ENZYMATIC DETERMINATIONS USING A ROTATING BIOREACTOR AND CONTINUOUS-FLOW/STOPPED-FLOW PROCESSING: DETERMINATION OF GLUTAMATE IN FOOD PRODUCTS

By

CHITRA JANARTHANAN

Master of Science

University of Madras

Madras, India

1987

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
DOCTOR OF PHILOSOPHY
December, 1996

ENZYMATIC DETERMINATIONS USING A ROTATING BIOREACTOR AND CONTINUOUS-FLOW/STOPPED-FLOW PROCESSING: DETERMINATION OF GLUTAMATE IN FOOD PRODUCTS

Thesis Approved:

Horacio A. Mottola

Thesis Adviser

Warren T. Ford

Nicol B. Reo

Sterling Burks by Horacio A. Mottola

Thomas C. Collins

Dean of the Graduate College

DEDICATED
TO
MY LOVING PARENTS

ACKNOWLEDGEMENT

I wish to express my sincere appreciation and gratitude to my adviser, Dr. Horacio A. Mottola, for his encouragement and advice throughout my graduate program. His valuable guidance, patience and support made it possible for me to achieve my goal. I am also grateful to the other committee members, Dr. Ziad El Rassi, Dr. Warren T. Ford, and Dr. Sterling Burks for their suggestions and support.

I am greatly indebted to Department of Chemistry and Dr. Lionel Raff for the financial support provided to me. I would also like to extend my thanks to the office staff, Carolyn, Cheryl, Cindy, Glenda, and June for their help. My appreciation to Mike Lucas and Bill Barnes for their assistance.

Words cannot express my admiration and gratitude to my father Janarthanan B., and my mother Dr. Dakshayani K. V., for their moral encouragement, constant support, and understanding. I am grateful to my sisters Amudha, Sakthi and brother Bagirathan for their confidence in me. Special appreciation goes to my grandmother (late) Kannamma, my aunt Dr. Rajakumari, uncles Dr. Varma and Kannan for their support. I would also like to extend my thanks to my cousins Sujatha, Suchitra, Deepak, and Sinduja for their moral support. To my loving husband Sivakumar for his encouragement and without whose help I would have never finished my thesis in time. My gratitude to parents-in-law Sambasivam and Jayalakshmi for their encouragement.

It would be a privilege to thank friends like Dale, Li, Lan, Robert, Lance, Kevin,

Mottora, and specially Kenneth without whom this endeavor would not have been as educational and stimulating. Special thanks to Mrs. Mottola for encouraging me and making me feel at home. My thanks also go to Gayatri, Sabbahath, Vanitha, Viji, Thumbiah, Shankar, Ramasubbu, John, Shelly, Ramanarayan, Cindy, Jagdeesh, and Sameer for their invaluable support.

TABLE OF CONTENTS

Chapter Page

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

Figure Page

CHAPTER I

INTRODUCTION

Glutamic acid in its free-form and as monosodium glutamate (MSG) is used as a flavor enhancer in foods prepared at home, restaurants, and in processed food. Possible mechanisms for the ability of MSG to stimulate taste receptors have been discussed by Cagan [1]. The chemical formula for glutamic acid is

$$HOOC - CH_2 - CH_2 - CH(NH_2) - COOH$$

It is a white solid with a gram formula weight of 147.13 g and melts at 205°C. The pK values for the ionizing groups of glutamic acid are 2.1 (α-COOH), 9.47 (α-NH_3^+), and 4.07 (side chain) [2]. Glutamic acid occurs in two optical isomeric forms, L- and D-. It is the L- form that possesses the flavor enhancing property.

Kikunae Ikeda, in 1908, distinguished and separated glutamate as the main agent in the sea tangles which the Japanese cooks had used for years as flavoring [3]. Monosodium glutamate is commercially manufactured by the fermentation of starch, molasses, or sugar [4]. The American food processing industry has used MSG widely since the late 1940s and the consumption in this country is estimated to be 28,000 tons per year [5]. Glutamate is a major component of all proteins, and occurs as a free acid in a variety of vegetables, meats, and seafood [6,7]. Tables I and II give the amounts of

glutamate in some foods, which vary between 6.7 to 658 mg/100 g in fresh food, and 0.05 to 6830 mg/100 g in processed food [13].

Glutamate plays an essential role in many metabolic processes [8] and acts as a donor of amino and methyl groups for synthesis of non-essential amino acids. It is used for transfer of energy, for transport of amino acids across cell membranes, and for removal of ammonia via the urea cycle. Glutamate also acts as a neurotransmitter and is a brain metabolite. There is evidence to suggest that it is responsible for 75% of the excitatory transmission in the brain [9]. Glutamate induced neuronal injury has been shown to be responsible for certain behavioral patterns such as retarded learning [10], and aggressive behavior [11].

The use of glutamic acid has become controversial in the past 30 years [12]. Some people who have consumed food containing MSG have suffered adverse effects such as stiffness, tension, and pain in the head, neck, and shoulders. Symptoms like these collectively were termed as Chinese Restaurant Syndrome. Studies have shown that injections of glutamate in laboratory animals have resulted in nerve cell damage in the brain.

A report from the Federation of American Societies for Experimental Biology (FASEB), in 1995, puts these concerns into perspective by showing that glutamic acid and its salts are safe for most people when consumed at customary levels [12]. Before this report, a citizen's complaint was filed in December 1994 with the U. S. Department of Health and Human Services of the Food and Drug Administration (FDA) to require that MSG be clearly labeled when used in food and also that the amount of free glutamic acid or MSG in such products be specified in grams, and such a food label should also

TABLE I

CONTENT OF L-GLUTAMIC ACID AND MSG EQUIVALENT (IN PARENTHESIS) IN FRESH FOODS[13]

Fresh Food	L-glutamate (mg/100g)	Monosodium glutamate (mg/100g)
Almond	39.8	(45.8)
Apple	11.7	(13.5)
Avocado	84.7	(97.4)
Broccoli	115.0	(133.0)
Capsicum (green)	30.7	(35.3)
Carrot	47.1	(54.2)
Corn	123.0	(142.0)
Egg	27.9	(32.1)
Garlic	112.0	(128.0)
Mushroom	192.0	(221.0)
Onion	102.0	(118.0)
Pea	69.4	(79.8)
Peanut	105.0	(121.0)
Potato	180.0	(208.0)
Soybean	63.2	(72.7)
Spinach	20.0	(23.0)
Strawberry	22.4	(25.8)
Tomato (green)	50.3	(57.9)
Tomato (red)	292.0	(336.0)
Walnut	658.0	(757.0)
Zucchini	6.7	(7.7)

TABLE II

CONTENT OF L-GLUTAMIC ACID AND MSG EQUIVALENT (IN PARENTHESIS) IN PROCESSED FOOD[13].

Processed Food	L-glutamate (mg/100mg)	Monosodium glutamate (mg/100g)
Asparagus (canned)	23.0	(26.5)
Baked beans (canned)	78.2	(89.9)
Beer (draught)	16.9	(19.4)
Bread (white)	22.4	(25.8)
Cheese (Camembert)	40.4	(46.5)
Cheese (Cheddar)	154.0	(177.0)
Cheese (Parmesan)	516.0	(594.0)
Chicken (barbecued)	143.0	(165.0)
Corn (canned)	49.6	(57.0)
Flour (wheat)	23.5	(27.0)
Hamburger (meat)	60.7	(69.8)
Mushrooms (canned)	34.0	(39.1)
Milk (pasteurized)	15.1	(17.4)
Olives (bottled)	21.4	(24.6)
Orange juice	27.3	(31.4)
Pizza	94.4	(109.0)
Potato crisps	198.0	(227.0)
Salmon (packed)	36.3	(41.8)
Sardines (canned)	22.3	(25.7)
Soup, chicken	205.0	(236.0)
Soy sauce	1090.0	(1253.0)
Stock cube (beef)	6827.0	(7851.0)
Sauce (tomato)	440.0	(506.0)
Tomato juice	109.0	(125.0)

bear an appropriate cautionary statement. Since the FDA failed to respond within the specified time (August 29, 1995), the citizen's group that filed the complaint has requested a federal court to step in on their behalf and require that all MSG in all the processed foods be labeled.

A statement released by a volunteer organization called the Truth in Labeling Campaign goes into detail about the various researches conducted by the FDA, the glutamate industry, and individuals; it also reports their results and consequences. They allegedly blame the FDA for dismissing the research that demonstrates the adverse reactions related to the use of MSG and for distorting the truth. The statement also criticizes FDA's response to the 1995 FASEB's report stating that the report released on August 31 is simply the latest illustration of promoting the fiction that MSG is safe [17].

Although the acceptable daily intake of MSG is 10.5 g [14], it was found that even a small dose of 2 g can induce asthma in some patients [15]. Schaumberg et al. [16] found that there were large variations in the oral threshold doses among individuals. For this reason it is important to determine the content of glutamic acid in foods and food products.

Table III summarizes some of the reviews on the safety of MSG. Many of these safety reviews were done because of the various MSG related adverse reactions. Based on the FASEB's report, the FDA would propose that if a significant amount of free glutamate is present in foods, then it should be declared on the label. This would allow the consumer to choose between foods containing insignificant amounts and those that might cause a reaction. If this is implemented, the food industries should then require appropriate analytical methods to determine glutamate.

