluene under reflux is said to deposit a monolayer of silane on the glass surface (84), while 3 to 8 molecular layers are formed when silylation is accomplished from aqueous alcoholic solutions. It has been pointed out by Johansson and others (85) that the degree of hydrophobility of a carrier has an influence on the enzyme uptake as well as the kinetics of the enzyme. There could also be problems due to partitioning of the substrate between the bulk of the solution and the hydrophobic carrier. The presence of multilayers, on the otherhand, could block the active site of the enzyme as a result of steric hindrance. In order to test if the presence of multilayers introduced any such problems, silylation was performed from aqueous alcoholic solution as well as from anhydrous toluene under reflux.

Four silanes, shown below, were evaluated for derivatization of the glass surface.



I: p-(Aminophenyl)trimethoxysilane
II: 3-(Aminopropyl)triethoxysilane

III: N-2-Aminoethyl-3-aminopropyl trimethoxysilane
IV: (Aminoethylaminomethyl)phenethyl-trimethoxysilane

The reasons for the selection of these silanes are as follows:

a) These are silanes of different chain lengths, chosen with the assumption that, overall, they will place the bulky enzyme at different lengths from the support matrix.

b) Silanes I and IV are aromatic, while II and III are aliphatic. These silanes would, therefore, shed light on the effect of aromaticity on the coupling process.

c) Silanes I and II are short and rigid while III and IV are long and flexible.

d) Silanes III and IV have four nitrogens capable of coupling to the enzyme, which could give a higher enzyme activity on the support.

4.2.(d) Procedure for Silylation from Toluene

A 1.0 mL portion of the respective silane (described earlier) was mixed with 9.00 mL of anhydrous toluene (dried over molecular sieves). To the above solution, 1.00 gram of porasil was added and degassed by application of vacuum, to fill all the pores in porasil with the silane solution. This mixture was refluxed for four hours followed by a brief wash with methanol. The porasil was then cured by heating it to 80°C for 12 hours.

4.2.(e) Procedure for Silylation from Alcoholic Solution

A 1.00 mL portion of the silane was mixed with 9.00 mL of 95% ethanol. A 1.00 gram portion of Porasil was added to this mixture and degassed by applying suction. This mixture was shaken in a mechanical shaker for two minutes, and the Porasil separated by filtration. The Porasil was then washed with a few milliliters of 95% ethanol and cured by heating to 110°C for 10 minutes.

4.2.(f) Procedure for Glutaraldehyde Coupling

To 0.5 gram of the silylated Porasil was added 20 mL of 2.5% solution of glutaraldehyde in 0.05 M phosphate buffer (pH 7.00). The solution-silica mixture was shaken in a shaker for 60 minutes, washed with plenty of distilled water to remove the unreacted glutaraldehye. Otherwise, the unbound glutaraldehyde could crosslink two enzyme molecules, thus, making it unavailable for bonding with the silylated silica.

4.2.(g) Procedure for Immobilization of Penicillinase

To 0.5 gram of the Porasil activated with the glutaraldehyde was added 10 mL of 0.05 M phosphate buffer (pH 7.00). The Porasil buffer mixture was cooled to 4°C in a refrigerator. The phosphate buffer was siphoned out of the vial containing the silica. The contents of one vial of penicillinase (1695 I.U./vial) were quantitatively transferred

using a minimum amount of phosphate buffer at 4°C, into the vial containing silica at 4°C. The vial was shaken in a shaker for four hours at 4°C. The silica was then washed successively with distilled water, 1.0 M KCl, and 0.05 M phosphate buffer (pH 7.00). The silica containing the immobilized penicillinase was then stored at 4°C in the above buffer for further studies. Reactions leading to the glass-penicillinase derivative are shown in Figure 14.

4.2.(h) Rate Studies with Immobilized Penicillinase

The initial rate of the penicillinase catalyzed hydrolysis of penicillin to penicilloic acid was used to measure the enzyme activity on the porasil.

The rationale for the initial rate measurement is as follows. Consider the conversion of a substrate 'S' into product 'P', in which an enzyme 'E' acts as a catalyst.

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} E + P$$

The initial velocity for this type of a system can be given by the Michaelis Menten equation (85).

$$V_o = \frac{V_{max}\,[S]}{K_m + [S]}$$

where V_o = initial velocity of the reaction
V_{max} = maximum velocity of reaction
= $k_{+2}[E_T]$, when the enzyme is saturated with substrate
E_T = total enzyme concentration
K_m = Michaelis Menten constant
= $\frac{k_{-1} + k_{+2}}{k_{+1}}$

Substituting for V_{max} in the Michaelis Menten equation the following

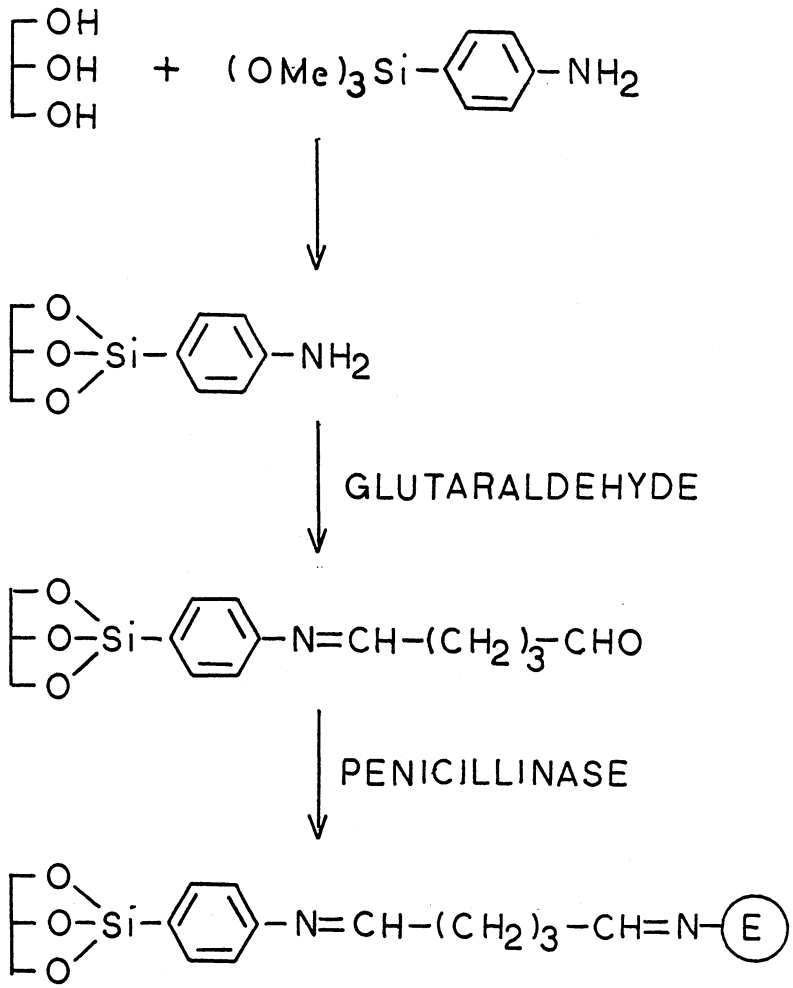


Figure 14. Synthetic Route for the Immobilization of Penicillinase

is obtained.

$$V_o = \frac{k_{+2} [E_T] [S]}{K_m + [S]}$$

When the substrate concentration is very high compared to K_m, the equation can be rewritten as follows.

$$V_o = k_{+2} [E_T]$$

Thus, the initial rate of an enzyme catalyzed reaction in the presence of a large excess of substrate is directly proportional to the total concentration of an enzyme. Therefore, the penicillinase activity on the various Porasils was indirectly measured, by measuring the initial rate of the penicillinase catalyzed hydrolysis of penicillin to penicilloic acid, in the presence of excess penicillin.

Procedure for the Measurement of Initial Rates. 0.500 Gram of the Porasil containing the immobilized penicillinase was taken in a 50 mL vial. 10.0 mL of 0.050 M phosphate buffer (pH 7.00, 0.10 M in KCl) was added to the vial, and a combination pH electrode was dipped into the solution. 10.0 mL of 0.100 M penicillin G solution was added to the vial and the rate of change of pH was monitored with a Radiometer pH meter.

While monitoring the initial rate of the reaction, a good mixing was achieved by bubbling nitrogen continuously into the solution. This study was conducted at room temperature. The decrease in pH was indirectly measured by following the change in voltage. As seen earlier in Chapter II, it is more sensitive to measure the change in voltage, rather than the change in pH.

Results and Discussion. The initial rates of the penicillinase

catalyzed hydrolysis of penicillins is shown in Table VI.

TABLE VI

INITIAL RATE STUDIES WITH PENICILLINASE IMMOBILIZED ON PORASIL

Silane Used	Rate (mv/min.)	
	Anhydrous Toluene	95% Ethanol
I	406 ± 18	485 ± 28
II	307 ± 32	306 ± 10
III	177 ± 18	132 ± 20
IV	130 ± 10	101 ± 17

There seems to be an inverse relationship between the rate of the reaction, and the length of the silane used in the silylation process, other variables being constant. Based on this, the use of short rigid silanes (I and II) that put the enzyme close to the support should be preferred to long and flexible spacers for the immobilization of penicillinase. Although silanes III and IV have two nitrogens capable of coupling, the rate is still very low. Due to the long length and flexibility of silanes III and IV, they could be coupled, intermolecularly, with glutaraldehyde. Even if they are free and immobilized the enzyme, they could put the enzyme so close to the matrix that the active site is not accessible to the substrate.

The deposition of multilayers of silane, if taking place, from 95% ethanol solutions does not pose any problems as evidenced by the similar rates when compared to silylation from anhydrous toluene. Silylation from 95% ethanolic solution is simple and rapid when compared to the tedious refluxing conditions from anhydrous toluene. As silane I (p-aminophenyl trimethoxysilane) gave the maximum rate, it was chosen for use in the immobilization of penicillinase in further studies.

4.2.(i) Effect of pH

pH has a marked effect on the activity of enzymes and hence, on the rate of an enzyme catalyzed reaction. At extremes of pH the enzyme may undergo an irreversible denaturation. The pH corresponding to the maximum activity is called the optimum pH, around which the enzyme activity is reversible. This is the pH which is of interest to the analytical chemist.

To study the effect of pH on the activity of the immobilized penicillinase, the manifold described in Chapter III was used. A 1 mM penicillin G in 1 mM phosphate buffer (I=0.10) was injected into a 1mM phosphate buffer carrier (I=0.10), passed through the SBSR, and the resulting signals recorded. Each time the phosphate buffers were adjusted to different pH values. A plot of the peak height versus pH is shown in Figure 15. A maximum occurs around a pH of 6. However, at pH values greater than 7, the peak height increases. This does not mean that there are several optimum pH's. There occurs a false minimum around a pH of 7. The minimum observed in this region is due to the maximum buffering capacity experienced around the pKa_2 of phosphoric acid (7.20) (86).