TABLE III

CHRONOLOGY OF RECOMMENDATIONS RESULTING FROM THE U. S. FOOD AND DRUG ADMINISTRATION, FDA, REVIEW AND ANALYSIS OF COLLECTED INFORMATION REGARDING MSG

Year	Comments
1959	FDA classifies MSG as a safe ingredient in processed foods.
1970	FDA sponsors an extensive review on the safety of MSG.
1980	A review by a committee of the Federation of American Societies for Experimental Biology, FASEB, concludes that MSG is safe at the levels normally used in processed food.
1986	A FDA's advisory committee concludes that MSG poses no threat to the public, but reactions of brief duration might occur in some people.
1987	A joint committee of the United Nations Food and Agriculture Organization and the World Health Organization places MSG in the safest category of food ingredients.
1991	A report by the European Communities' Scientific Committee for Foods reaffirms MSG as safe.
1992	The American Medical Association states that glutamate is not a significant health hazard.
1995	Based on the report from FASEB, FDA is planning to propose that foods containing free MSG (not bound in proteins along with other amino acids) declare that on the label.

Generally food industries analyze thousands of samples a day. Hence, in order to declare the content of a substance, it should need methods that are simple, fast, selective, sensitive, efficient, and which can be automated. The monitoring of glutamate is also essential for fermentation control during its production. So far several methods have been reported for this purpose; some of these methods need pre-treatment and/or are time consuming.

The aim of this work was to develop continuous-flow methods using a bioreactor for the determination of glutamate. The continuous-flow approach [18] is the most flexible way to perform a number of operations necessary in a chemical determination since manual handling of thousands of samples is time and labor consuming. The continuous flow allows the incorporation of the necessary apparatus in a way that determinations may be performed on small sample volumes and with limited consumption of reagent.

For industrial applications, continuous flow is advantageous since the process can be monitored regularly by injecting standards. Moreover, by combining with a stopped-flow operation, it is possible: (1) to increase the sensitivity of a measurement by increasing the residence time, and thus the conversion of the monitored species, and (2) to record the reaction rate that serves as the basis for the analytical result. In such a case the increase in signal with time at early times approximates a straight line, the slope of which will reflect the initial reaction rate from which the concentration of the analyte can be determined [18]. The reproducibility is achieved by operating the pump with a microprocessor controlled system as was used in this work.

The methods developed and described here are highly selective to glutamate

since they use enzymes. Enzymes are powerful natural catalysts and they are selective for the structures of the substrate so that only one chemical present in a mixture of similar chemicals is converted to a single product at a measurable rate. The selectivity of the enzyme is due to its ability to bind the substrate and arrange reactive groups so that a certain reaction transition state is selected. Enzymes are also stereoselective, such that chiral carbon atoms can be distinguished (e. g., the enzymes used in the work reported in this thesis are specific for the L- isomer of glutamate).

The activity of an enzyme can be controlled and retained by immobilizing it on a matrix [19]. Immobilization offers many advantages, such as

- repeated use of expensive enzymes,
- easy separation and recovery of products with minimum contamination,
- accessible incorporation into a flow system.

The present work proposes two methods for the selective determination of glutamate by electrochemical detection. In both cases, the enzymes were immobilized on controlled pore glass that was affixed onto a rotating disc. This approach offers the advantages of the immobilized enzymes without the problems of the packed-column reactors which normally are used in conjunction with continuous-flow processing [20]. The rotation of the disc makes utmost use of the active sites of the enzyme.

The first method uses the enzymes glutamate dehydrogenase and diaphorase for the indirect determination of glutamate. Nicotinamide adenine dinucleotide (NAD^+) is used as a coenzyme for this system, which is reduced (to NADH) during the reaction. The current produced due to the re-oxidation of NADH in the presence of a mediator is the measured parameter. The second method is a direct method in which the enzyme

glutamate oxidase is used. The current produced during the anodic oxidation of one of the products, hydrogen peroxide, is detected in this case. The development, optimization and characterization of both of the above mentioned approaches are discussed in detail.

Both these methods employ immobilized enzymes, continuous-flow operation, and a bioreactor that, due to its unique design and operation uses a small amount of enzyme, but very efficiently. Therefore these methods can be automated and a large number of samples can be analyzed in a specified time with the same enzyme preparation. The development of these techniques should help provide the food industries with a simple, fast, economic, selective, and efficiently automated mode of glutamate determination.

CHAPTER II

ANALYTICAL CHEMISTRY OF GLUTAMATE: A LITERATURE REVIEW

Several analytical methods have been developed for the determination of glutamate. These existing methods can be broadly classified as enzymatic and non-enzymatic.

Non-Enzymatic Methods

Some of the most common non-enzymatic methods available are, high performance liquid chromatography (HPLC), gas chromatography (GC), GC with mass spectrometry (GC - MS), thin layer chromatography (TLC), paper chromatography (PC), ion-exchange chromatography (IC), coulometry, amino acid analyzer, capillary electrophoresis (CE). Among these the most frequently used methods are HPLC, GC-MS, CE and are briefly discussed below.

High Performance Liquid Chromatography

This is the most frequently used technique in amino acid determinations. A number of methods utilizing HPLC are available in the literature for the determination of glutamate and almost all of them involve pre-column derivatization followed by

separation with reversed-phase liquid chromatography. Most of these methods involve fluorescence, ultraviolet absorption, or electrochemical detection.

For fluorescence detection, reagents are chosen which form fluorescent products but are not fluorescent themselves. This minimizes interference and reduces the need for separation of the derivative from the reagent [49]. The reagent most commonly used for fluorescence detection is *o*-phthaldialdehyde (OPA) and some of its derivatives [21]. *o*-phthaldialdehyde with *N*-isobutyl-L-cysteine is used with a methanol - acetate mobile phase, and a linear gradient system to reduce the retention time and stabilize the derivatives [22, 23], *o*-phthaldialdehyde with *N*-acetyl-L-cysteine is also used with gradient elution using a methanol-acetate buffer as mobile phase [24, 25]. The OPA reagents react with the primary amino acids to produce highly fluorescent compounds: 1-alkylthio-2-alkyl substituted isoindoles [50].

Other derivatizing agents that are generally used are 9-fluoro methyl chloroformate [26 - 30], naphthalene 2-3-dicarboxaldehyde [32], and dialkyl amino naphthalene sulphonyl chlorides. The 9-fluoro methyl chloroformate reagent forms stable adducts and avoids the use of noxious thiol derivatizing reagents such as 2-mercaptoethanol or 2-methyl- 2-propanethiol [26]. This method uses isocratic elution with buffered mixture of acetic acid-methanol-acetonitrile as mobile phase, and the minimum detectable quantity is about 5 pmol of the adduct [31]. Naphthalene 2-3-dicarboxaldehyde [32] reacts with amino acids in the presence of cyanide ions to form an intensely fluorescent 1-cyanobenz (f) isoindole [33]. In another process called dansylation the amino acids are derivatized with dialkyl amino naphthalene sulphonyl chlorides that yield fluorescent sulfonamides [34, 61].

The most common pre-column derivative for ultraviolet detection with gradient elution is phenyl isothiocyanate (PITC) [35 - 42]. Free amino acids and hydrolyzed samples (samples are hydrolyzed to separate the amino acids that are bound in the form of peptides) are dissolved in a mixture of methanol - water - triethyl amine and are dried. The derivatizing agent is added to the dried sample and dried under vacuum for 15 - 20 min. This derivative has sufficient sensitivity, requires short run times, and is stable [43]. Another derivatizing agent used is 6 aminoquinolyl-N-hydroxy succinimidyl carbamate [44, 45].

For electrochemical detection, the reagents used are OPA [46], OPA in the presence of sulfite ions with isocratic elution [47], and PITC [48]. Almost all these systems require gradient elution. Typical mobile phases consist of buffered acetonitrile or buffered methanol with controlled pH. In the systems mentioned above the retention time for glutamic acid ranged between 5-25 min. The HPLC systems offer good detection limits and different amino acids can be separated and determined at the same time. The disadvantage of this approach is that it requires prior derivatization, and that it is time consuming. Although a gradient system provides separation with good resolution, the re-equilibrium step required by the column between samples is time consuming.

Gas Chromatography

Gas chromatography (GC) and GC-mass spectrometry (GC-MS) are two techniques used to analyze for amino acids. In particular, GC-MS provides a powerful tool for amino acid determination. This approach has high sensitivity, inherent

specificity of selected ions, and the ability to simultaneously quantify naturally occurring and isotopically enriched substances. Both these methods require pre-treatment of samples to remove proteins and for the purification of the amino acids, since only pure amino acids can be determined using GC/MS. Proteins are removed commonly by precipitation, but centrifugation, dialysis, or ultra filtration are also used [51] The amino acids are usually purified by ion-exchange chromatography [52]. Since amino acids have low volatility, they must be derivatized into volatile compounds to be separated by GC. Acid-catalyzed esterification of the carboxylic moiety of amino acids is commonly employed for the determination of amino acids [53].

Derivatization procedures which have been reported include those using pentafluoro propionic anhydride with hexafluoroisopropanol [54], *N (O)*-tert-butyl dimethanol [55], *N*-acyl alcohol esters, and trimethylsilyl derivatives. The minimum detectable quantity is between 0.010 to 0.0010 femtomoles [56]. Rapid derivatization as *N (O, S)*-ethoxycarbonyl esters provide good resolution and fast determination [57]. A simple single step derivatization of amino acids as their tert-butyl dimethyl silyl (TBDMS) derivatives in tandem with MS enable fast separation and determination at nanomoles and picomoles quantities based on their retention capacity [58, 59].

Many types of GC columns and detectors are available. The best columns for amino acids are reported to be capillary columns containing a layer of barium carbonate coated with either methyl siloxane or methylphenyl siloxane [60]. The disadvantage of GC is that purification and derivatization of amino acids are required.

Other chromatographic techniques like thin layer chromatography, and ion-exchange chromatography are also used. Ascending paper chromatography is also used

as a method for the separation of amino acids, and the feasibility of its use on a semi-quantitative basis has been demonstrated [72, 73].