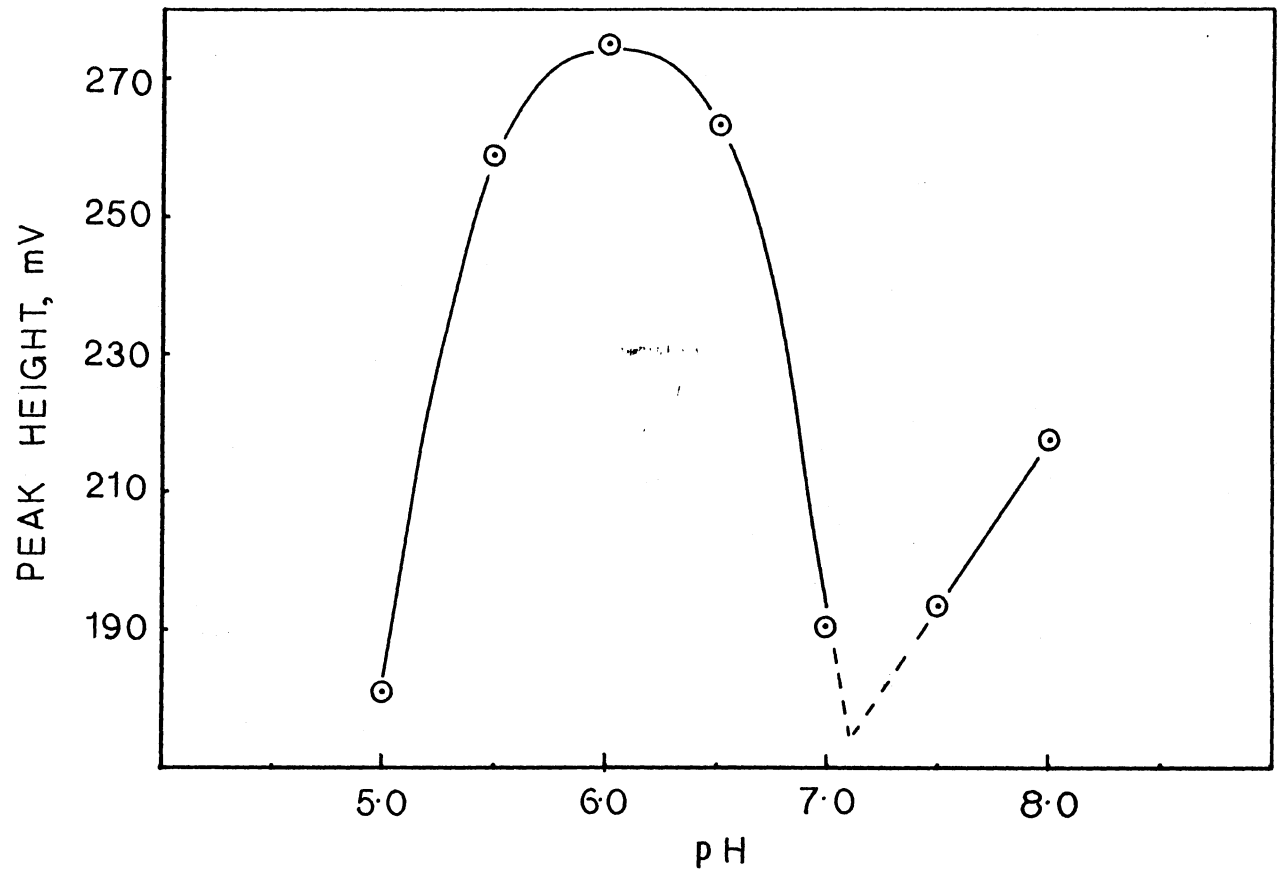


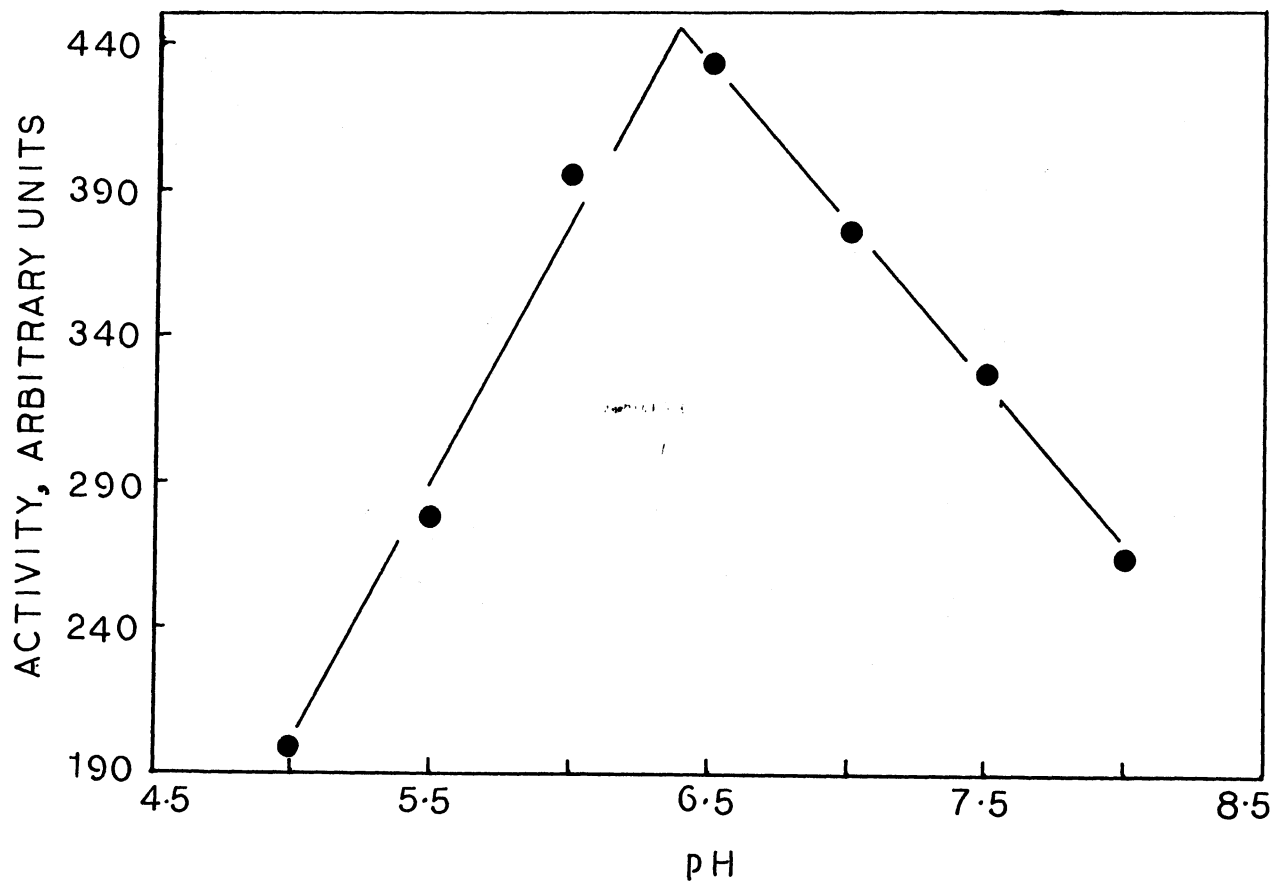
Figure 15. Effect of pH on the Peak Height

To get a meaningful pH optimum, the buffering capacity at different values of pH have to be considered. In highly buffered solutions, as will be seen later, peak width will be very small. Therefore, a plot of peak height/width (which takes into consideration the buffering action) against pH, should give one optimum pH, as shown in Figure 16. The peak height/width, corresponding to maximum enzyme activity, passes through a maximum around a pH of 6.4 which coincides with the pH optimum of free penicillinase (87). The change in activity of an enzyme with pH is due to the amphoteric nature of the amino acids which make up the enzyme, specifically those affecting the binding of the substrate (88). To ensure stability of the immobilized penicillinase and achieve maximum sensitivity, solutions were maintained at a pH of 6.4 in all further studies.

4.2.(j) Effect of Temperature

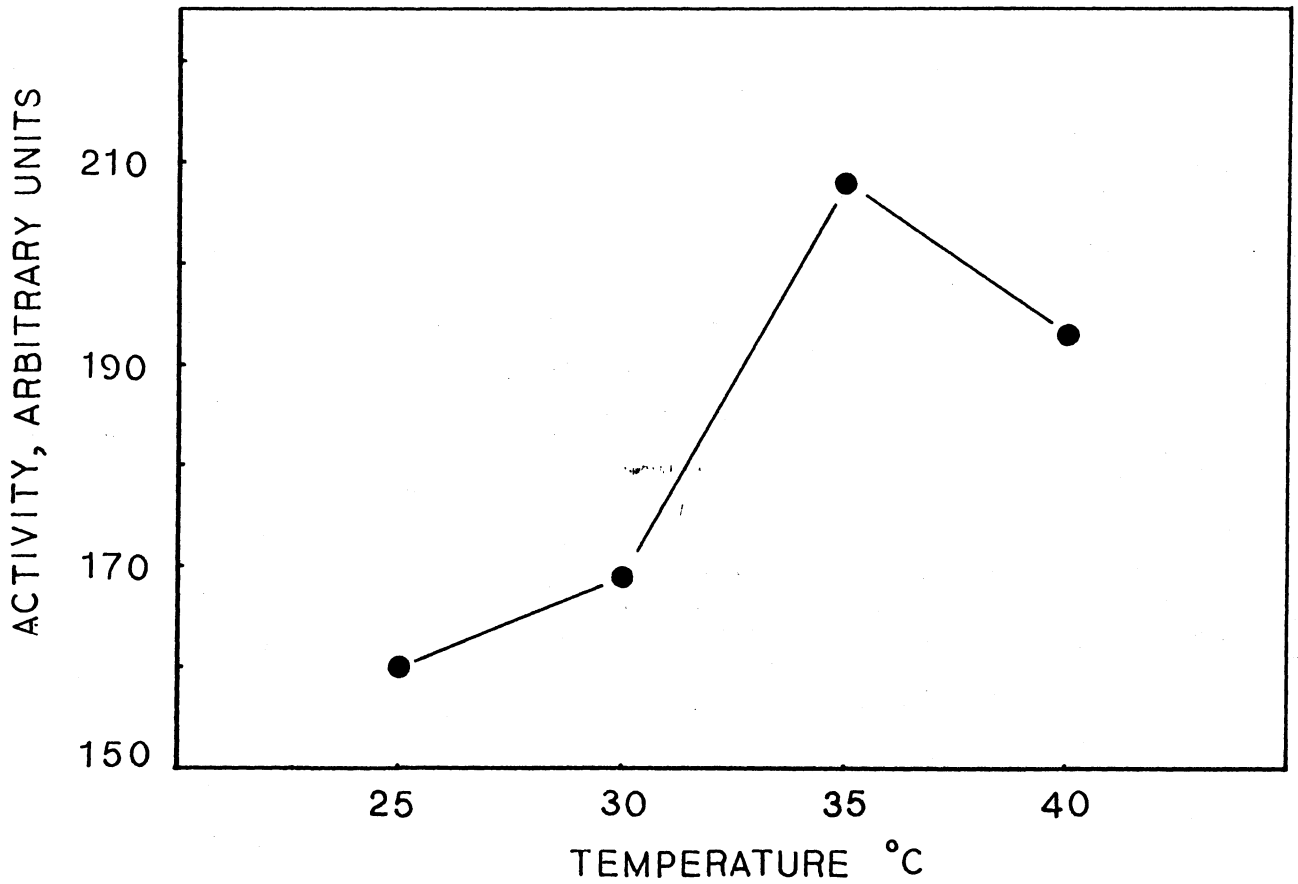
Temperature has an influence on the rate of an enzyme catalyzed reaction. The effect of temperature on the activity of immobilized penicillinase is depicted in Figure 17. Penicillinase exhibits maximum activity around 35°C. The observed change in the activity of the enzyme may be due to a change in the velocity of the breakdown of the enzyme-substrate complex. The decrease in activity at very high temperatures was a result of enzymatic breakdown. Usually enzymatic analyses are carried out at the optimum temperature (temperature at which activity is maximum).

The optimum temperature was not maintained in the flow system, due to the problem of dissolved N_2 getting released at temperatures higher than 30°C. In all the studies involving the determination of penicillins



Activity = Peak Height/Width

Figure 16. Effect of pH on Peak Height/Width



Conditions: 1) 1 mM phosphate buffer, pH 6.40, I=0.10
2) Substrate 1 mM penicillin G in above buffer

Figure 17. Effect of Temperature on the Activity of Immobilized Penicillinase

in real samples, the solutions were thermostated to 25°C.

4.2.(k) Stability of Immobilized Penicillinase

As discussed earlier in Chapter II, penicillinase in solution is stable for only a few hours at room temperature. A concentrated solution (10 mg/mL), on the other hand, is denatured in 15 minutes (69).

The long term stability of immobilized penicillinase is depicted in Figure 18. It retains more than 97% of its initial activity even after ten months of usage. Although the mechanism of the increased stability due to enzyme immobilization is not known, it is believed that the covalent binding of an enzyme to a matrix prevents denaturation by preventing the unfolding of the enzyme. It is accepted that unfolding of the protein is the major cause of enzyme denaturation (81). The loss of activity of an immobilized enzyme can occur due to microbial attack.

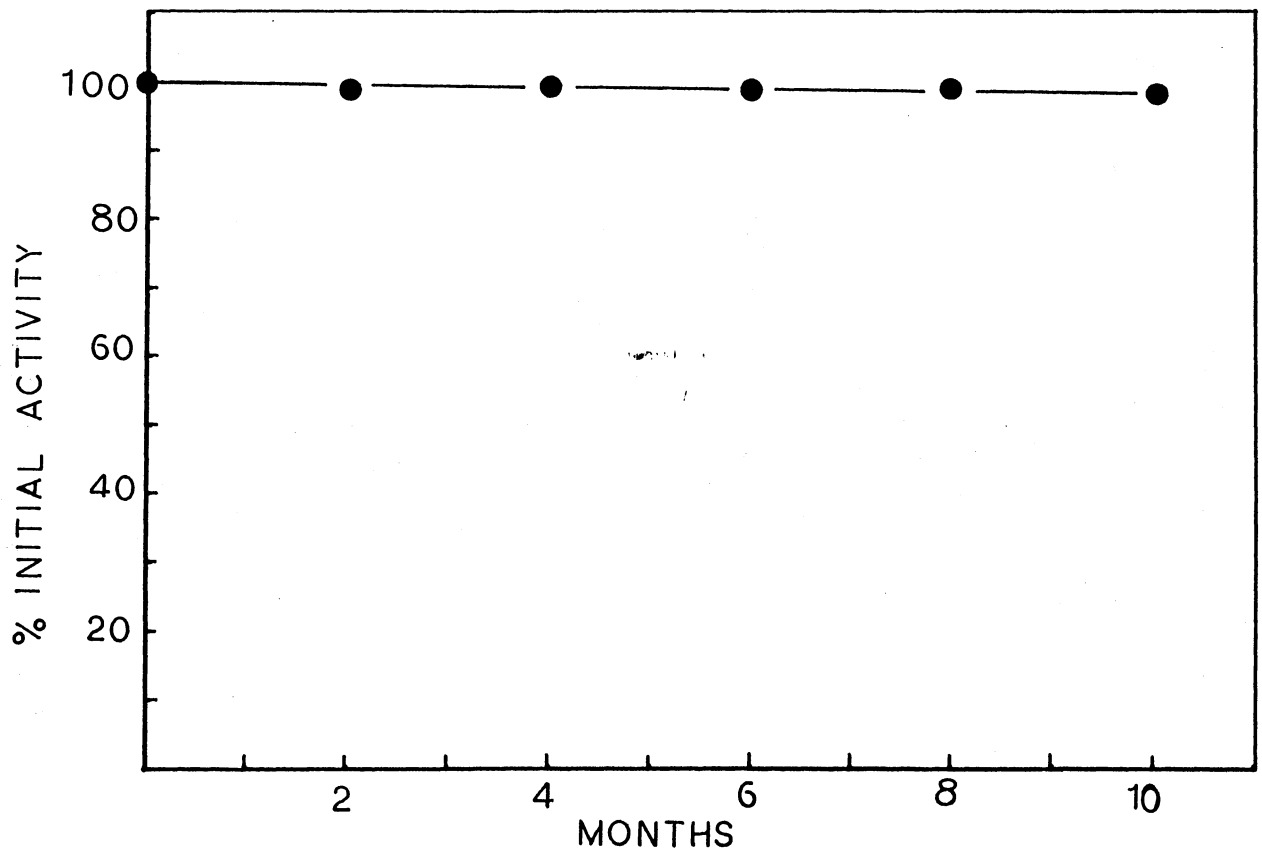


Figure 18. Long Term Stability of Immobilized Penicillinase

CHAPTER V

DESIGN OF PENICILLINASE REACTORS

5.1 Introduction

As discussed in Chapters I and II, the use of packed columns introduce pressure drop across the column. Peristaltic pump, the heart of a flow injection system, cannot be easily used with a packed reactor. Attention has been focused in this study, on the development of a new reactor configuration without much pressure drop. Glass was selected as the carrier because: (a) glass open tubular reactors of any inner diameter can be easily drawn with a capillary drawing machine; and (b) penicillinase immobilized to glass is very stable. Stabilities of eight months have been reported (17).

Several factors have to be taken into consideration in the design of an enzyme reactor for use in a continuous-flow system. According to enzyme kinetics, a high local activity of enzyme in the reactor is necessary for the determination of substrates (81). The high activity of enzyme should be present in the shortest length of a reactor as possible to decrease dispersion of the sample plug. It has been shown that dispersion increases with the length of the reactor (89). As discussed in Chapters I and II, an enzyme has to be covalently coupled for use in a continuous-flow system. Summarizing, the ideal characteristics of a reactor for use in a continuous-flow system are: (a) covalent

attachment of enzyme to the reactor; (b) high local activity of enzyme; (c) minimum length of reactor; and (d) low pressure drop across the reactor

The reactor configurations studied include: (a) glass open tubular reactor; (b) single bead string reactor (glass beads in glass tubing); (c) single bead string reactor (glass beads in teflon tubing); and (d) multicapillary coil.

5.2 Preparation of Glass Open Tubular Reactor

Iob and Mottola used glass open tubular reactors (OTR) for the immobilization of uricase (90). They used the procedure of Onuska and coworkers (91) to roughen the inner surface of glass capillary tubing. Roughening of the inner surface of glass capillary is necessary to increase the surface available for the immobilization of the enzyme in the reactor.

A pyrex glass tubing [9 mm outer diameter (O.D.) and 7 mm inner diameter (I.D.)] was drawn on a Hewlett Packard (Model 1045A) capillary drawing machine to yield a glass capillary of 1.4 mm I.D. Whiskers were grown inside the capillary following the procedure of Onuska and coworkers. During the process of whisker growth, the metal ions from the bulk of the glass migrate to the surface (92). These metal ions were leached out with concentrated HCl at 80°C overnight. This is necessary to release the silanol groups for silylation. The coil was washed successively with 20% HCl (to prevent readsorption of metal ions) and water.

2.0 mL of p-(aminophenyl)trimethoxysilane (the silane that immobilized the maximum amount of penicillinase) was added to 18.0 mL of 95% ethanol. Fifteen minutes was allowed for the hydrolysis of silane

to silanol. The coil was filled with the silanol solution and allowed to stand for five minutes. The solution was removed, and the capillary rinsed briefly with a few milliliters of 95% ethanol. Curing was accomplished by heating the coil to 110°C for 10 minutes, with nitrogen flowing through the coil.

A 2.5% solution of glutaraldehyde in 0.05 M phosphate buffer (pH 7.00) was pumped through the coil for one hour. The unbound glutaraldehyde was removed by aspirating distilled water through the coil.

About 835 units of penicillinase in a minimum volume of 0.05 M phosphate buffer (pH 7.00) was injected into the coil at 4°C and the coil stored in the refrigerator overnight. The coil was then rinsed with water, 1.0 M KCl, and 0.05 M phosphate buffer (pH 7.00).

5.3 Preparation of Single Bead String Reactors

The single bead string reactor (SBSR) was introduced by Reijn and co-workers (93) to increase radial mixing in a sample plug, for use in Flow Injection Analysis (FIA). A single bead string reactor was constructed for two reasons: (a) the beads would provide more surface area for the immobilization of the enzyme; and (b) the single bead string reactor offers tenfold decrease in dispersion when compared to an open tubular reactor of the same dimensions (93).

5.3.(a) Glass Beads Packed in Glass Tubing

1.0 mm glass beads (Propper Manufacturing Corporation, Long Island, New York) were leached with concentrated HCl in a soxhlet extraction apparatus overnight. This enabled the removal of all the metal ions from the surface of the beads. The beads were then washed with water,

methanol, acetone, and dried at 140°C for one hour. After this treatment, the beads were coated with a saturated solution of $NH_4F \cdot HF$ solution. The methanol, on the beads, was evaporated by applying vacuum for 5 to 6 hours. The beads were then packed in a glass tube, the tube sealed by fusing the ends, and heated to 450°C for three hours. The beads were then washed with methanol and subjected to the soxhlet leaching procedure with concentrated HCl as described earlier.

The silylation was performed with p-(aminophenyl)trimethoxysilane similar to the procedure with the glass open tubular reactor.

The beads were then packed in a 1.4 mm I.D. glass capillary coil (1 m long, coil diameter-6 cm) that had been etched with $NH_4F \cdot HF$ and silylated using p-(aminophenyl)trimethoxysilane. The packing resulted in a zigzag pattern, as shown in Figure 19, which was responsible for the increased radial mixing in the reactor.

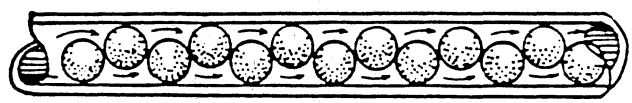


Figure 19. Schematic Diagram of a SBSR

The beads were held in place in the reactor by fusing a piece of capillary, whose O.D. was smaller than the I.D. of the reactor, on both the ends of the SBSR.

Coupling of penicillinase to the inner walls of the glass capillary

tubing and the bead surface was performed as described in Chapter IV.

5.3.(b) Glass Beads in Teflon Tubing

In earlier studies with glass open tubular reactors and glass single bead string reactors, it has been shown that the use of glass beads in glass tubing reduces the amount of enzyme immobilized on the walls of the capillary tubing (94). It was pointed out that the immobilization of enzyme on the beads alone may be sufficient. This led to the development of a single bead string reactor using teflon tubing. A 1.3 mm I.D. teflon tubing was packed with 1.00 mm glass beads, that had been previously silylated. A small piece of teflon tubing was inserted at both the ends of the reactor to hold the beads in place. The immobilization of penicillinase was performed as described earlier.

5.4 Preparation of Glass Multicapillary Coil

As an alternative to packing glass beads in a tube to increase the surface area, the packing of capillaries inside a tube was considered. A pyrex glass tubing (9.0 mm O.D. and 6.3 mm I.D.) was cleaned with a cycle of solvents (methanol, dichloromethane, and acetone) as described by Onuska and co-workers (91). The cleaned glass tubing was drawn into 1.0 m long glass capillaries (2.6 mm O.D. and 1.68 mm I.D.). Seven of these capillaries were then packed into a 1.0 m long glass tubing described above. The capillaries drawn had an O.D. such that there was a snug fit inside the glass tubing.

The packed tube was then cleaned with the cycle of solvents, dried and drwan with the Hewlett Packard capillary drawing machine to give a capillary tubing (1.75 mm O.D. and 1.33 mm I.D.) with seven capillary

tubings packed inside as shown in Figure 20.

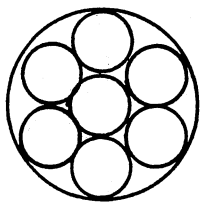


Figure 20. Cross-sectional View of a Multicapillary Coil

The coiling on the machine could not be done automatically because of the large mass of glass. Therefore, the above multicapillary was drawn out into a straight tube, 1 m in length, and then manually coiled in the Hewlett Packard coiling tube.

The multicapillary coil was then subjected to all the steps, already described for the open tubular reactor.

5.5 Observations on the Preparation of Reactors

The preparation of the glass open tubular reactor was the simplest of all the reactors. The single bead string reactor with glass tubing and the glass multicapillary coil had a severe strain on the glass and snapped very easily. The multicapillary coil was very brittle, probably from the ammonium bifluoride etch. The single bead string reactor with teflon tubing was easy to construct and handle. This single bead string reactor could be coiled to any coil diameter, which was not possible with

the glass tubing.

5.6 Results and Discussion

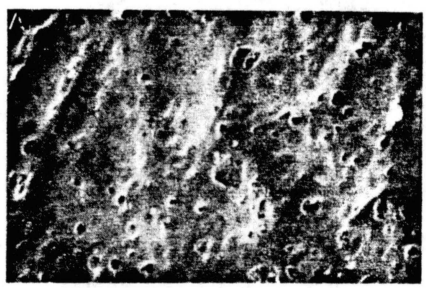
5.6.(a) Effect of Ammonium Bifluoride Etching

The increase in the available surface area on the beads and in the inner walls of capillary tubing due to the ammonium bifluoride treatment is shown in Figure 21.

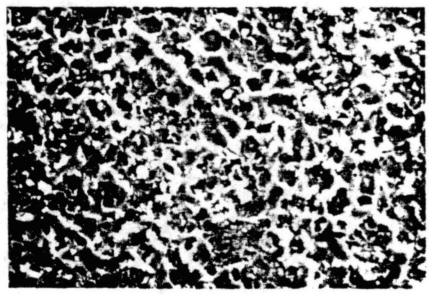
Although the mechanisms of the etching process has not been solved, it is believed that ammonium bifluoride on heating releases HF gas which then reacts with the glass to form "whiskers" in glass tubing and "volcanic crater erosion" on the glass beads (94). The net result as shown in Figure 21 was a tremendous increase in the surface area available for the immobilization of penicillinase.

5.6.(b) Comparison of Open Tubular Reactor and Single Bead String Reactor

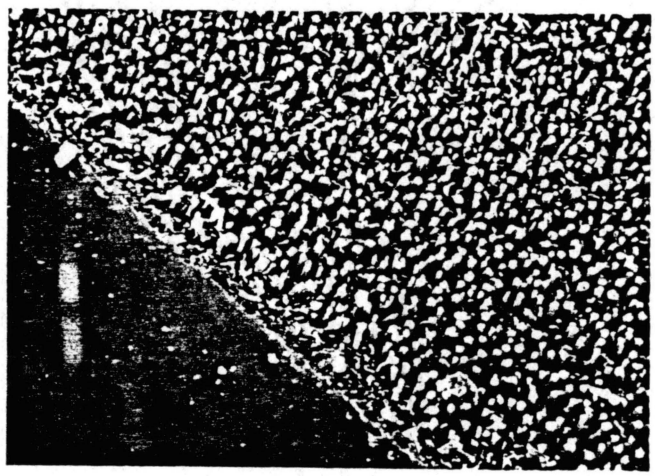
Table VII summarizes the performance of an open tubular reactor when compared to a single bead string reactor of the same dimensions. The peak for a single bead string reactor was 2.5 times higher than an open tubular reactor, offering a much better sensitivity. The width at half peak height was also smaller enabling a greater sampling rate. The large radial dispersion introduced by the geometry of the packing was responsible for the reduction in peak width. The increase in area under the peak can be explained as due to a combined effect of radial dispersion and increased surface area (available for enzyme coupling).



A



B



C

A: 5000 magnification of an untreated bead
B: 5000 magnification of an etched bead
C: 2500 magnification of a glass capillary (untreated surface is shown on the bottom left-hand corner)

Figure 21. Scanning Electron Micrographs of Etched Glass Beads and Capillaries

TABLE VII

COMPARISON OF GLASS OPEN TUBULAR REACTOR
AND SINGLE BEAD STRING REACTOR

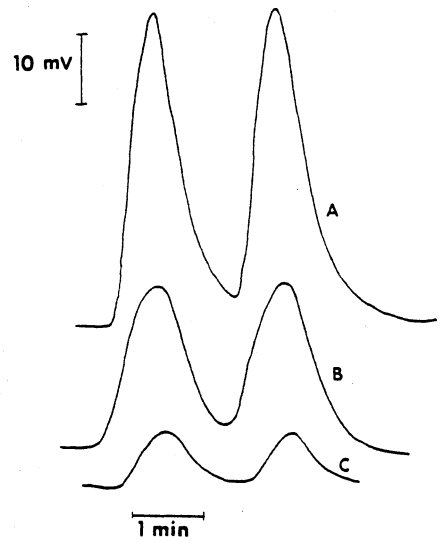
	Open Tubular Reactor	Single Bead String Reactor
Peak height, mv	17.6	44.0
Width at half peak height, arbitrary units	9	6
Area under the peak, arbitrary units	330	585

Carrier: 5.0×10^{-4} M phosphate buffer, ph 7.04
Sample Injected: 1.00 mL of 8.0×10^{-4} M Penicillin V

A comparison of signals obtained with and without enzyme on the glass beads is shown in Figure 22. These curves show that the use of glass beads in a glass tubing reduces the amount of enzyme immobilized on the walls of the capillary tubing. This emphasized that immobilization of enzyme on the beads alone is sufficient, which led to the development of the single bead string reactor with teflon tubing and glass beads, as mentioned earlier.

5.6.(c) Comparison of Diffusion in Reactors

Prior to the immobilization of penicillinase in the various reactors, a study was conducted to measure the extent of dispersion. The continuous flow manifold used was the same as in Figure 10. The carrier solution was a 1.00×10^{-3} M phosphate buffer (pH 7.00, 0.1 M in KCl).

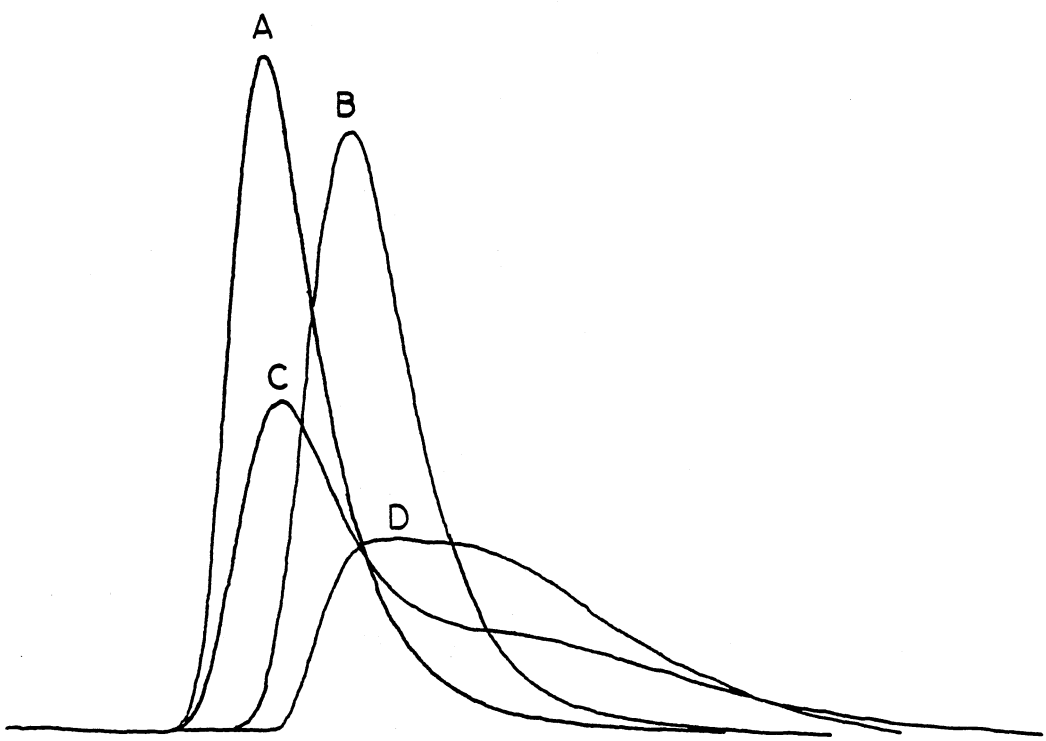


A: Single bead string reactor with enzyme immobilized on both the walls and the beads.
B: Same reactor as in A but with the beads removed.
C: Same reactor as in B but packed with untreated glass beads.

For the three curves, the carrier (5.0×10^{-4} M phosphate buffer, pH 7.00, and 0.10 M KCl), and the injected sample (1 mL, 0.8 mM Penicillin V in the same buffer) were the same.

Figure 22. Signals Obtained with Open Tubular Reactor and Single Bead String Reactor

A pressurized reservoir was used to pump the carrier. The injected sample was a 194 μL pH 5.80 phosphate buffer (1.00×10^{-3} M and 0.100 M in KCl). The injected buffer passed through the reactor and was sensed by the capillary pH electrode. The resulting transient signals were recorded on a strip chart recorder. A large dispersion was reflected as short and broad peaks. Typical signals obtained with the reactors are shown in Figure 23 and the data summarized in Table VIII.