Capillary Electrophoresis

The basic principle of capillary electrophoretic separation is according to the charge-to-mass ratio of the substance. Separation proceeds in a capillary field with an appropriate buffer under high voltage, using an in-line or off-line detection system. Capillary zone electrophoresis (CZE) is the most commonly used technique in CE. Compounds can be separated rapidly and easily.

The separation by CZE is based on the differences in the electrophoretic mobilities; resulting in different velocities of migration of ionic species in the electrophoretic buffer contained in the capillary. The separation mechanism is mainly based on differences in solute size and charge at a given pH [62]. Here, the amino acids can be determined directly or indirectly by derivatizing before separation and determination. Jorgenson and Lukacs [63] did the pioneering works on CZE by demonstrating high resolution of fluorescent derivatives of amino acids, and since then CZE has been widely used for this purpose.

Amino acids can be derivatized as fluorescein isothiocyanate derivatives [64] and can be determined with laser induced fluorescence detection. This is very sensitive, but the presence of excess reagent interferes with the determination. Another derivatizing agent used is fluorescein thiohydantion used with laser induced detection of the derivatives [65].

A post-column derivatization reaction that results in chemiluminescence emission

is also used. For example Zhao et al. [66] determined amino acids as their isoluminol thiocarbomyl derivatives with a minimum detectable quantity of 30 femtomoles. Amino acids can also be determined as dansylated derivatives [67], phenylthiohydantoin derivatives [68], or OPA derivatives [69].

Another approach is to use a background electrolyte containing a UV-absorbing species that is displaced by the analyte ion in the sample zone. Oefner [70] determined amino acids within 60 s by using chromate ion in the carrier. Here, 5, 5 diethyl barbiturate was added to avoid a gradual shift of pH. This method has better selectivity, sensitivity, and speed compared to the other methods that has been described before with the capillary electrophoresis technique.

Zhou [71] used zwitterionic buffers with amperometric detection at a copper micro electrode. The buffer depresses the osmotic flow thereby resulting in a better separation of amino acids and an increase in the S/N ratio by reducing the noise due to the high current used in the separation. Though the method is sensitive, and in some cases separation can be done within 60 s, there can be limitations due to the smaller diameters of the capillaries used and their capacity to dissipate heat. The selection of stationary phases and mobile phases available are less compared to the conventional separation techniques, and although it is efficient for the determination of DNA fragments, it is not superior to HPLC in its quantitative capabilities [62].

Miscellaneous Methods

Glutamic acid has been determined using a series-electrode piezoelectric quartz crystal sensor [74]. Here, the amino acid is treated with formaldehyde and condensation

occurs between the amino and aldehyde groups. The result of this reaction is that the amino group is masked and its basicity disappears, hence the solution shows acidic properties [75]. Frequencimetric titration with NaOH as titrant is used for the determination of glutamate with this sensor. The lowest titratable concentration of glutamic acid is 0.070 mM. Though this sensor is sensitive, it is not selective for glutamate.

Another approach using a chemical sensor for glutamate determination is based on near-infrared spectroscopy [76]. Glutamate can also be determined by titrimetry with a base [77], and by indirect atomic absorption spectrophotometry [78]. Most of these techniques require separation before determination. In some cases, derivatization is a must before determination, and all these procedures are invariably time consuming.

Enzymatic Methods

Enzymes are a large and ubiquitous class of protein molecules that act as biocatalysts. They are distinguishable from each other by their active sites, into which only particular substrates can fit. The primary structures of enzymes are due to the sequence of different amino acids linked to each other by peptide bonds. The size, shape, and function of the enzyme are affected by the number of amino acids, their position, and their interaction in forming bonds.

Enzymes affect the rate of a reaction without themselves undergoing any permanent change and can often catalyze reactions 10^8- 10^{11} times more rapidly than the corresponding non-enzymatic catalysts. As many as 10^6 substrate molecules, consequently can be metabolized per enzyme active site and per minute. These rates are

achieved even though the enzyme catalyzed reaction requires milder conditions than some non enzymatic methods. The range of reactions catalyzed by enzymes is broad and these reactions are usually specific or highly selective, and they are especially preferred when low substrate concentrations are encountered.

Even though enzyme-catalyzed reactions have been used for analytical purposes for many years, their early use has been limited due to their instability. Out of the different methods available to prolong the activity of the enzymes, two such methods are discussed below as they were used in this work.

Improvement of Enzyme Stability by Immobilization

In aqueous solutions enzymes lose their catalytic ability relatively fast, and neither can the enzymes be recovered nor their activity regenerated [79]. This difficulty can be minimized either by protein engineering or by immobilization on inert supports.

Protein engineering can be used to prevent destabilizing processes that normally result in enzyme deactivation. The structural integrity of an enzyme can be strengthened by design. In stabilization through design process, the amino acid residues that provide only weak contributions to the conformational stability of the protein are replaced by ones that provide stronger interactions. Another approach that enhances the stability of a protein is to replace a flexible residue such as glycine, which requires greater free energy than other, sterically constrained residues in order to restrict its conformation [80]. Provided the substitutions do not introduce undesirable steric interactions, the mutated protein will have a decreased entropy of unfolding, resulting in stabilized protein. In second approach the stability is increased by cloning [81].

Immobilization has been defined as the process whereby the movement of enzymes, cells, etc. in a given space is completely or severely restricted usually resulting in a water insoluble form of the enzyme [82]. The methods available for immobilization can be broadly classified and sub-classified as:

Physical Methods

- entrapment
- adsorption

Chemical Methods

- chelation
- covalent binding
- crosslinking

The covalent and crosslinking methods are briefly discussed here since they have been used in this work.

Covalent Binding. Because of their primary structure, enzymes have different functional groups that can be used for immobilization. Only a few supports, however, have reactive groups for direct coupling of enzymes like maleic anhydride-based polymers, and methyl acrylic anhydride-based polymers. Supports that do not posses active groups, but have hydroxyl, amino, or carboxyl groups, can be activated before immobilization by undergoing reactions like diazotization, alkylation, arylation, amide-bond formation, Schiff's base formation [84, 85], Ugi reaction [86], amidination, or thio-disulfide interchange [87]. This is a wide spread approach where the enzymes are

covalently bonded to the matrices. The selection of conditions for immobilization is difficult compared to other methods and the immobilization should not involve the functional groups essential for the catalytic action of the enzymes. Zaborsky [83] has devised different methods by which the activity of the immobilized enzyme can be enhanced. The wide variety of binding reactions and of carriers with functional groups capable of covalent coupling, or susceptible to being activated to give such groups, makes this a generally applicable method of immobilization [82].

Crosslinking. The use of this method was first reported by Quicho and Richards [88]. The process involves the formation of covalent bonds between enzyme molecules in the presence of bi- or multifunctional reagents, leading to three dimensional crosslinked water insoluble aggregates. Optimum conditions can be obtained only by a trial and error method. Homobifunctional reagents, hetero bi-, or heteromultifunctional reagents can be used. The reagents containing carboxyl, diazo, isocyanate, alkyl iodides, or iodoacetamide groups can be used as crosslinking agents. The most common reagent used for this purpose is glutaraldehyde.

The reaction with glutaraldehyde was originally thought to involve Schiff's base formation between selected amino groups of the enzyme and the aldehydic groups of the reagent (Scheme A, Figure 1), but the exceptional stability of glutaraldehyde in basic media led to another scheme, also shown in Figure 1(Scheme B). This suggests that the reaction involves the conjugate addition of amino groups of enzyme to the ethylenic double bonds of the α, β- unsaturated oligomers found in the commercial aqueous solutions of glutaraldehyde used. This is supported by the fact that reactivity of freshly distilled glutaraldehyde is lower towards enzymes compared to the aged commercial