A: SBSR (glass beads in teflon tubing)
B: SBSR (glass beads in glass tubing)
C: Multicapillary coil
D: Open tubular reactor

Figure 23. Comparison of Diffusion in Reactors

TABLE VIII

DISPERSION IN REACTORS

Reactor	Peak height mv. (SD)	Peak area cm^2 (SD)	Peak width cm (SD)
SBSR[1]	99.5 (0.3)	23.1 (0.3)	7.73 (0.7)
OTR[2]	42.7 (0.3)	24.4 (0.3)	11.9 (0.6)
MCC[3] (Channels open)	74.1 (6.7)	20.5 (8.1)	11.0 (1.4)
MCC[4] (Channels closed)	76.1 (6.6)	25.1 (0.8)	12.2 (0.7)

1: Single bead reactor
2: Open tubular reactor
3: Multicapillary coil (channels around the inner capillaries open)
4: Multicapillary coil with channels closed
Carrier solution: 1.00×10^{-3} M phosphate buffer, pH 7.00, 0.100 M in KCl
Injected Sample: 1.00×10^{-3} M phosphate buffer, pH 5.80, 0.100 M in KCl, 194 µL

In unsegmented continuous-flow analysis, peak height is the most useful peak parameter for the purpose of quantification (89). Single bead string reactor, not only gives the maximum peak height, but also a good reproducibility. This shows that the injected sample plug undergoes minimum dispersion. The minimum peak width was obtained with the single bead string reactor. Therefore, the single bead string reactor should give the maximum sampling rate.

Although the precision was about the same in an open tubular reactor, when compared to a SBSR, the peak height was only about 43% of

SBSR. This would reduce the sensitivity in half. The decrease in peak height was due to the great longitudinal dispersion suffered in an open tubular reactor. Taylor (95) and Aris (96) studied the dispersion in open tubular reactors, under conditions of laminar flow. They showed that the velocity of the fluid at the center of the tube is two times that at the wall. This is the major contributor to peak broadening in flow injection analysis. Taylor (95) and Aris (96) also showed that a decrease in the radius of the reactor reduces the dispersion. A decrease of radius would, however, reduce the available surface area.

An increase in surface area was accomplished, while decreasing the radius, by the use of the multicapillary coil. The main drawback with the multicapillary coil was the presence of channels surrounding the capillaries. The flow rate in the channels was so slow that double peaks (or split peaks) were obtained in a flow injection analysis system.

The decrease in dispersion was evidenced by taller peaks, when compared to an open tubular reactor. Although bigger signals were obtained, it cannot be used in flow injection analysis because of the poor reproducibility in the signal heights, as well as area.

An attempt was made to block the channels by plugging the channels with wax. Only one side could be plugged by inserting thin wires into the capillary tubing and sucking molten wax. This, however, did not improve the precision, which might be due to the suction of solution at the reactor exit by capillary action (where channels were open). From the viewpoint of dispersion alone, single bead string reactor appears as the best reactor configuration.

5.6.(d) Comparison of Reactors with Immobilized Penicillinase

The reactors described in the beginning of this chapter, with covalently bound penicillinase, were tested in the unsegmented continuous flow manifold described in Chapter III. The results are summarized in Table IX.

TABLE IX

PERFORMANCE OF REACTORS IN A CONTINUOUS-FLOW SYSTEM

Reactor	Peak Height mv.	Peak Width min.
OTR	54.2	2.0
MCC	91.0	2.4
SBSR (Teflon tubing)	185.0	1.5

Carrier: 1.00×10^{-3} M phosphate buffer
Sample Injected: 1.00×10^{-3} M Penicillin G in above buffer, volume - 194 µL
Flow Rate: 1.6 mL/min

The results follow the same trend as discussed earlier. The peak height enhancement in the SBSR, when compared to an OTR, was 340% against 230% when dispersion alone is considered. This can be explained as due to a decreased dispersion, and increased enzyme activity as a result of

greater surface area. Thus, the single bead string reactor using teflon tubing is easy to construct, easy to use (not fragile), can be coiled to increase secondary flow, thereby decreasing dispersion. Consequently, the single bead string reactor prepared by packing glass beads into a teflon tubing was adopted for use in the continuous flow determination of penicillins described in Chapter VI.

CHAPTER VI

STUDIES WITH A SBSR IN A CONTINUOUS-FLOW SYSTEM

The penicillinase-catalyzed hydrolysis of penicillin to penicilloic acid involves the release of protons. The simplest detector is a glass pH electrode. It has to be pointed out that other UV-Vis detectors using dye coupling reactions cannot be used directly in conjunction with an immobilized enzyme reactor. The adsorption of dyes onto enzymes could create complications. They could only be used in a post column reactor, which increases dispersion of the sample. However, pH sensing has its own disadvantages such as: (a) the resulting pH change could affect the enzyme activity; (b) the pH response is a logarithmic function of the analyte concentration and hence, is hard to reproduce; and (c) any buffer present in the sample or carrier may affect the response (97).

Rusling and co-workers (17) used a slightly buffered carrier solution, to produce a useful range of linear response for analytical determinations. The reason for the linear response at low concentrations of analyte has already been explained in Chapter II. The same principle was used to produce a linear response with substrate concentration.

6.1 Effect of Buffer Capacity

The nature of the buffer plays an important role in the

stabilization of an enzyme (81). Phosphate or Tris buffers of the same pH do not have the same surface tension, charge and other properties, that may affect the conformation of the enzyme and its activity and stability. The use of a phosphate buffer has been found to be successful for immobilized penicillinase (17). As a result, it was retained for use in the continuous-flow system.

The buffer capacity of the phosphate buffer is important, as the change of pH due to enzymatic hydrolysis is monitored in the continuous-flow system. The single bead string reactor using teflon tubing was used in the continuous-flow manifold described in Chapter III. Various concentrations of penicillin G, in the same buffer as the carrier solution, were injected into the SBSR and the resulting transient signals recorded on a strip-chart recorder. This study was conducted at five concentrations of buffer. A plot of peak height as a function of penicillin G concentration at various buffer concentrations is shown in Figure 24.

The potentiometric response of the system, E_p, as already seen in Chapter II, can be give as

$$E_p = 59.2 \times \frac{V_m \Delta t S}{K_m \beta}$$

where the terms have their usual meanings. This equation predicts that if V_m/K_m and β remain constant or suffer small changes in compensating directions, the system response will be a linear function of penicillin concentration. The equation has been found to hold good at low concentrations of penicillin. At higher substrate concentrations, V_m/K_m is lowered because of a drop in the enzymatic activity due to a decrease in pH. When β, the buffer capacity, is high, the change in pH is small.

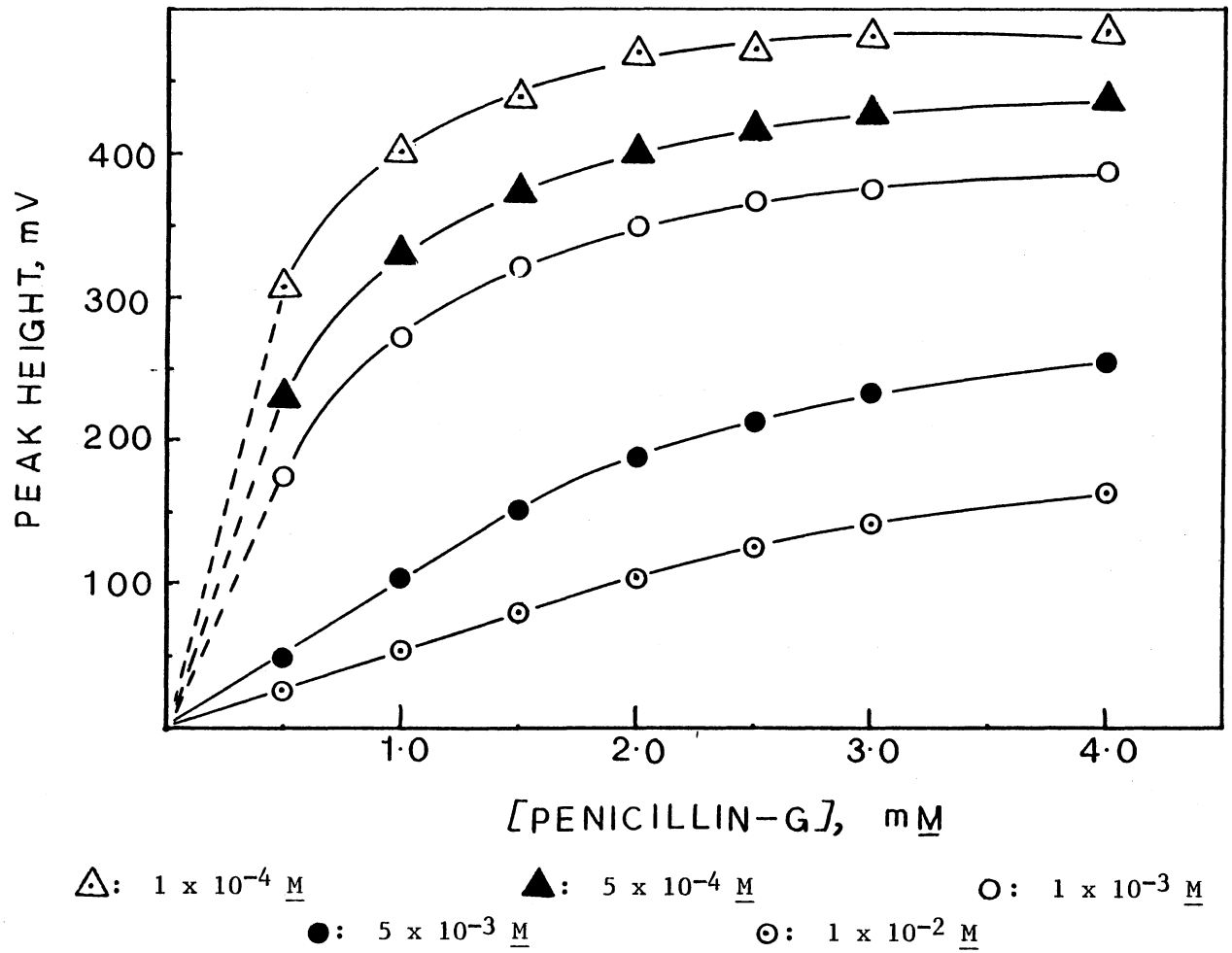


Figure 24. Effect of Buffer Concentration on the Working Curve

This accounts for the larger linear range at high concentrations of buffer. Also, as predicted by the above equation, the response is smaller when β is high. Buffer capacity, therefore, can be used to alter the sensitivity of the determination, as well as the linear range of the working curve.

The effects of buffer capacity on the height and width of the signal are shown in Figures 25, 26, and 27.

The reason for the increase in peak height with decreasing buffer concentration has been explained earlier. The buffer capacity cannot be decreased indefinitely to increase the sensitivity. As shown in Figure 24, peak width increases with decreasing buffer concentration. This can be explained as follows. In a highly buffered carrier, the buffer is able to penetrate deeper into the same plug and neutralize the hydrogen ions produced as a result of enzymatic hydrolysis. This results in narrower peaks. However, in a slightly buffered solution, the buffering action is experienced only at the edges of the sample plug, resulting in broader peaks.

In order to arrive at a compromise between sampling rate and sensitivity, the peak height/width was plotted against the concentration of phosphate buffer used to make up the solutions. The maximum was obtained around a buffer concentration of 1.00×10^{-3} M. This optimum buffer concentration was used in further studies.

6.2 Effect of Sample Size

> Changing the injected sample volume is a powerful way to change dispersion. An increase in peak height and in sensitivity of measurement is achieved by increasing the volume of injected sample solution (89, p.19).

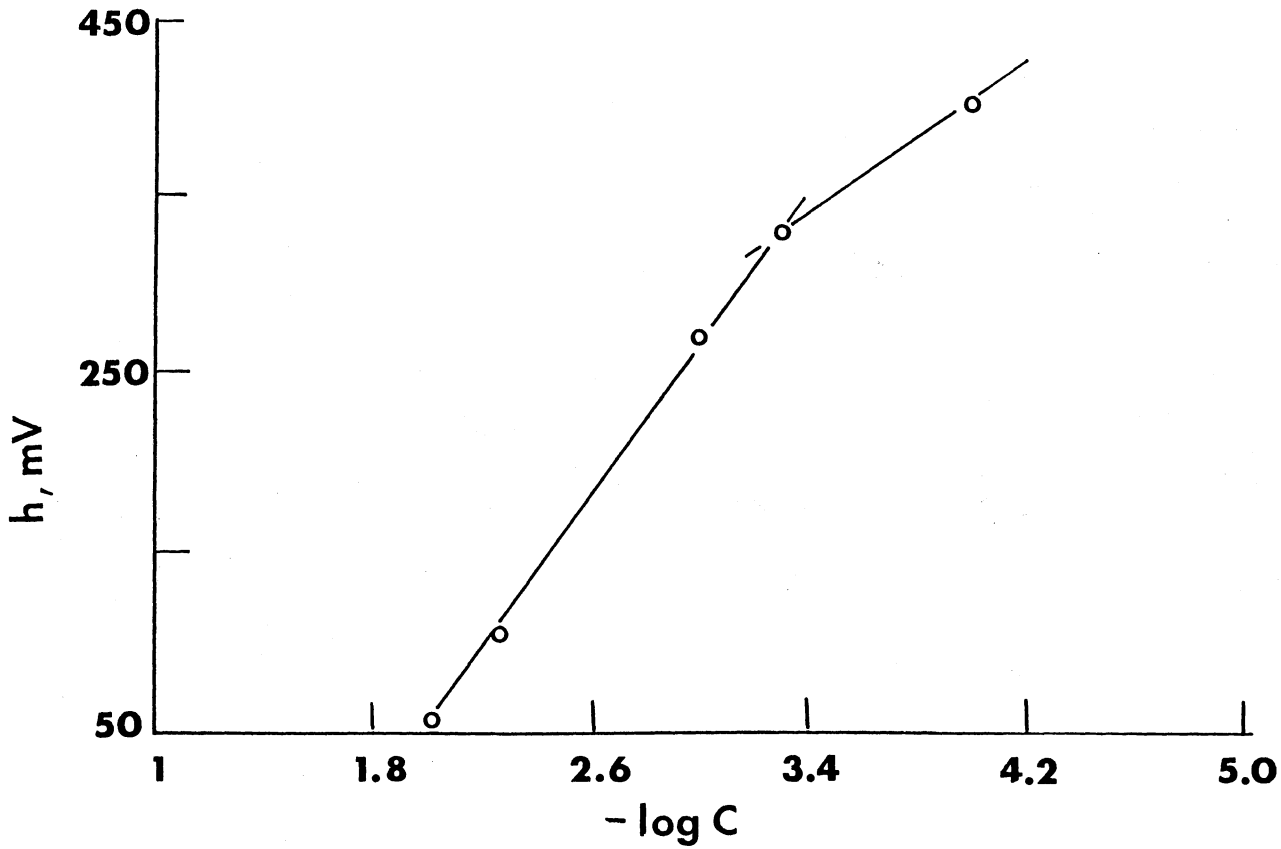


Figure 25. Effect of Buffer Concentration on Signal Height

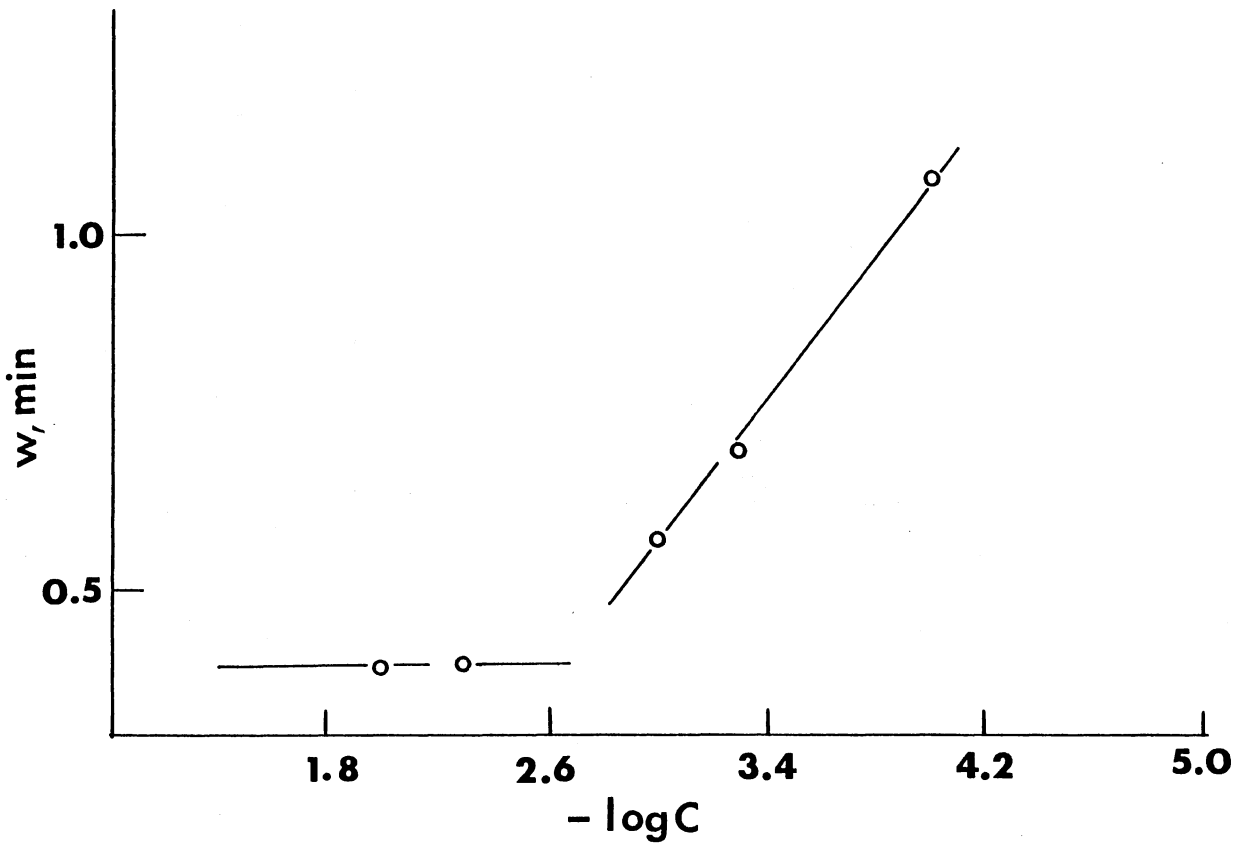


Figure 26. Effect of Buffer Concentration on Signal Width

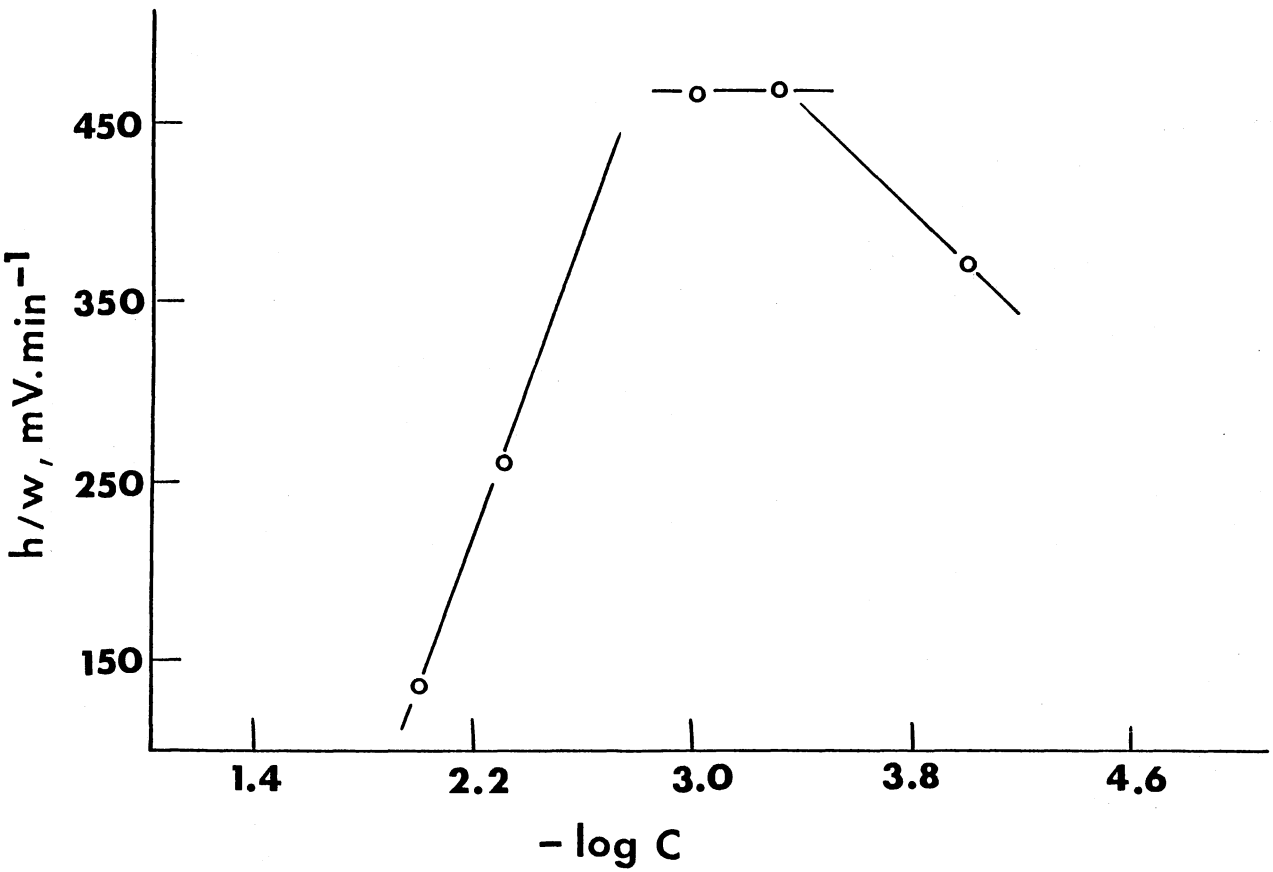


Figure 27. Effect of Buffer Concentration on Signal Height/Width

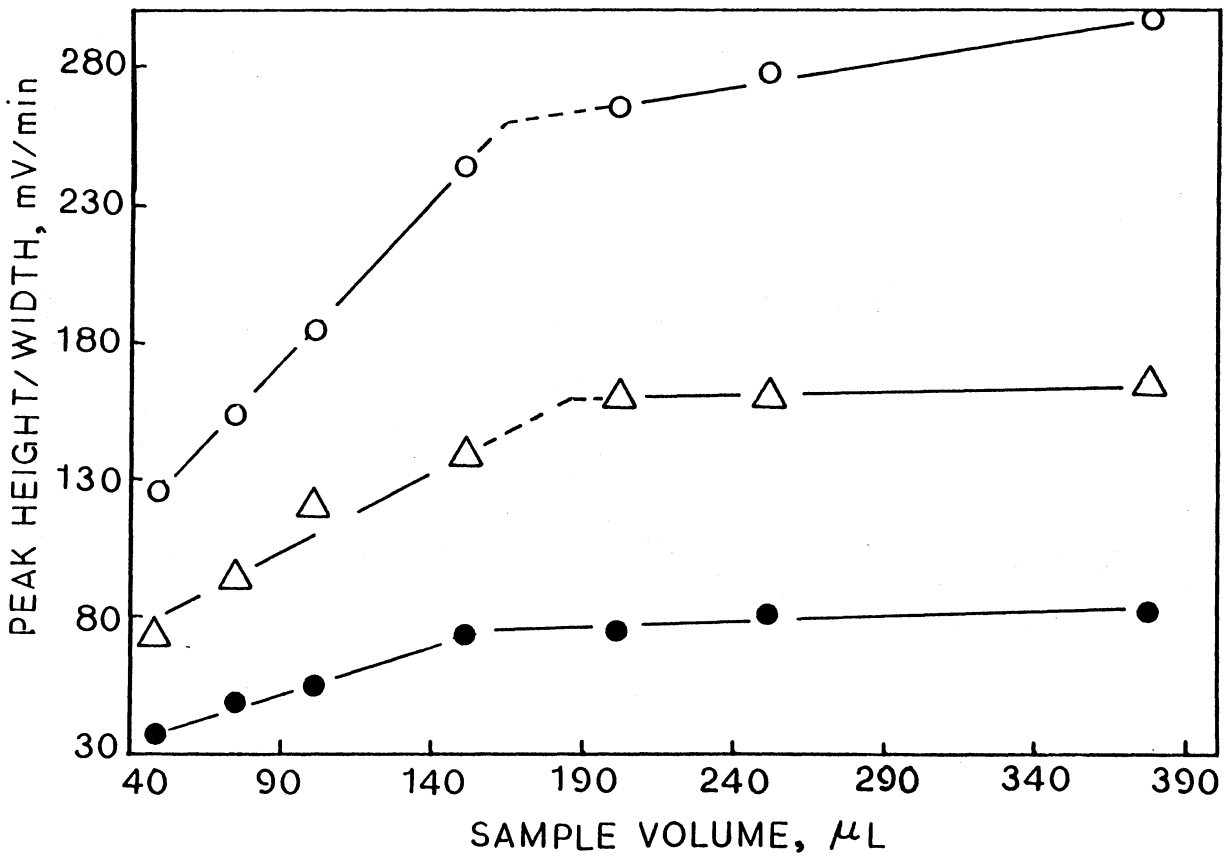
The authors, however, did not mention the detrimental effects of increasing the sample volume. As a direct consequence of introducing a longer sample plug, the peak width increases.

The continuous-flow manifold described in Chapter III was utilized to study the effect of sample size on peak height and peak width. The volume of penicillin G injected was varied, and the peak height and width of the transient signal was measured. This study was conducted at three different concentrations of penicillin G to cover the major portion of the working cruve in the determination of penicillins. In order to reach a compromise between sensitivity and sampling rate, peak height/width was plotted against the volume of penicillin G injected, as shown in Figure 28. A sample volume between 145 and 185 µL can be used as the optimum range. Based on the needs of the chemist (sensitivity or sampling rate), the volume of sample injected can be increased or decreased, about this optimum range.

6.3 Effect of Flow Rate

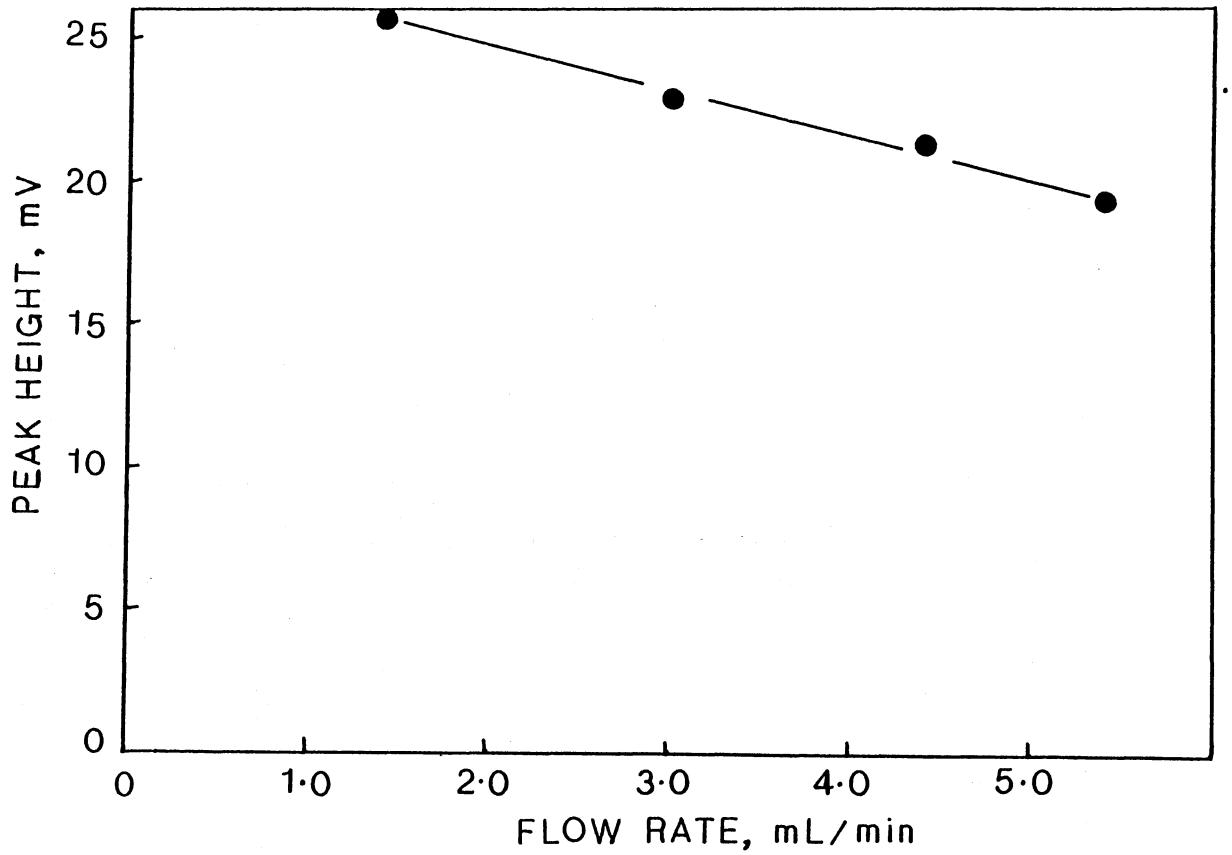
Flow rate of the carrier solution affects the sensitivity, as well as the sampling rate. To study the effect of flow rate, a 0.100 mM solution of penicillin G was injected into the manifold, described in Chapter III, and the signals recorded. The effect of flow rate on peak height, peak width, and peak height/width is shown in Figures 29, 30, and 31 respectively.