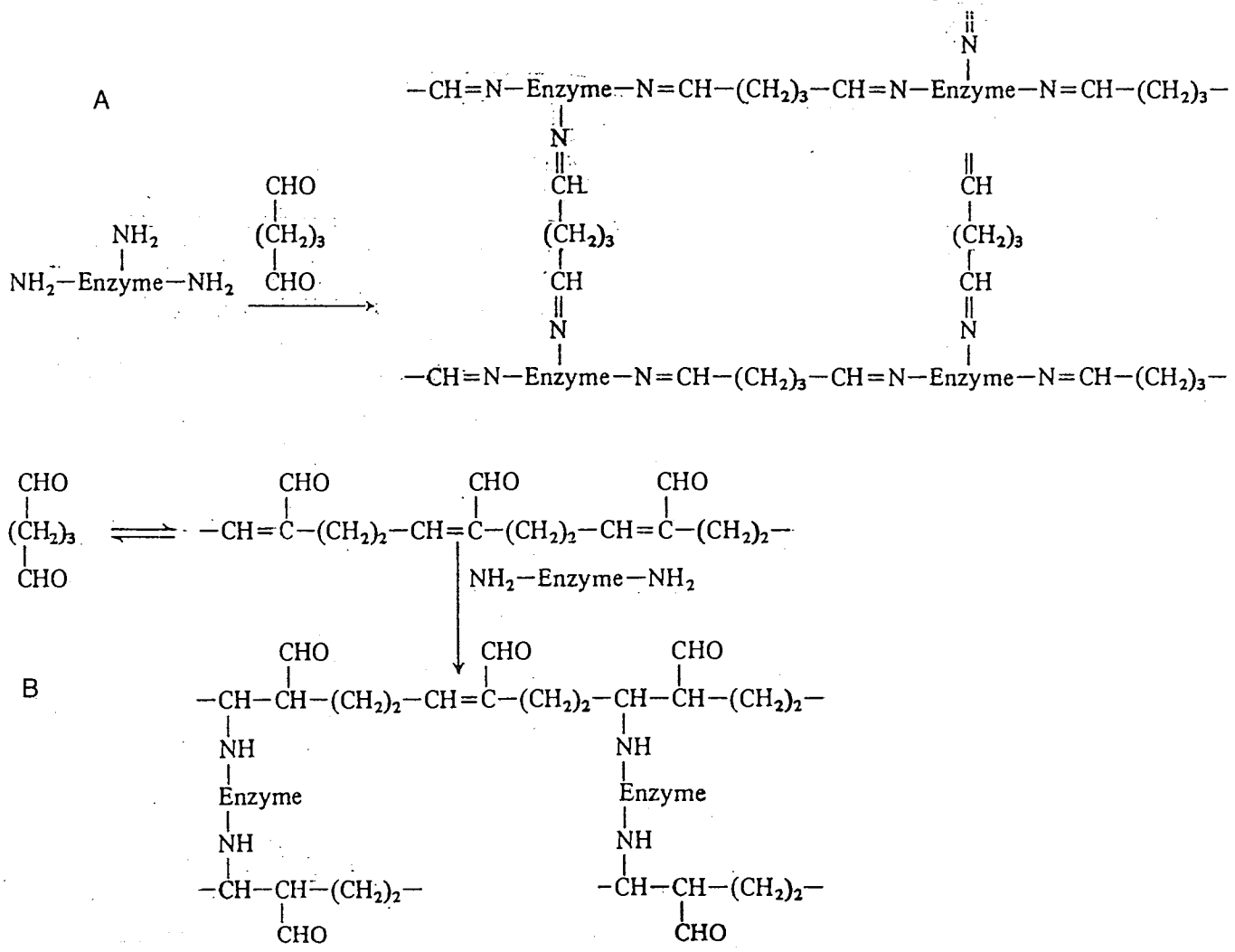

Figure 1. Schemes A and B for Crosslinking of Glutaraldehyde

product [89]. The formation of aggregates depends on the concentration of enzyme, reagent, pH, and ionic strength of the solution [89].

So far the use of enzymes as catalysts and the methods for their stabilization had been discussed in a brief and general manner. The use of enzymes in their immobilized form for the determination of glutamate is considered in the following sections. In particular, the methods employing the enzymes glutamate decarboxylase, glutamate oxidase, and glutamate dehydrogenase for the determination of glutamate are investigated.

Determination Using Glutamate Decarboxylase (EC 4. 1. 1. 15)

The reaction of glutamate in the presence of water and catalyzed by glutamate decarboxylase (GlDC) to form γ-aminobutyric acid and CO_2 is as shown:

$$\text{L-glutamate} + H_2O \xrightarrow{\text{GlDC}} \gamma\text{-aminobutyric acid} + CO_2$$

The liberated CO_2 can be detected by immobilizing the enzyme at the tip of a CO_2 gas-sensing electrode [90]. The glutamate diffuses through the membrane and the gas can be detected by a commercial CO_2 gas-sensing electrode. The linear response was found to be from 0.10 mM to 10 mM. This sensor, however, has a long response time and poor selectivity.

A CO_2 optrode was constructed by Wolfbeis and his co-workers [91] by covering the fiber-optic sensor with a membrane-immobilized glutamate decarboxylase. The production of carbon dioxide was determined by a pH sensitive fluorescent dye, 1-hydroxypyrene-3, 6, 8-trisulphonate, entrapped by the membrane. The change in the

signal was found to be related to the concentration of glutamate in the linear range of 0.10 mM to 2.5 mM.

Determination Using Glutamate Oxidase (EC 1. 4. 3. 11)

This enzyme (GOX) catalyzes the reaction of glutamate in the presence of oxygen and water to produce α-ketoglutarate, NH_3, and H_2O_2. The concentration of glutamate can be determined by measuring the concentration of the ammonia, the H_2O_2 produced, or the oxygen consumed.

$$\text{L-glutamate} + O_2 + H_2O \xrightarrow{\text{GOX}} \alpha\text{-ketoglutarate} + NH_4^+ + H_2O_2$$

Determination Based on Oxygen Consumption. The oxygen consumption was followed by using either an electrode or an optrode. Wolfbeis et al. [91] developed an oxygen optrode. Here, the oxygen sensitive optrode was covered with a membrane containing the immobilized enzyme. The decrease in oxygen partial pressure was determined by dynamic quenching of the fluorescence of an oxygen sensitive indicator dye. The glutamate linear range for this sensor is 0.02 mM to 1.0 mM.

An O_2 consumption-based determination [92] has been developed by immobilizing the enzyme glutamate oxidase on an Immobilon-AV affinity membrane (size exclusion membrane to reduce interference) attached to an oxygen electrode. The detection limit was found to be 4.0 μM. The sensor was stable for approximately 200 determinations.

Glutamate oxidase (from *Streptomyces Platensis* NTU 3304) has been used to determine glutamate by monitoring the consumption of O_2 [93]. The enzyme was

immobilized onto a cellulose triacetate membrane which was held at the surface of an oxygen sensing electrode with a piece of polyethylene net secured by an O-ring. A polycarbonate ultra filtration membrane was used on the top of the enzyme membrane for protection. A linear relationship was observed between the decrease in the dissolved O_2 and the concentration of glutamate in the range 0.12 mM to 0.84 mM.

Determination Based on the Measurement of the Ammonium ions Produced. The ammonium ions produced by the reaction can also be monitored to determine glutamate. A fiber optic ammonia sensor was developed by Arnold and Ostler [94]. The internal solution was composed of ammonium chloride and *p*-nitrophenol which is a pH indicator dye. The pH change is measured as a change in absorbance. Another enhanced ammonia sensor has been developed by using chlorophenol red and bromothymol blue in the internal solution of a fiber-optic sensor [95].

Another sensor was designed for fluorescence detection [96]. This sensor was constructed by trapping, with a gas permeable membrane, a thin layer of an internal solution containing ammonium chloride at the tip of a fiber-optic probe. The ammonia diffuses through the membrane and reacts with the dye forming the non-protonated form of the dye, which is fluorescent. The limit of detection for glutamate with this sensor is 0.10 μM at pH 7.80. The main problem associated with using ammonia sensors is that they are pH dependent. At pH less than 8.0, for example, only 3% of the total ammonium nitrogen is in the form of detectable ammonia.

Determination of H_2O_2 Produced. Several biosensors have been developed based on the detection of H_2O_2. Most of them are based on electrochemical detection of hydrogen

peroxide given by the equation below [97 - 105].

$$H_2O_2 \rightarrow O_2 + 2H^+ + 2e^-$$

A micro enzyme electrode was prepared, for example, by crosslinking L-glutamate oxidase from *Streptomyces P-106* with glutaraldehyde on an aminopropyl-platinized platinum wire [106]. The H_2O_2 is detected amperometrically at 0.5 V vs. SCE. The linear range is reported to be from 20.0 μM to 2.0 mM.

Yao et al. [107] have developed and compared an enzyme electrode and an enzyme reactor based on immobilized L-Glutamate oxidase. The hydrogen peroxide produced is monitored amperometrically in the range of 5.0 μM to 1.0 mM. The enzyme reactor was found superior with regard to sensitivity and speed. Another enzyme electrode was prepared by gelatin entrapment of glutamate oxidase [108]. The sensor responded linearly to glutamate in the range 0.0010 mM to 1.0 mM with a response time of about two minutes.

The common interference in most of the H_2O_2 -based sensors is ascorbic acid. One method to reduce the interference is to add a membrane like a Nafion membrane [109] or to form a membrane on the electrode by electropolymerization [97]. The interference of ascorbic acid and other electro active compounds can be eliminated, for instance, by a film of 1,2-diaminobenzene deposited on the working electrode by electropolymerization.

Another way to remove this interference is to use a mediator like tetrathiafulvalene (TTF), which favors the use of a lower applied potential [110]. The sensor can be based on a carbon paste electrode containing the electron transfer media,

TTF, and the enzyme immobilized on the electrode surface. The enzyme is crosslinked with glutaraldehyde and the matrix held onto the electrode surface by an electrochemically deposited polymer of resorcinol and 1, 3-phenylenediamine. The incorporation of the mediator permits the sensor to operate at 0.15 V vs. an Ag/AgCl reference electrode. Other mediators used are 1, 1-dimethyl ferrocene [105] and a ferrocene modified siloxane ethylene oxide polymer [111].

Hydrogen peroxide can also be detected by chemiluminescence. A reaction scheme often used is based on glutamate oxidase and peroxidase-catalyzed oxidation of luminol by hydrogen peroxide [112]. The enzyme reactor is prepared by immobilizing glutamate oxidase on controlled pore glass packed into a column. The light emission generated during the determination was conducted through a fiber-optic cable to a photomultiplier tube. The linear range for determination is between 0.70 μM to 60 mM of glutamate. The problem with this arrangement is the high pH required for chemiluminescence at which the enzyme is not stable.

Another chemiluminescent reaction used for H_2O_2 determination is based on the reaction of hydrogen peroxide with acridinum ester to produce light [113]. The reaction scheme is as follows.

$$\text{L-glutamate} + O_2 \rightarrow \alpha\text{-ketoglutarate} + NH_3 + H_2O_2$$

$$H_2O_2 + \text{acridinium ester} \rightarrow \text{n-MA} + CO_2 + \text{light}$$

The hydrogen peroxide generated from the first reaction reacts with the ester to form light. The second reaction requires a high pH for maximal light production also, detrimental to enzyme activity. The enzyme is immobilized on porous-glass beads

packed into a column. Hydrogen peroxide is produced when glutamate passes through this column and is mixed with the ester. Sodium hydroxide was added before this mixture reaches the detector. The problem with this detector is the interference from ascorbic acid.