Peak height is affected less when compared to the peak width. This finding agrees with that of Reijn and co-workers (93), who stated that peak height was not affected when the flow rate was decreased in a SBSR. They were considering only dispersion in a SBSR.



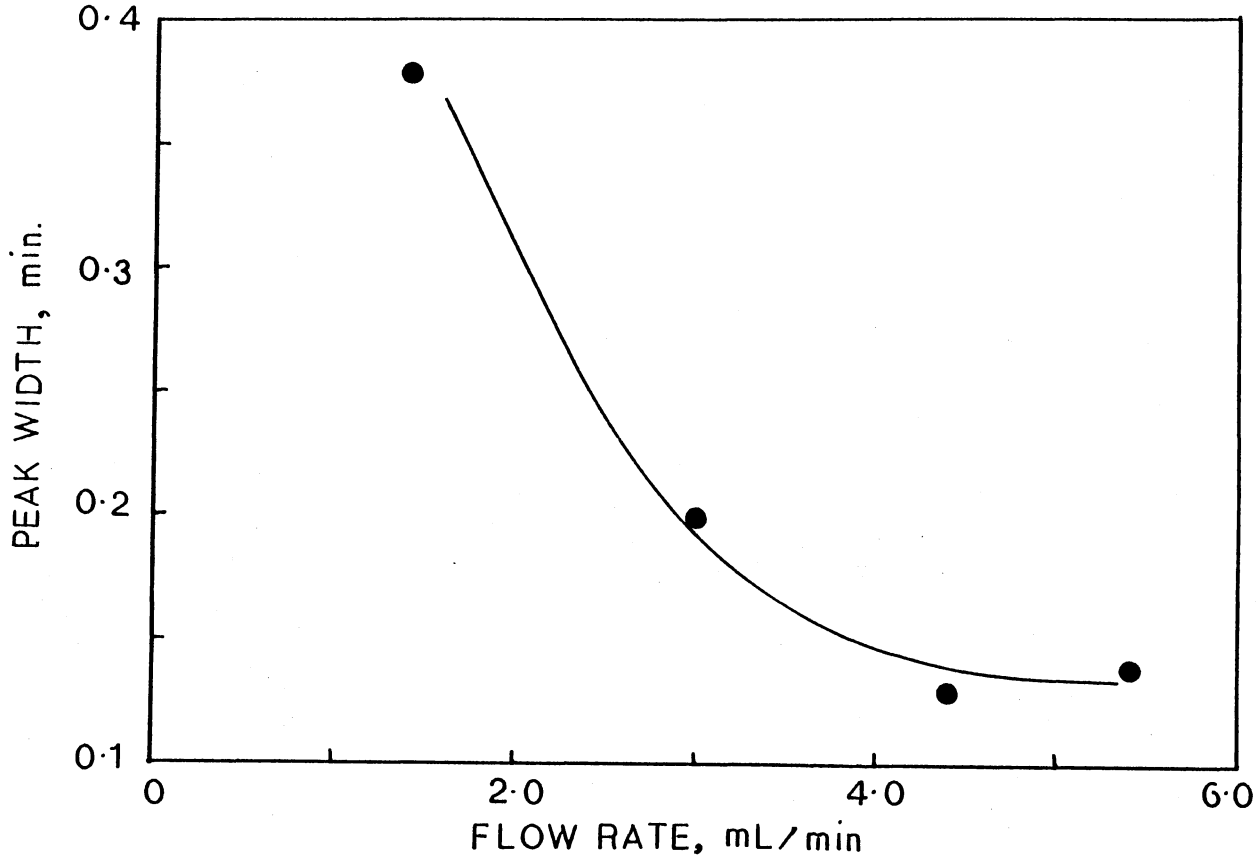
△: 0.1 mM Penicillin G ○: 0.2 mM Penicillin G ●: 0.05 mM Penicillin G

Figure 28. Effect of Sample Size on Peak Height/Width



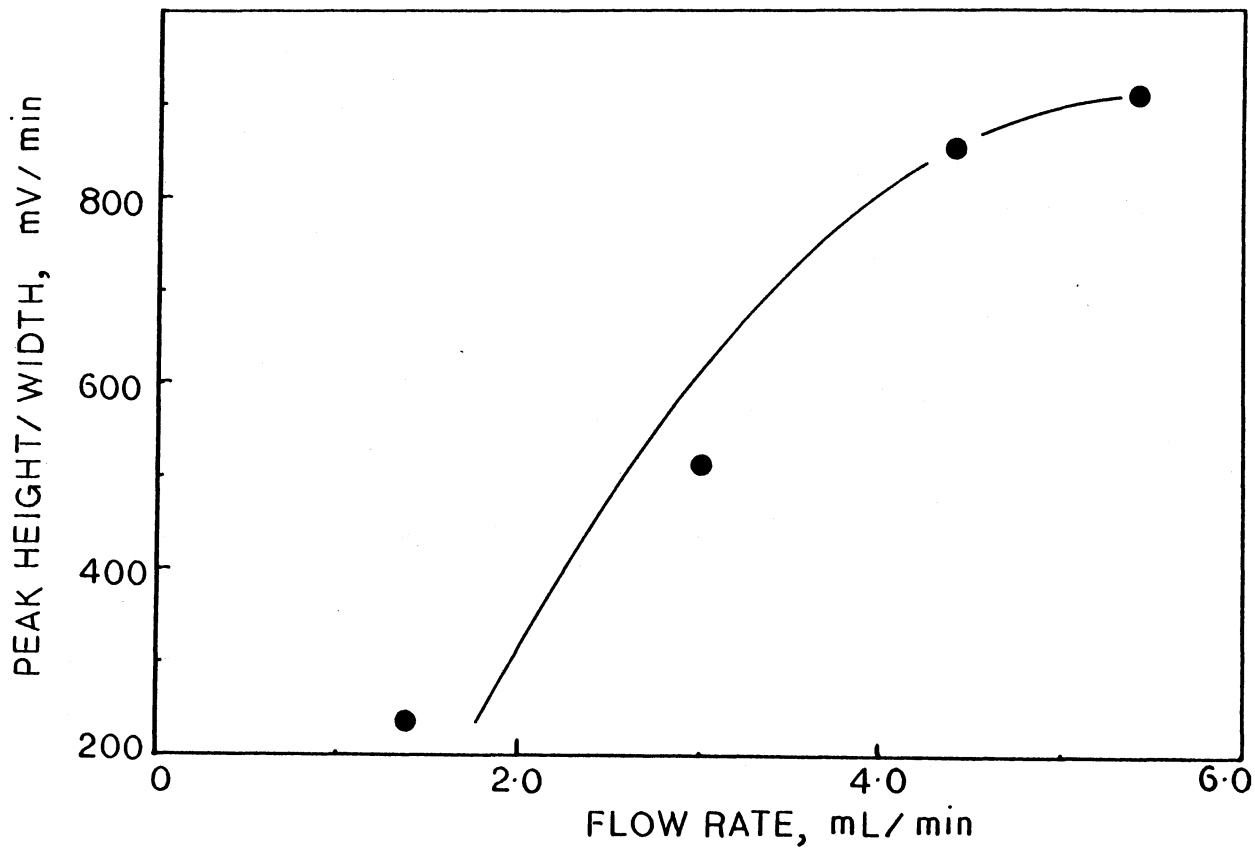
Carrier: 1 mM Phosphate buffer, pH 6.4 (I=0.10)
Sample: 0.1 mM Penicillin G in the same buffer

Figure 29. Effect of Flow Rate on Peak Height



Carrier: 1 mM Phosphate buffer, pH 6.4 (I=0.10)
Sample: 0.1 mM Penicillin G in the same buffer

Figure 30. Effect of Flow Rate on Peak Width



Carrier: 1 mM Phosphate buffer, pH 6.4 (I=0.10)
Sample: 0.1 mM Penicillin G in the same buffer

Figure 31. Effect of Flow Rate on Peak Height/Width

In this study, there was a slight decrease in peak height. This can be explained as due to a decreased reaction time with increasing flow.

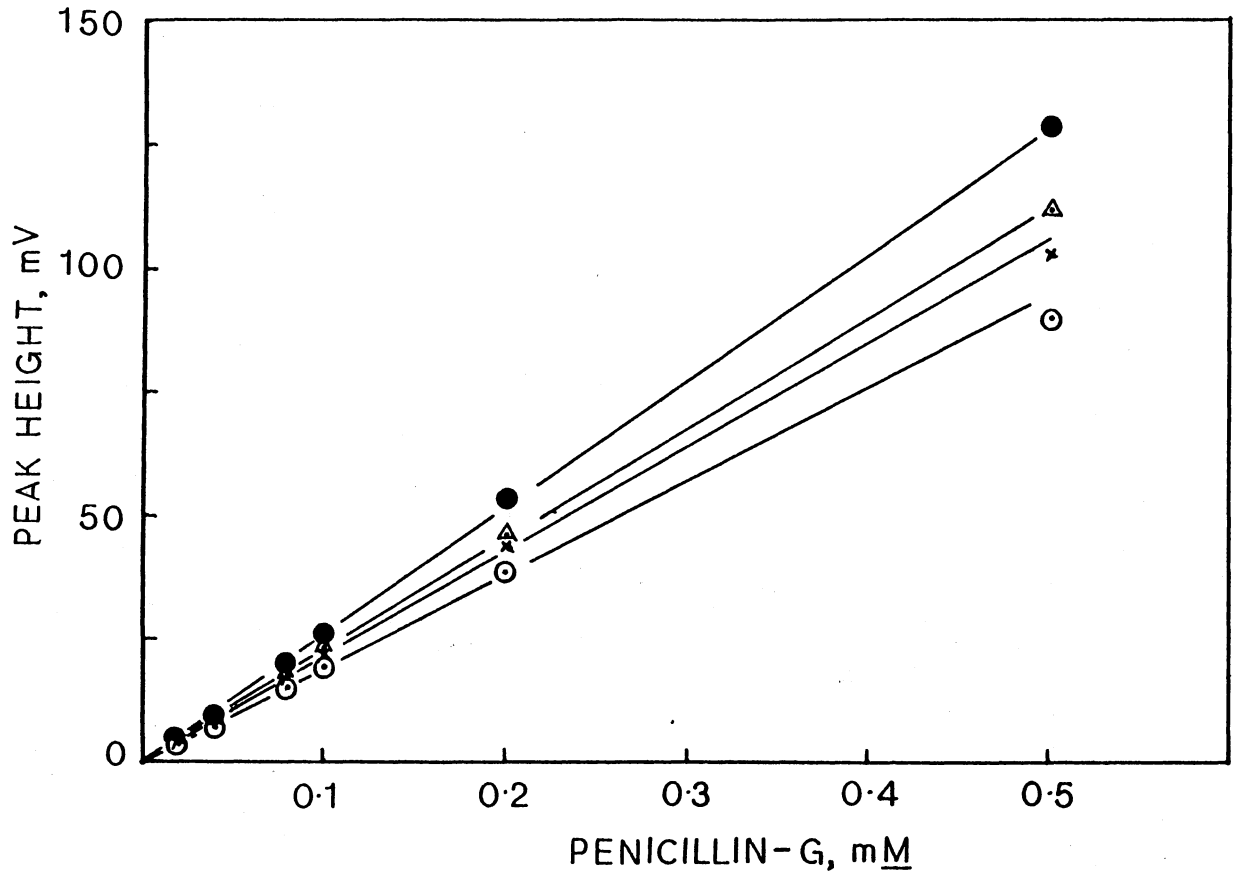
As illustrated in Figure 32, changing the flow rate of carrier from 1.4 mL/min to 5.4 mL/min does not bring about a significant change in sensitivity. To achieve a high sampling rate, a flow rate of 4.0 mL/min was adopted for the determination of penicillins in real samples.

6.4 Determination of Penicillins in Real Samples

The last objective of this study was to use the best immobilized enzyme reactor for the determination of penicillins in real samples. The optimum conditions (concentration of buffer, pH, temperature, sample size, and flow rate) described earlier were adopted for the determination of penicillins. The manifold described in Chapter III was used for the determinations. Various concentrations of penicillin G in 1 mM phosphate buffer were intercalated into the continuous-flow system and the signals recorded.

Typical signals obtained with the injection of penicillin G standards are shown in Figure 33. Each injection was performed in triplicate. As can be seen from the tracing of the signals, excellent reproducibility can be achieved. A working curve was prepared by plotting these peak heights versus the concentration of penicillin G, as shown in Figure 34. The working curve was linear up to a concentration of 0.5 mM penicillin G.