Determination with Glutamate Dehydrogenase (EC 1. 4. 1. 3)

Glutamate is converted to α–ketoglutarate in this reaction catalyzed by glutamate dehydrogenase. Being a dehydrogenase NAD^+ is a cofactor for this enzyme.

$$\text{L-glutamate} + NAD^+ \xrightarrow{\text{GlDH}} \alpha\text{–ketoglutarate} + NH_4^+ + NADH$$

Glutamate can be directly determined by monitoring NADH. This can be done by fluorescence [114, 115]. The enzyme GlDH was immobilized on isothiocyanate-modified CPG and packed into a column [116]. The NADH produced from the reaction was then monitored fluorometrically at an excitation wavelength of 340 nm and an emission wavelength of 460 nm. The working curve is linear up to 0.2 mM.

An indirect method using a fiber-optic biosensor was developed by Wang [117]. Here the NADH produced in the glutamate reaction is oxidized back by transferring the electrons to a non-fluorescent compound, resazurin, in the presence of the enzyme diaphorase to generate a highly fluorescent compound, resorufin. Both enzymes were immobilized on a thin layer of a Polyvinyl alcohol membrane at the end of a fiber optic probe. The generated fluorescence intensity was found to be directly proportional to the concentration of glutamate. The linear range is between 1.0 μM to 10.0 μM. The response time was comparitively faster at higher glutamate.

Glutamate can also be determined by monitoring NADH spectrophotometrically. Here, the NADH is reacted with a dye, for example iodonitro tetrazolium (INT) to form a colored product, formazon, which can be detected spectrometrically [118, 119]. NADH was also detected by treating it with bacterial bioluminescent enzymes immobilized on a separate nylon coil [120].

Electrochemical methods can also be used for the detection of NADH, but the direct oxidation of NADH at an electrode suffers from high overvoltage [121] and electrode fouling [122]. This makes the detection system susceptible to interfering reactions, high background currents, and a variation of the response factor with time. Therefore a mediator is normally used which facilitates the oxidation of NADH back to NAD^+. Different mediators, like phenazine methosulfate [123], Meldola Blue [124] Methylene Blue [125], phenoxazines, and phenothiazines [126] have been used. Some of the mediators were found to be inactive in buffers like glycine - NaOH [128]. Most of them are not stable, especially in the pH range required for operation of glutamate dehydrogenase [126, 127].

The goal of this research discussed in this thesis is to develop detection methods for glutamate that complements existing techniques. Glutamate was chosen because its role as a flavoring agent for decades. Moreover, because of its alleged part in the Chinese Restaurant Syndrome, a brief discussion of which is presented in Chapter I. Food industry would probably need methods that are simple, fast, selective, sensitive, efficient, and methods that can be automated.

Two electrochemically based detection strategies are discussed here. The first approach that was investigated brings together the use of immobilized glutamate

dehydrogenase, diaphorase, and the oxidation of co-factor with the help of a mediator. The second approach is based on the detection of the H_2O_2 produced by the oxidation of glutamate by glutamate oxidase. The specific details of each approach are discussed at the beginning of the respective chapters, and the conclusions of these methods are presented at the end of this dissertation.

CHAPTER III

DETERMINATION OF GLUTAMATE USING THE ENZYMES GLUTAMATE DEHYDROGENASE (EC 1.4.1.3) AND DIAPHORASE (EC 1.8.1.4)

INTRODUCTION

Coupled Reactions

Coupling a main enzyme-catalyzed reaction with an indicator reaction is common in enzymatic methods. This is often practiced to indicate (or) amplify the change in the reaction. The most popular of these coupling of reactions uses an organic dye in the indicator reaction. The reaction between the dye and a product of the main reaction can be followed. For example in the determination of glucose using glucose oxidase (EC 1.1.3.4) and peroxidase (EC 1.11.1.7), the oxidized (or reduced) form of the dye can be monitored spectrophotometrically:

$$\text{glucose} + O_2 + H_2O \xrightarrow{\text{Glucose oxidase}} \text{gluconic acid} + H_2O_2 \qquad (1)$$

$$H_2O_2 + \text{dye (reduced form)} \xrightarrow{\text{Peroxidase}} \text{dye (oxidized form)} + H_2O \qquad (2)$$

or electrochemically, as illustrated by the determination of L-arginine using arginase (EC 3.5.3.1) and urease (EC 3.5.1.5) as in:

$$\text{L-arginine} \xrightarrow{\text{arginase}} \text{L-ornitnine} + \text{urea} \quad (3)$$

$$\text{urea} \xrightarrow{\text{urease}} 2NH_4^+ + HCO_3^- \quad (4)$$

and monitoring with a cation-selective electrode responsive to NH_4^+ [129]. The change in potential with time is measured, which is proportional to the concentration of arginine.

When dehydrogenases are used as catalysts, a cofactor is needed that can act in an intermediary capacity in the electron transfer process. Nicotinamide adenine dinucleotide (NAD^+) is one such coenzyme that is used in these reactions. The structure of NAD^+ is shown in Figure 2. It acts by (reversibly) accepting hydrogen at the fourth position of the nicotinamide ring. Increased sensitivity and lower limits of detection can be achieved by coupling another reaction in which NADH is oxidized back to NAD^+.

Scheme of Detection

The method proposed here uses the set of coupled reactions illustrated in Figure 3. A reaction catalyzed by the enzyme diaphorase (Dia) is coupled with a reaction catalyzed by glutamate dehydrogenase (GlDH) to increase the sensitivity. Nicotinamide adenine dinucleotide (NAD^+) acts as a coenzyme for the GlDH-catalyzed reaction. Normally oxidoreductases have prosthetic groups bound to them that help the enzyme to catalyze by acting in an intermediary capacity in the electron transfer process. But GlDH, not being a flavoprotein cannot catalyze the reaction on its own unless NAD^+ is present. The reaction sequence is shown below

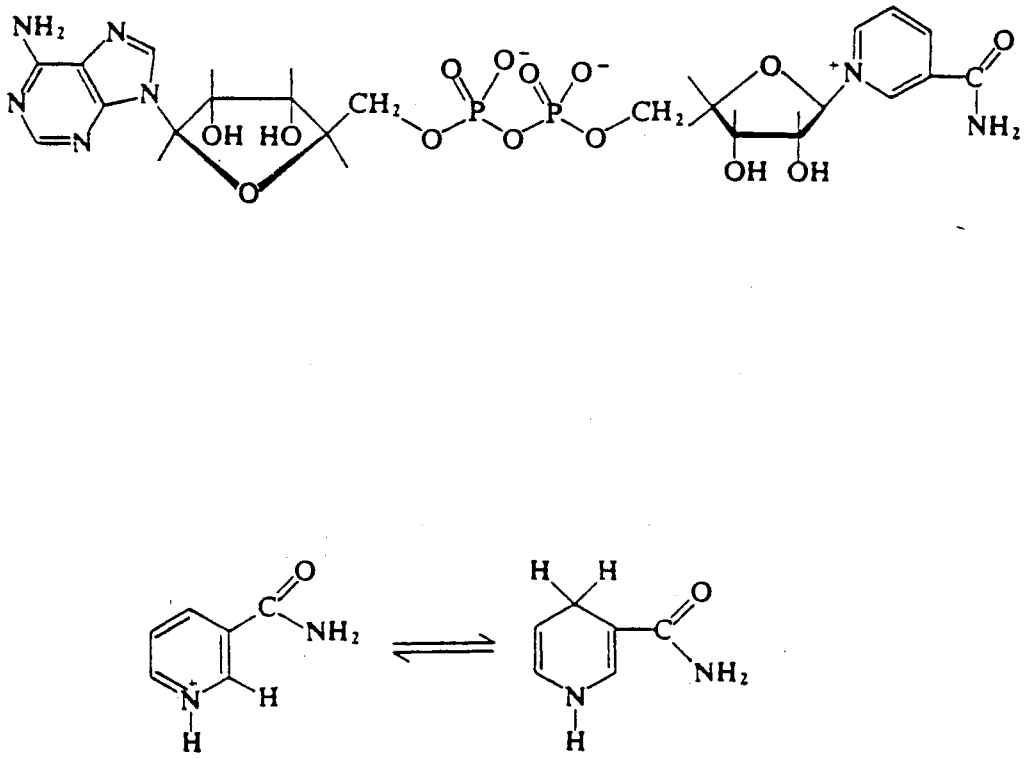

Figure 2. Structure of NAD^+ and of Its Reduced Form, NADH

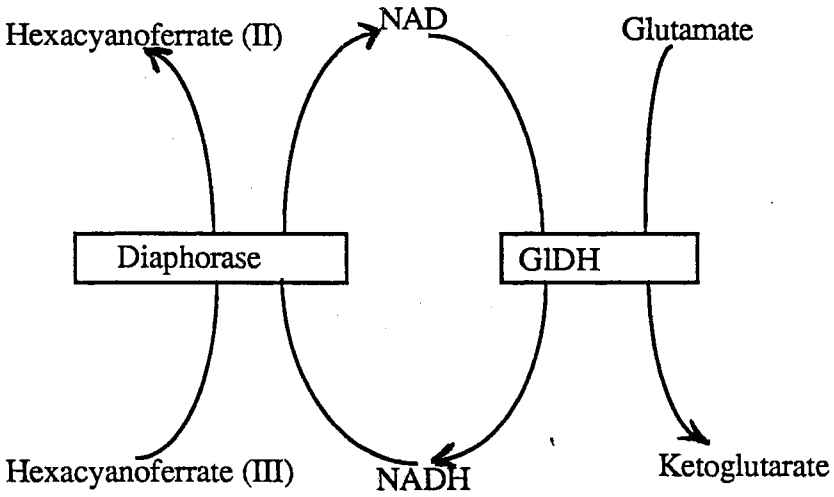

Figure 3. Scheme of Reactions for the Determination of Glutamate Using a Two-Enzyme System.

$$\text{L-glutamate} + NAD^+ \xrightarrow{GlDH} \alpha\text{-ketoglutarate} + NH_4^+ + NADH \qquad (5)$$

$$NADH + Fe(CN)_6^{3-} \xrightarrow{Dia} NAD^+ + Fe(CN)_6^{4-} \text{ (indicator reaction)} \qquad (6)$$

The indicator reaction can be split into two reactions:

$$NADH + \text{Med (ox)} \xrightarrow{Dia} NAD^+ + \text{Med (red)} \qquad (7)$$

and the electrode reaction:

$$\text{Med (red)} \rightarrow \text{Med (ox)} + e^- \qquad (8)$$

As can be seen in reaction (5) L-glutamate is oxidized to α-ketoglutarate, and in that process NAD^+ gets reduced to NADH. In reaction 2, the NADH is re-oxidized to NAD^+, reducing the mediator (Med) in the process. The current produced by the oxidation of the mediator is followed. The mediator is generally used to avoid side reactions that occur because of the direct oxidation of NADH at the electrode surface, and moreover to reduce the applied potential. Here, hexacyanoferrate(III) acts as the mediator. The hexacyanoferrate(II and III) system is an attractive oxidation-reduction couple as it is stable in the presence of oxygen and it is electrochemically reversible.