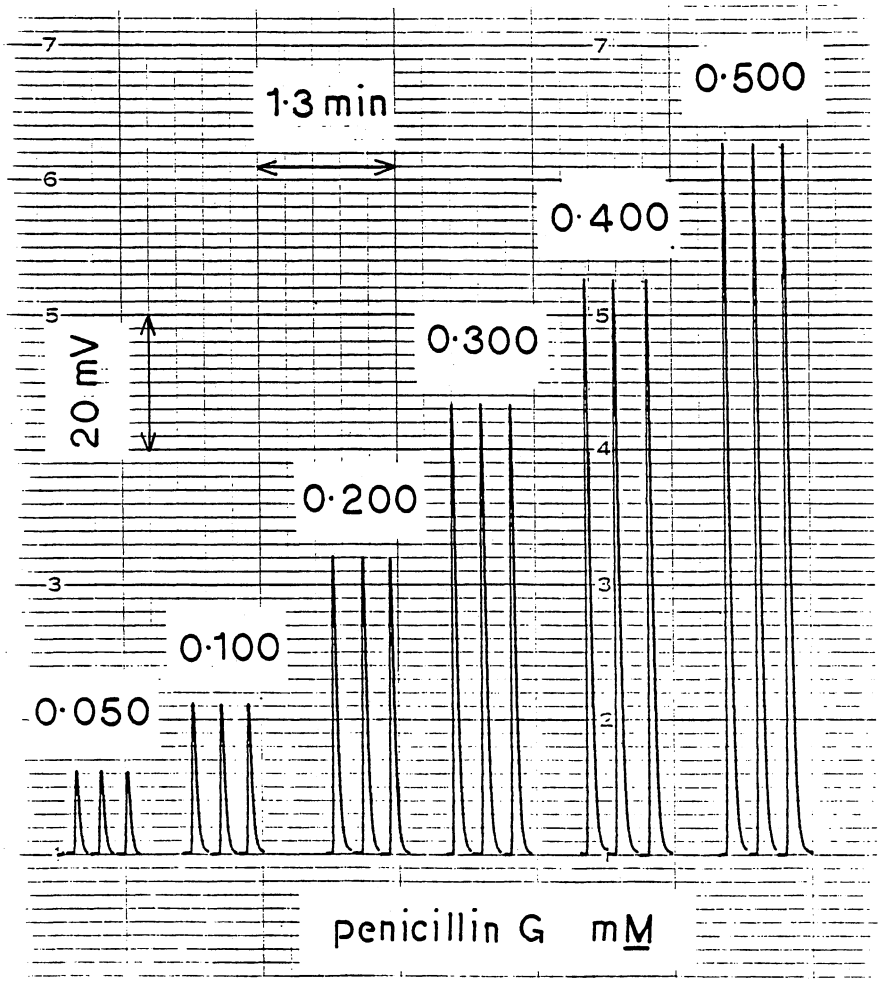
Penicillin G tablets and injectables were made up in 1 mM phosphate buffer, pH 6.4 (I=0.10). A 4000 fold dilution for tablets and a 1000 fold dilution for injectables were necessary to bring them into the



--: 1.4 mL/min --: 3.0 mL/min --: 4.4 mL/min --: 5.4 mL/min

Carrier: 1 mM Phosphate buffer, pH 6.40 (I=0.10)
Sample: Penicillin G in the same buffer

Figure 32. Effect of Flow Rate on the Working Curve



Carrier: 1 mM Phosphate buffer, pH 6.40 (I=0.10)

Figure 33. Typical Flow Injection Signals Obtained with Penicillin G Standards

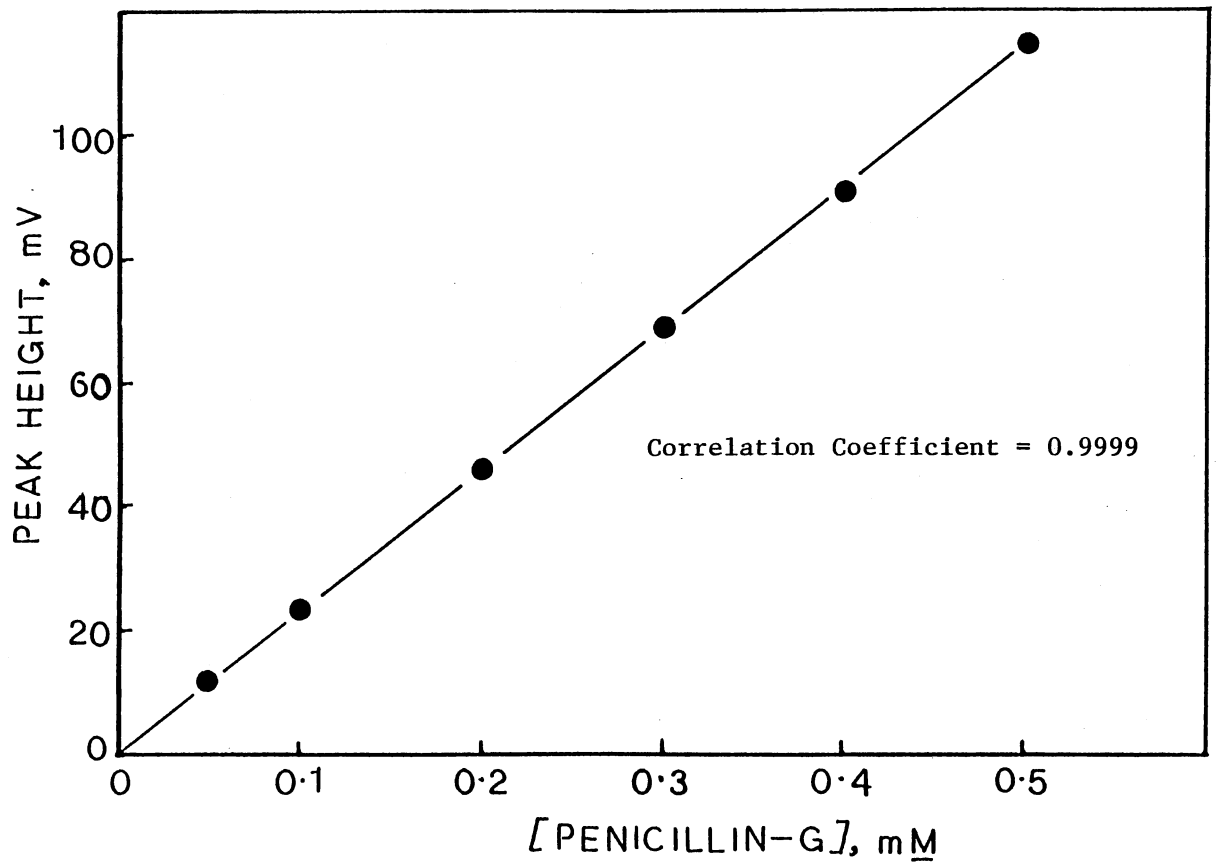


Figure 34. Working Curve for the Determination of Penicillin G

concentration range of the working curve. Due to the presence of additives and buffers in the pharmaceutical products, the pH of the sample was different from that of the carrier solution. Therefore, a blank was run by injecting these samples into a blank SBSR of the same dimensions as the enzyme reactor and recording the signals. The signal height due to the enzymatic hydrolysis was obtained by injection of samples into the enzyme reactor and recording the signals. As much as 150 injections per hour can be performed with this method.

The difference in signal height, of sample and blank, was used for interpolation in the working curve. The results for the determination of penicillin G in tablets and injectables are summarized in Table X. Inspite of the high dilutions involved, the results agree very closely with that of the manufacturers of the tablets and injectables. The buffers and additives in the preparations did not interfere in the determination.

TABLE X

DETERMINATION OF PENICILLIN G IN TABLETS AND INJECTABLES

Sample	Value Determined (units)	Manufacturer's Value (units)
Tablets (Squibb)	251,000	250,000
Injectables (Eli Lilly)	204,000	200,000

Penicillin V was also determined in fermentation broths. A sample of fermentation broth, from Eli Lilly, was diluted 100 fold with 1 mM phosphate buffer, pH 6.40 (I=0.10). The diluted sample was then injected into the manifold described in Chapter III. Typical flow injection signals obtained with broths are shown in Figure 35. The signal height of the blank was subtracted from that obtained when the broth was injected into the enzyme reactor. This difference in signal heights was used for interpolation in the working curve. The working curve used for the determination of penicillin V in broth is shown in Figure 36. The results are summarized in Table XI.

TABLE XI

DETERMINATION OF PENICILLIN V IN FERMENTATION BROTHS

Method Used	Value Determined	Value Reported by Eli Lilly
Eli Lilly (Chemical)	-	12.58 mg/mL
Eli Lilly (HPLC)	-	12.19 mg/mL
Immobilized enzyme reactor	12.3 ± 0.1 mg/mL	

The value determined using the enzymatic method in this study falls between the chemical and HPLC assays of Eli Lilly. The presence of other buffers and additives in the broth did not interfere in the

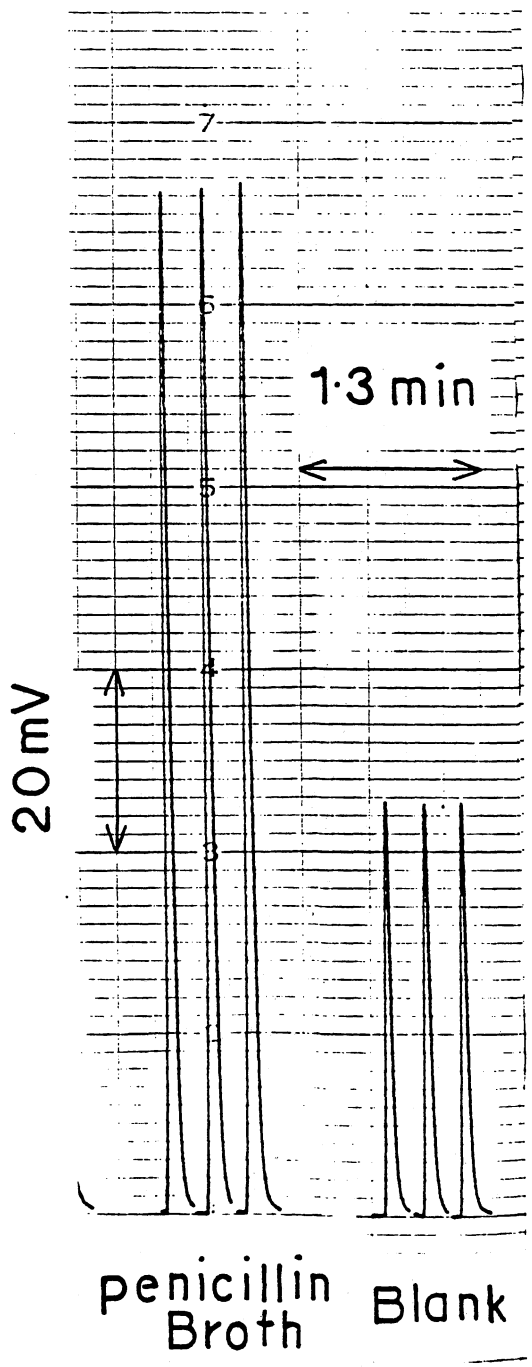


Figure 35. Typical Signals Obtained with Penicillin Broths

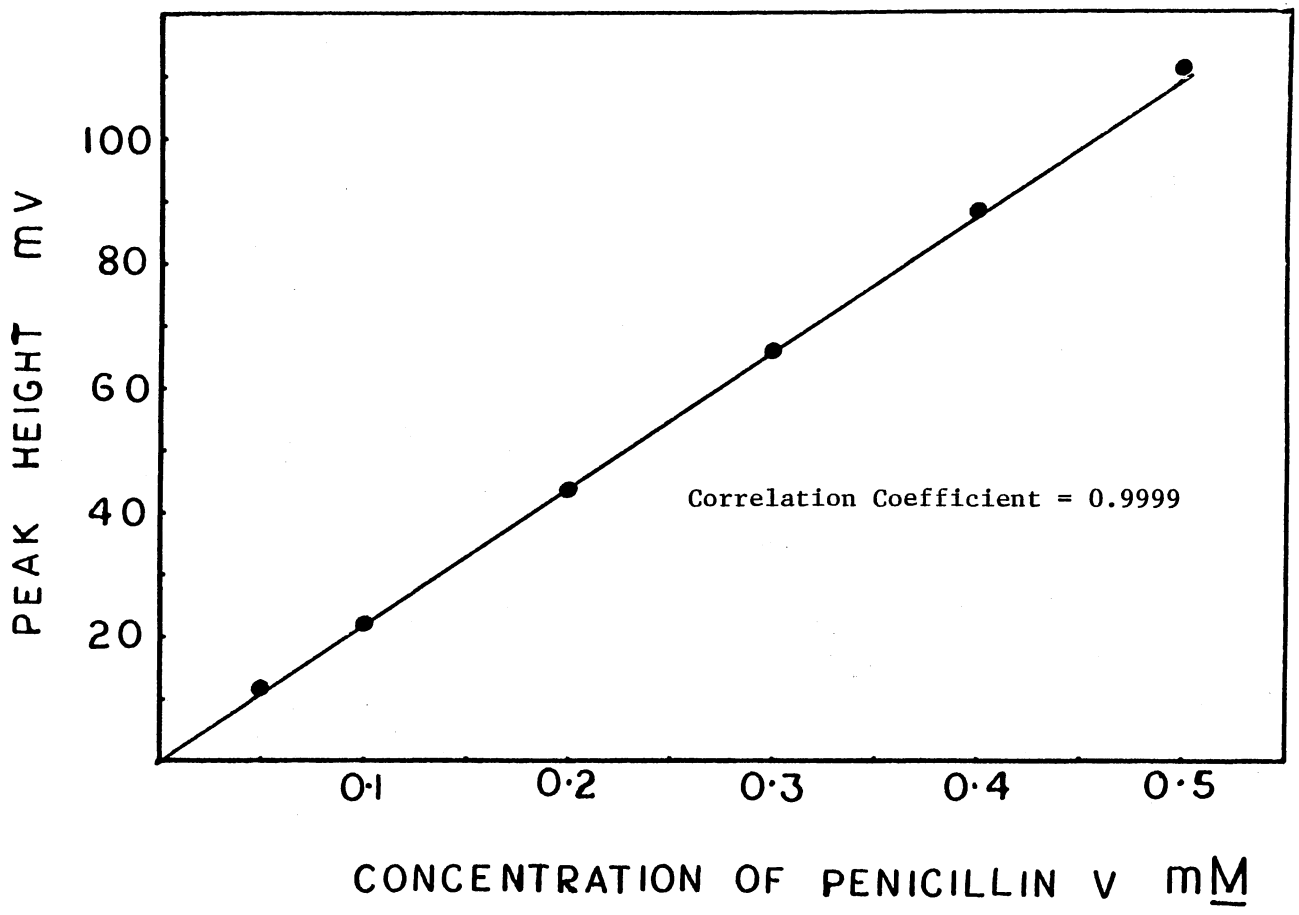


Figure 36. Working Curve for the Determination of Penicillin V in Fermentation Broths

determination of penicillins. More samples could not be run because of the difficulty in getting these samples. The samples had to be shipped at a temperature of 0°C.

6.5 Design of a Flow Cell for pH Measurement in Continuous-Flow

The use of the Radiometer glass capillary pH electrode with simple peristaltic pumping is not satisfactory. Peristaltic pumping introduces noise due to pulsations. The frequency of the noise increases with the speed of the pump. The noise was attributed to the streaming potential generated when a liquid was forced through a narrow capillary by a pressure gradient (98). Van den Winkel and co-workers (98) suggested the use of a large quantity of electrolyte in the carrier solution to decrease the noise. In continuous-flow systems with immobilized enzymes, the ionic strength cannot be increased indefinitely, since this could decrease the stability of the preparation. Van den Winkel and co-workers (98) also suggested the introduction of air bubbles into the carrier stream and venting it out prior to reaching the detector. The bubbles serve to dampen the pulsations and insulate the electrostatic noise generated by the pump rollers.

Ruzicka and co-workers (99) used bubbles to provide electrical insulation between the rollers and the ion selective electrode. They also used a pulse suppressor at the electrode exit. All these devices complicate a simple continuous-flow manifold.