The current produced during the oxidation of hexacyanoferrate(II) to hexacyanoferrate(III) can be detected at a platinum electrode. One special aspect here is the cycling of NAD^+/NADH between the two enzyme-catalyzed reactions which results in the regeneration of NAD^+. The initial rate of the reaction (the slope of the signal vs. time profile) was used for the determination of glutamate, as this kinetic approach is more sensitive and convenient in continuous-flow/stopped-flow processing. A brief

account of this approach is presented below.

Initial Rate Method of Determination

In general for the conversion of the substrate [S] to product [P], the following sequence applies:

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P \qquad (9)$$

Almost immediately after the enzyme-catalyzed reaction (9) begins, the rate of change of concentration of the enzyme-substrate complex with time is essentially zero, that is, the enzyme-substrate complex achieves a steady-state condition. The rate equation for the intermediate is,

$$d[ES]/dt = k_{+1}[E][S] - k_{-1}[ES] - k_{cat}[ES] = 0 \qquad (10)$$

and permits to solve for [ES]. Since $[E]_0$, the total enzyme concentration, can be given at any time as the sum of [E] and [ES]; we have:

$$d[ES]/dt = k_{+1}[E]_0 - (k_{+1}[S] + k_{-1} + k_{cat})[ES] = 0 \qquad (11)$$

Dividing the above equation by k_{+1} and solving for [ES], results in:

$$[ES] = \frac{[E]_0[S]}{\frac{(k_{-1} + k_{cat})}{k_{+1}} + [S]} \qquad (12)$$

and the rate equation for the formation of product can be written as:

$$IR = k_{cat}[ES] = \frac{k_{cat}[E]_0[S]}{\frac{(k_{-1} + k_{cat})}{k_{+1}} + [S]} \quad (13)$$

The use of the initial rate (IR), which is the measurement of rate before more than 10% of the substrate has been converted to product [130], minimizes the complications that arise due to the effects of reversible reactions, and inhibition of enzyme by products. When all of the enzyme is in the [ES] form, then the maximum velocity is given by

$$(IR_{max}) = k_{cat}[E]_0 \quad (14)$$

Substituting in equation (13) results in:

$$IR = \frac{IR_{max}[S]}{K_M + [S]} \quad (15)$$

This equation is called the Michaelis-Menten equation, and K_M is the Michaelis-Menten constant. Numerical values of IR_{max} and K_M can be derived by following a transposition of this equation:

$$\frac{1}{IR} = \frac{1}{IR_{max}} + \frac{K_M}{IR_{max}} \cdot \frac{1}{[S]} \quad (16)$$

which can be plotted as 1/IR vs. 1/[S]. This plot is called the Lineweaver-Burk plot. The slope of this line is K_M/IR_{max}, and the intercept is $1/IR_{max}$ as shown in equation (16). The values of IR_{max} and K_M can then be calculated from the plot. A plot of initial rate vs. substrate concentration is shown in Figure 4. As predicted by equation (15), at low substrate concentration, the rate is directly proportional to substrate concentration,

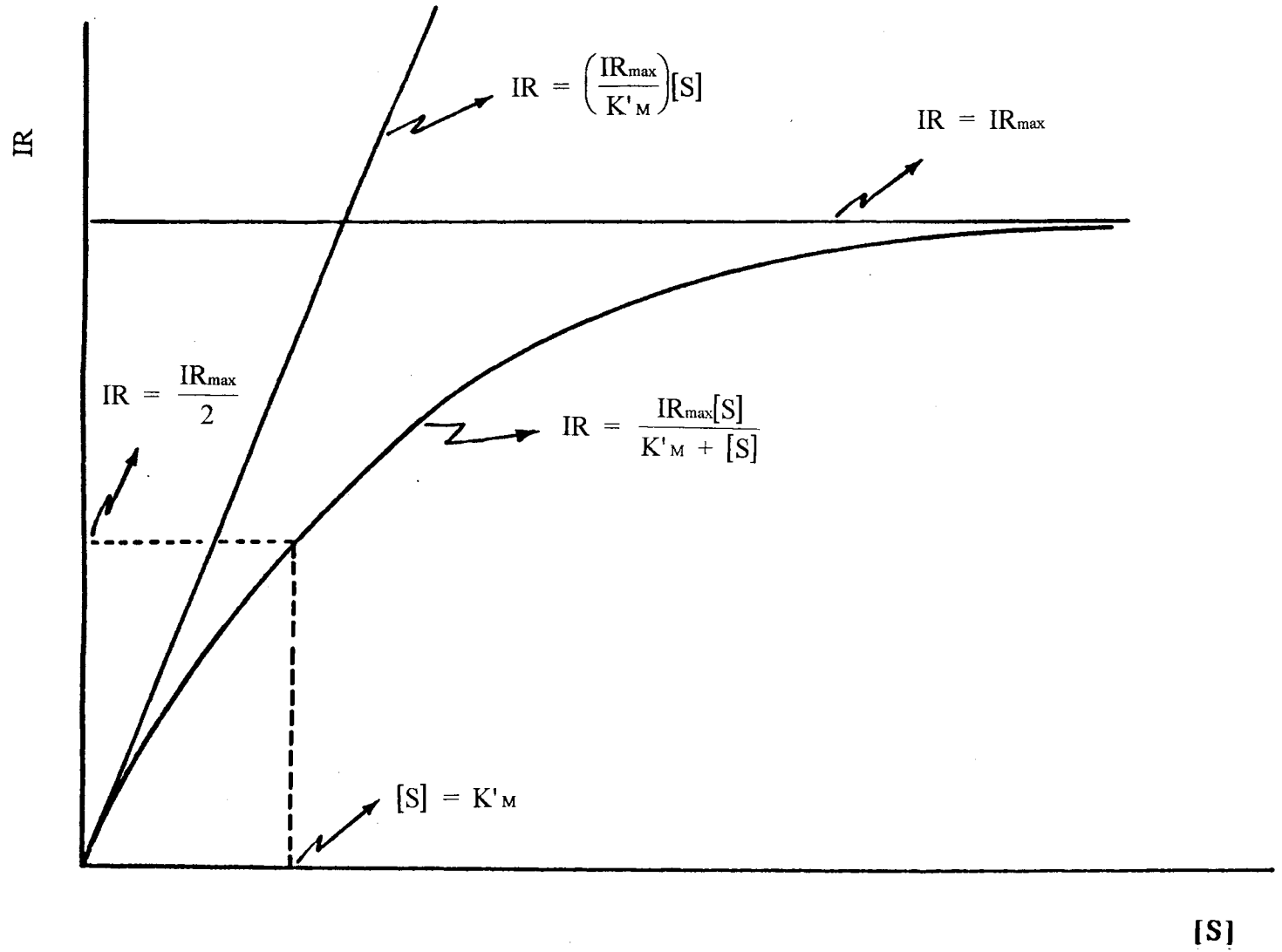

Figure 4 Plot of Initial Rate vs. Substrate Concentration for enzyme-catalyzed reactions following Michaelis-Menten kinetics.

that is;

$$IR = \left(\frac{IR_{max}}{K_M}\right)[S] \qquad (17)$$

Therefore the linear region at low substrate concentration in the plot of initial rate vs. substrate concentration can be employed for the determination of the analyte.

When the enzyme is immobilized the K_M value either decreases or increases in comparison with the K_M value of the native enzyme, for example, K_M of lactate dehydrogenase was found to decrease on immobilization and for L-amino acid oxidase was found to increase [131]. This K_M is called the apparent Michaelis-Menten constant, represented by K'_M and can be calculated experimentally from the Lineweaver-Burk plot. One of the main effects on the value of K'_M is the diffusional constraint for the substrate to approach the enzyme active site and for the products to exit. An effective way to deal with this situation is discussed later in this chapter.

EXPERIMENTAL

Instrumentation

The general experimental setup (Figure 5) for the determination of glutamate consists of a pump that can be programmed for continuous-flow/stopped flow processing, a home-built electrochemical cell, an amperometric detector, and a recorder. All the components are described in detail here.