In the continuous-flow manifold described in Chapter III, the problem of pulsations was eliminated by the use of nitrogen pressure to move the carrier solution. The drawback of this system was the

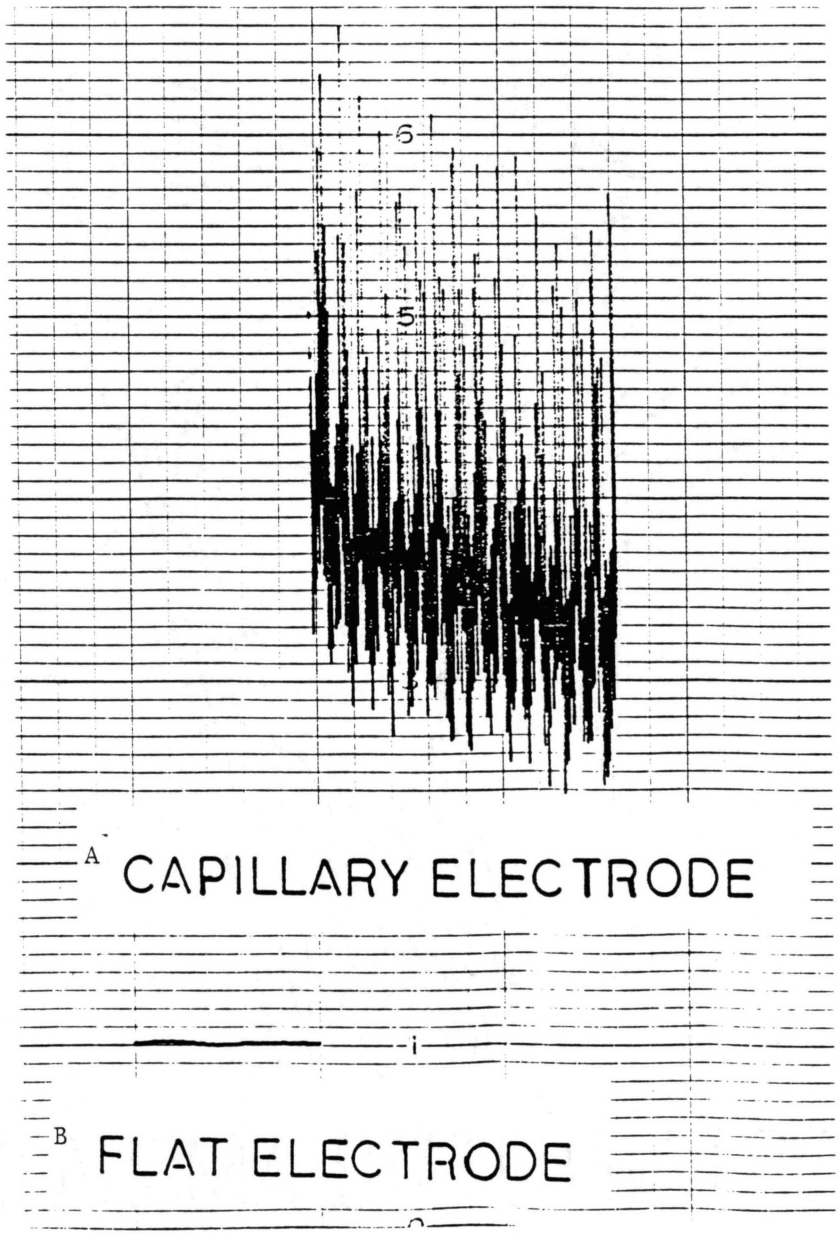
necessity to clean and rinse the reservoir whenever carrier solutions had to be changed. All these problems, apart from the high cost of the capillary glass pH electrode, led to the development of a new flow through cell using a flat surface pH electrode (100).

The flow cell, as described in Chapter III, was constructed from plexiglass to fit a gel - filled flat surface combination pH electrode. The baseline obtained with this flat electrode in a continuous-flow system with peristaltic pumping is compared with that obtained from the capillary electrode in Figure 37. The baseline obtained with the new flow cell is noise free, unlike that of the capillary electrode.

Van den Winkel and co-workers (98) used a flat surface fluoride ion selective electrode connected to a saturated calomel reference electrode by means of a capillary tubing, to study the effect of streaming potentials associated with peristaltic pumping. They showed that the streaming potentials were generated at the capillary tubing connecting the electrodes. The streaming potentials increased with the length of the tubing used, and decreased with the radius of the tubing.

In the new design of the flow-through pH detector, the streaming potentials were completely eliminated because of the absence of any capillary tubing connecting the reference electrode and the indicator electrode (combination electrode).

To compare the flow injection signals of the two electrodes using a peristaltic pump, the continuous-flow manifold described in Chapter III was modified by substituting a peristaltic pump for the pressurized reservoir. A pH 7.00 buffer was injected into a carrier buffer (pH 4.00), passed through the reactor and the signals recorded. Peaks



A: Baseline obtained by using Radiometer glass capillary pH electrode (G299A) and a Radiometer saturated calomel reference electrode (K401)

B: Baseline obtained from a Sensorex (450C) combination electrode with the new flow cell

Carrier: 1 mM Phosphate buffer, pH 6.4 (I=0.10)

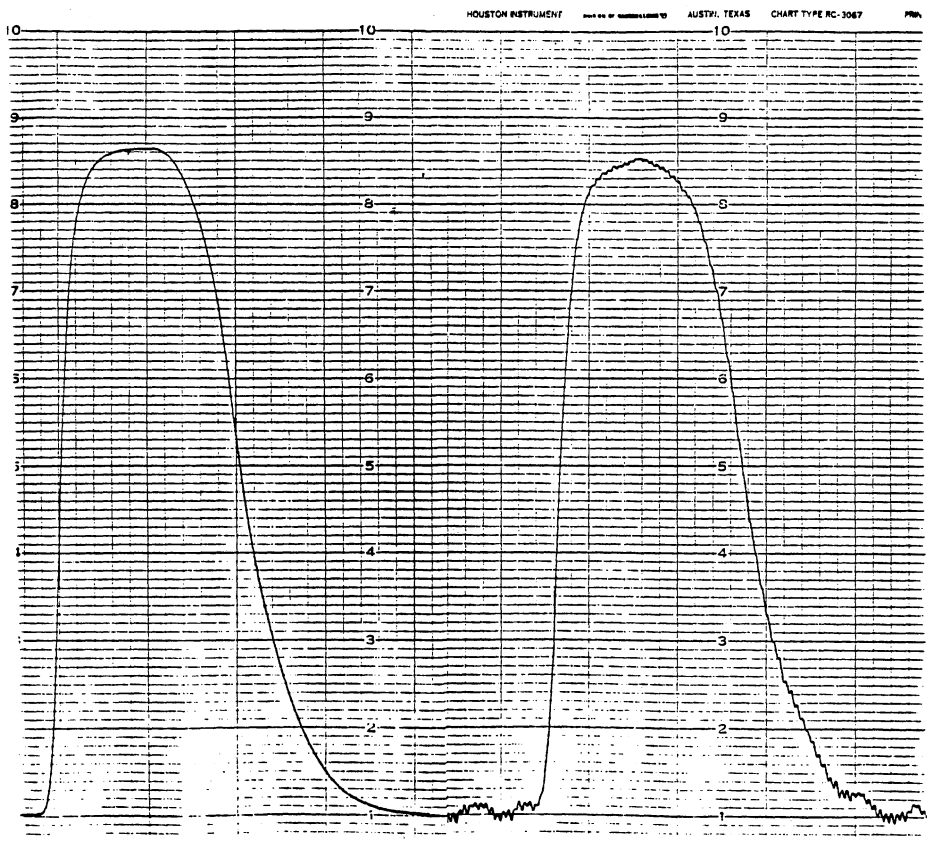
Figure 37. Noise Due to Streaming Potentials

obtained with the new electrode are compared with those of the capillary electrode in Figure 38. The peak heights from both the electrodes are comparable, while the baseline obtained using the capillary electrode is noisy. The noise level seems to be low because of the large span (2 V) used on the recorder, to accomodate the signal.

The effect of other signal parameters are summarized in Figure 39. The response time of both the electrodes are comparable as evidenced by the time taken for the signal to reach a steady state from the point of injection. The peak width from the new flow cell is marginally larger than that due to the capillary electrode. This is probably due to the larger cell volume (50 μL). The main advantage of this new flow cell is its low cost and the absence of streaming potentials.

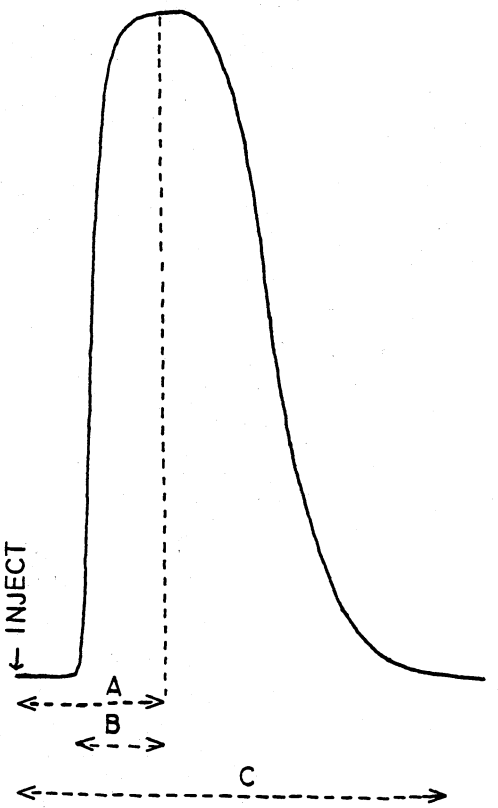
Figure 40 shows the working curves for the determination of penicillin G obtained with the capillary electrode and the flat electrode, using the manifold described in Chapter III. The sensitivities (slope of the working curve) are about the same for both the electrodes.

The new flow cell, when used in conjunction with a flat surface combination pH electrode, provides an inexpensive alternative to the use of the glass capillary electrode. The new flow cell can be used with peristaltic pumps for the continuous monitoring of pH.



Carrier: pH 4.00 buffer
Sample injected: pH 7.00 buffer

Figure 38. Flow Injection Signals from Capillary Electrode and Flat Electrode



	A min	B min	C min	NOISE	COST $
FLAT ELECTRODE	0·23	0·177	0·679	LOW	70
CAPILLARY ELECTRODE	0·22	0·155	0·650	HIGH	270

Figure 39. Comparison of Radiometer Capillary Electrode and the Flat Electrode in Continuous-Flow

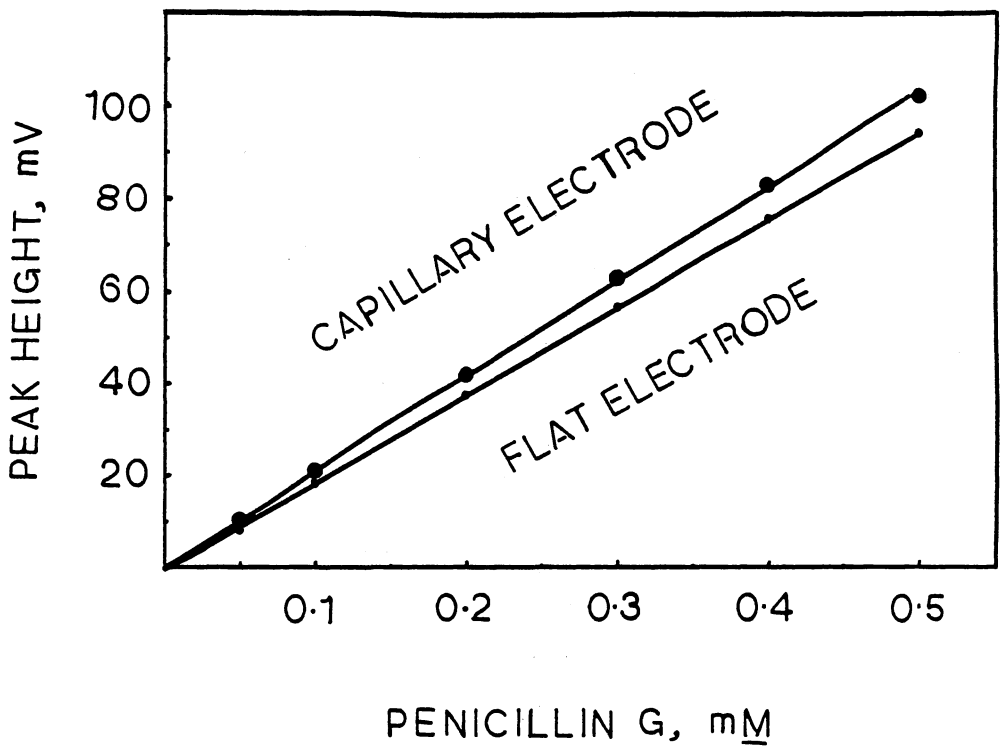


Figure 40. Working Curves Obtained with the Capillary Electrode and the Flat Electrode

CHAPTER VII

CONCLUSIONS

The enzyme penicillinase has been immobilized by a variety of methods, most of them being physical. Physical methods of immobilization are unsuitable for use in flow injection analysis. The study has shown that the use of short, rigid silanes, like p-(aminophenyl)trimethoxysilane, for the immobilization of penicillinase gives maximum activity on glass. Silylation of a glass surface can be performed easily in 95% ethanolic solution, as compared to the slow refluxing conditions from anhydrous toluene.

The use of a packed reactor with peristaltic pumping is unsatisfactory, due to back pressure problems. The use of a new reactor design (SBSR) enables the use of it in continuous-flow systems with peristaltic pumping. The geometry of the design allows minimum dispersion of the sample plug. The high surface area on the glass beads, made possible by an etching process, provides maximum activity of penicillinase after covalent attachment of the enzyme. It has also been shown that the use of SBSR constructed with teflon tubing, with enzyme immobilized on glass beads alone is satisfactory.

Simple signal parameters like peak height and width can be used for the optimization of several conditions like concentration of buffer, pH, flow rate of carrier, sample volume, and temperature in a flow

injection system. This optimization makes it possible to strike a compromise between sensitivity and sampling rate in a flow injection system.

In enzymatic reactions involving the consumption or release of protons, potentiometric monitoring can be easily used, and should be preferred over dye coupling reactions. Problems like electrostatic noise due to the pump rollers in peristaltic pumps, and streaming potentials can be avoided by the use of the new flow cell in combination with the flat surface electrode.

The flow injection manifold is very simple and can be assembled in any small laboratory with minimum cost. This flow injection system can be used to determine a wide variety of penicillins, that are susceptible to attack by penicillinase, in the most complex samples like fermentation broths and pharmaceutical preparations. This simple method provides results that agree very closely with that of the well established HPLC method. Minimum sample preparation is needed, unlike in the other methods. For instance, in HPLC, either protein precipitation with trichloroacetic acid or extraction with solvents is necesary before the samples could be injected into the column.

The use of the immobilized penicillinase in a continuous-flow system combines the selectivity of the enzyme with the speed of continuous-flow analysis. By making use of this system, as many as 150 injections per hour can be made. The immobilized enzyme has been found to retain 97% of its initial activity even after 10 months of usage, during which thousands of determinations can be performed. As the enzyme is reused several times during the lifetime of the reactor, the cost per determination is reduced drastically.

If this continuous-flow system is completely automated with an auto sampler and a data processing system, it has the potential of becoming a powerful analytical tool in the pharmaceutical industry, for the routine determination of penicillins.

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