The different solutions were pumped into the electrochemical cell with a FIAtron SHS - 200 (FIAtron Systems, Milwaukee, WI) solution handling unit. This unit has a

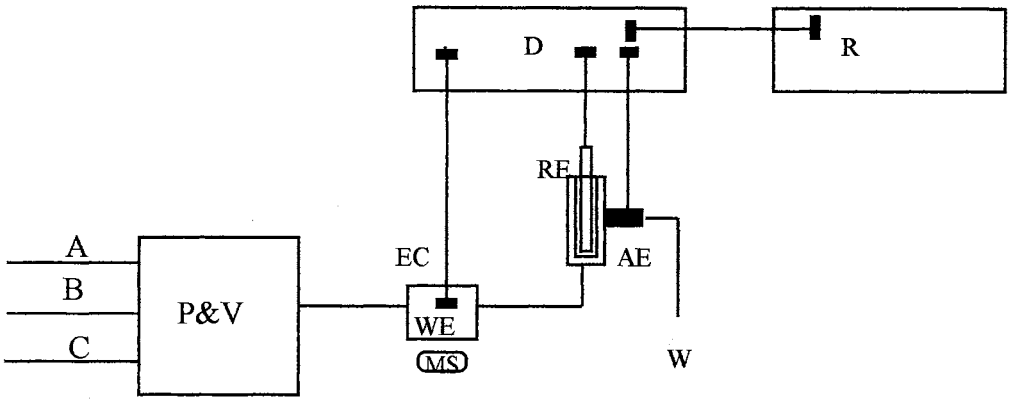

Figure 5. Schematic Diagram of the Continuous-Flow/Stopped-Flow/ Continuous-Flow System.

A Intake of reagent (NAD^++ $[Fe(CN)_6]^{3-}$)

B Intake of sample

C Intake of carrier

P&V Pump and valve unit (FIAtron SHS-200)

EC Electrochemical cell (bioreactor)

WE Pt-ring working electrode

RE Reference electrode (Ag/AgCl/ 3.0 M NaCl)

AE Stainless steel tube (auxiliary electrode)

W Waste outlet

D Potentiostat/amperometric detector (LC-4B, Bioanalytical Systems Inc.)

R Strip-chart recorder (Hewlett Packard)

MS Magnetic stirrer

multi-channel peristaltic pump, and a dual channel sample injector that is an all Teflon device consisting of electrically driven four-way solenoid valves of micro miniature construction. All components are under software control and are readily programmable from a soft touch front panel keyboard. The value of any programmable parameter can be displayed on a four digit LED read out and can be reprogrammed using the front panel.

The sample injection system has two sample loops. With the help of the valves, both sample and reagent can be pumped through, with the carrier. The different operational modes available allow the user to chose the mode of operation. In the stopped-flow mode used for this work, the unit pumps a programmable volume of the sample and the reagent (if needed) into the cell. The sample-reagent mixture remains stationary in the cell for a programmed time interval during which the measurement is made. All the tubing used in the pump was made of Tygon (Fisher AccuRated 1.0 mm i. d., Fisher Scientific, Pittsburgh, PA). The rest of the tubing for the setup was 1.0 mm i. d., PTFE (Cole Palmer, Chicago, IL).

Figure 6 illustrates the design of the flow-through/electrochemical cell containing a circular platinum electrode and the rotating reactor. The main body of the cell is made of Plexiglas. The circular ring electrode is embedded into the lower half of the cell around the rotating disc in a symmetrical position.

The reactor is a Teflon disk in which a small magnetic stirring bar has been embedded (Teflon-coating micro stir bar from Markson Science, Inc., Phoenix, AZ). A small needle like projection was located at the center of the disk. This rests in a specially designed grove that permits smooth rotation of the reactor. The reactor is driven by a

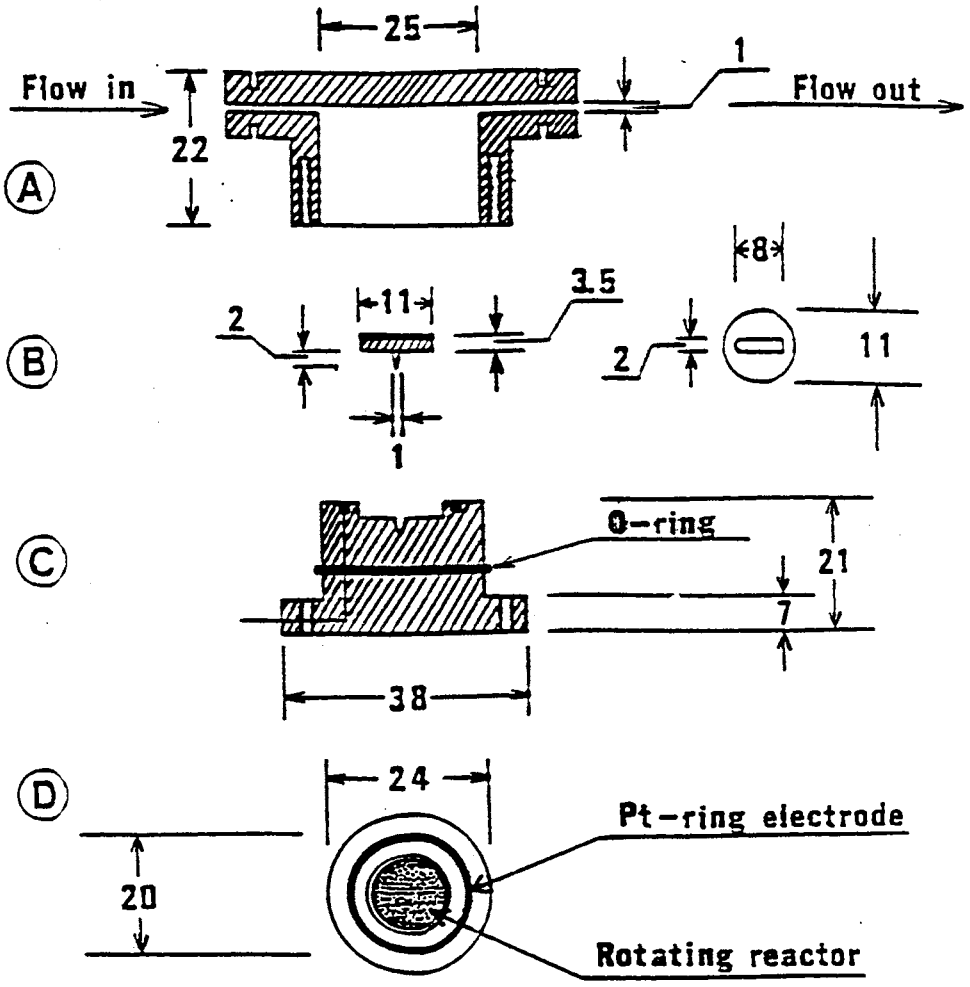

Figure 6. Schematic Representation of the Flow Cell (All Dimensions in mm).
A: Upper cell body outlining the flow-through reaction/detection chamber.
B: Rotating reactor showing the positioning needle point.
C: Lower half of the cell exhibiting the well for the rotating reactor, and the Pt-ring electrode.
D: Top view of the cell.

laboratory magnetic stirrer (Dayton Electric, Chicago, IL) located underneath the electrochemical cell. The rotation velocity was controlled by means of a variable transformer. The relationship between the voltage settings and the revolutions per minute of the reactor was determined by filming the rotation of the reactor with time and by playing back at slow motion [134].

All the pH measurements were made using a Model 601 A digital pH meter (Orion Research, Cambridge, MA). The meter is equipped with an epoxy-body combination electrode (Sensorex, Westminster, CA).

The signals were obtained from an amperometric detector. The purpose of this amperometric unit is to apply a fixed potential between working and reference electrode and measure the resulting current at that potential. This unit (Model LC - 4B, Bioanalytical Systems Inc., West Lafayette, IN) has a mode switch that selects the function to be monitored by the internal digital voltmeter and displayed on the front panel window. Any applied potential value between 0 and ±1.99 V can be applied. An operating baseline can be established by nullifying the steady-state background current using the offset control. The sensitivity of the controller output can be selected by a range switch. The output can be "conditioned" using a filter switch. The available time constants help in filtering the noise and thereby to work under optimum conditions. Connections to the working cell and the recorder can be made from the rear panel.

The signals were recorded using a single pen drive strip-chart recorder (Model 7127 A, Hewlett-Packard, San Diego, CA). The chart transport system has 4 selectable speeds for the chart rolls. The input module to the recorder has 16 spans from 1.0 mV to 100 V with 1 M input resistance at the null on all fixed and variable spans.

Chemicals

Glutamic dehydrogenase (EC 1.4.1.3; from Bovine liver), Diaphorase (EC 1.8.1.4; from *Clostridium Kluyveri*), aminopropyl-CPG (700Å), methionine, isoleucine, tryptophan, histidine, and ß-Nicotinamide adenine dinucleotide were bought from Sigma, St. Louis, MO. Glutamic acid and glutathione were obtained from Fisher Scientific, Springfield, NJ. The rest of the amino acids were purchased from Nutritional Biochemicals Corporation, Cleveland, OH. Dibasic sodium phosphate was from CMS, Houston, TX, monobasic sodium phosphate and hexacyanoferrate(III) were from Mallinckrodt, St. Louis, MO, and glutaraldehyde (25% w/w aqueous solution) was from Aldrich, Milwakee, WI. Seasonings, sauces, and other food products were purchased from a local grocery store.

Reagents

All the solutions were prepared in deionized distilled water (Wheaton Autostill 1.5, Wheaton Instruments, Milleville, NJ). Buffer solutions were prepared by dissolving the appropriate amounts of monobasic and dibasic phosphate in de-ionized distilled water. The main reagent consisted of NAD^+ (1.0 mM) and hexacyanoferrate (III) (1.0 mM), and was prepared in the working buffer solution which was a phosphate buffer (pH 7.80).

Enzyme Immobilization

Aminopropyl-CPG was used to immobilize GlDH and diaphorase. The following

procedure was used for the pre-treatment of the aminopropyl-CPG. The CPG was spread on one side of the double sided tape and was allowed to react with 2.5% glutaraldehyde solution (pH 10.0, carbonate buffer) for 2 h. After this, the CPG was washed thoroughly with pH 7.00 phosphate buffer. For GlDH, a 125 µl aliquot of the enzyme suspension (pH 7.50) was added to the CPG and the reaction was allowed to continue overnight at 4 °C. In case of Diaphorase about 4.5 mg of the enzyme was dissolved in 150 µl of pH 7.00 buffer (phosphate) and was reacted with the pre-treated aminopropyl-CPG overnight at 4 °C. A simplified scheme of the immobilization chemistry is shown in Figure 7. After this, the immobilized enzymes were washed with phosphate buffer (pH 7.50) and stored in the same buffer at 4 °C for further use. This enzyme preparation was found to be stable for about two weeks.

Measurement of Initial Rate

For measurements of the initial rate, a continuous-flow/ stopped-flow/ continuous-flow method was used. In the continuous-flow step the carrier solution (0.10 M phosphate buffer, pH 7.80) was pumped through the system. During this time the sample and the reagent were loaded, mixed, and carried to the optimum position in the cell. During the stopped-flow step, the pump was stopped for a programmed interval of time allowing the reaction to take place. The different time intervals used in this work are tabulated in Table IV.

The resulting signal produced by the oxidation of the hexacyanoferrate(II), that was formed during the reaction, was recorded on a chart paper. The signal was recorded in the form of mV which is directly proportional to the current produced during the

For aminopropyl CPG (APCPG):

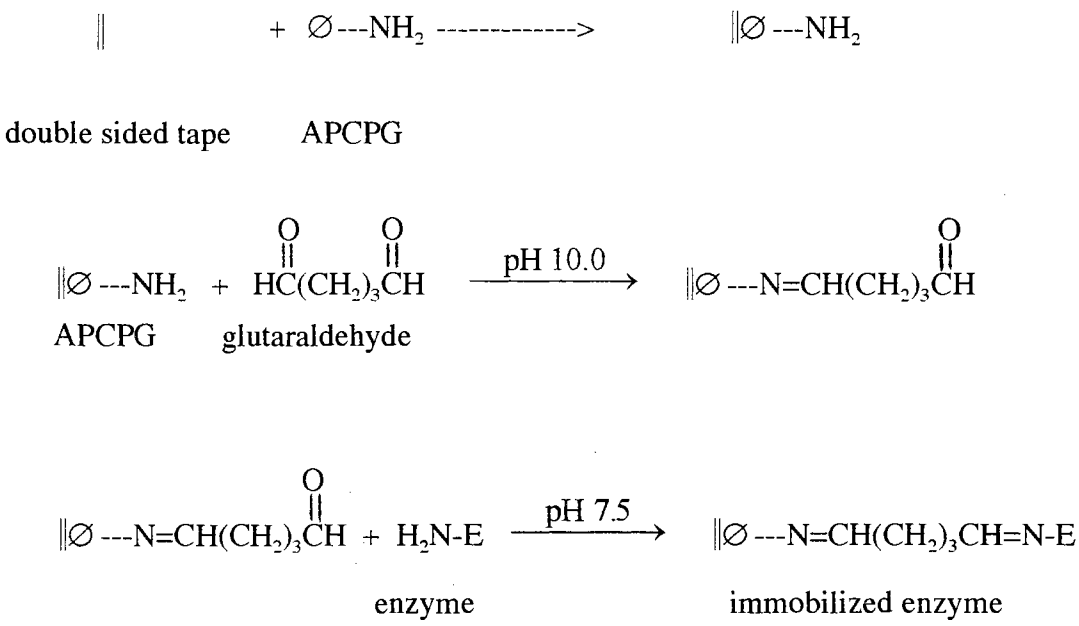

Figure 7. Simplified Scheme of Immobilization Using the Glutaraldehyde Attachment.

TABLE IV

TIME INTERVALS USED IN THE CONTINUOUS FLOW/STOPPED FLOW/CONTINUOUS FLOW OPERATION AS PROGRAMMED WITH THE SHS-200 UNIT.

TIME INTERVAL (s)	OPERATING CONDITIONS	PUMP
70 (T1)	carrier passed through the system until sample introduction	ON
30 (T2)	sample loading	ON
25 (T3)	reagent loading; both sample and reagent transported by carrier to the cell	ON
75 (T4)	stopped flow cycle repeated for introduction of next sample	OFF

reaction, and in amperometry the current is directly proportional to the concentration of the electroactive species. Hence

$$\Delta mV/\Delta s \propto \Delta[Fe(CN)_6^{3-}]/\Delta t \qquad (18)$$

and the rate given by $\Delta[Fe(CN)_6^{3-}]/\Delta t$ was determined by measuring the slope of the initial part of the peak (Figure 8). It was found that, for this system the maximum response takes 6 minutes. So the rate measured during the first 30 s can be used as the initial rate of the main reaction as this will fall well within 10% of the reaction.

RESULTS AND DISCUSSION

Advantages of Using a Rotating Reactor [132, 133]

When enzymes are used as catalysts, the design and performance of a reactor are of major importance, that is, the design should utilize the available active sites of the enzyme effectively. The most widely used reactors in continuous-flow systems are packed-column reactors. These consist of columns packed with particles on which the biocatalyst has been immobilized. The popularity of this form of bioreactor stems from the ease with which it can be prepared and integrated into the continuous-flow arrangements. The goal of this discussion is to introduce an alternate form of reactor, the rotating reactor. The rotating reactor is easy to prepare, and operate. Moreover, it permits to utilize the active sites of the immobilized enzymes effectively.

For a bioreactor in conjunction with continuous-flow, the mass transport and the chemical kinetics are the physico-chemical factors dictating the degree of utilization of these active sites.

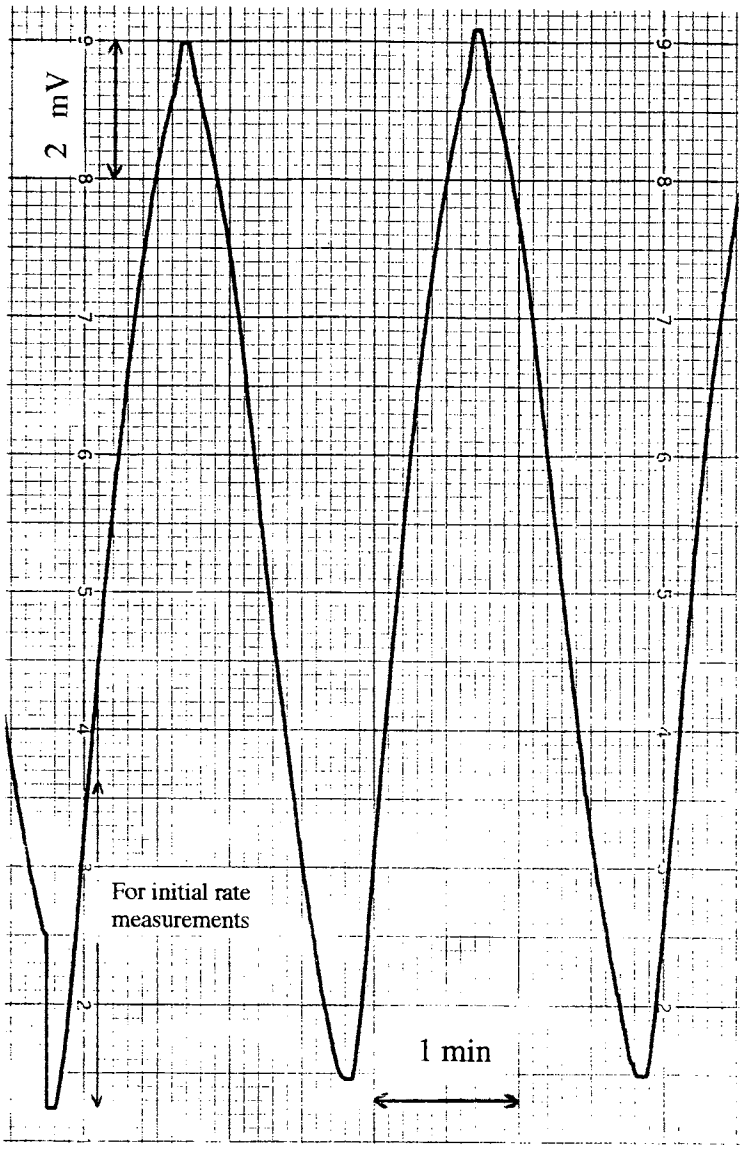

Figure 8. Typical Signals Obtained from the GlDH/Dia System

Mass Transport. In flow systems, the transport of the solute from the bulk of the solution to the immobilized enzyme site (solid surface) can be considered as crossing a stagnant layer at the surface (Figure 9). The rate of mass flow of solute is obtained from

$$d[S_s] /dt = D / \delta ([S_b] - [S_s]) \quad (19)$$

where D is the molecular diffusion coefficient of the solute, δ is the thickness of the stagnant (diffusion) layer, S_b is the solute concentration in the bulk of the solution, and S_s is the solute concentration at the surface of the immobilized enzyme. It is evident from the above equation that the arrival of solute or substrate molecules can be increased either by decreasing the thickness of the diffusion layer and/ or by increasing the concentration gradient ($[S_b] - [S_s]$). Such an arrival can be influenced by the flow. The role of the flow is not only that of transporting the components of the system, but also to facilitate diffusional interface mass transfer.

Experimental conditions in unsegmented continuous-flow systems result in predominantly laminar flow of the sample plug containing the analyte. In laminar flow, each layer of solution flows in parallel paths and the solute diffuses through each layer and the diffusion layer to reach the active site, while in turbulent flow solutes diffuse directly from the bulk solution through the diffusion layer. Hence it is reasonable to assume that the concentration gradient developed within the diffusion layer boundaries is larger under turbulent than laminar flow conditions. Nevertheless turbulent flow does not have the advantage offered by the laminar flow, like, long residence time within the packed column, and if small sized particles are used for packing to make up for the short residence time in case of turbulent flow, problems due to back pressure arise.

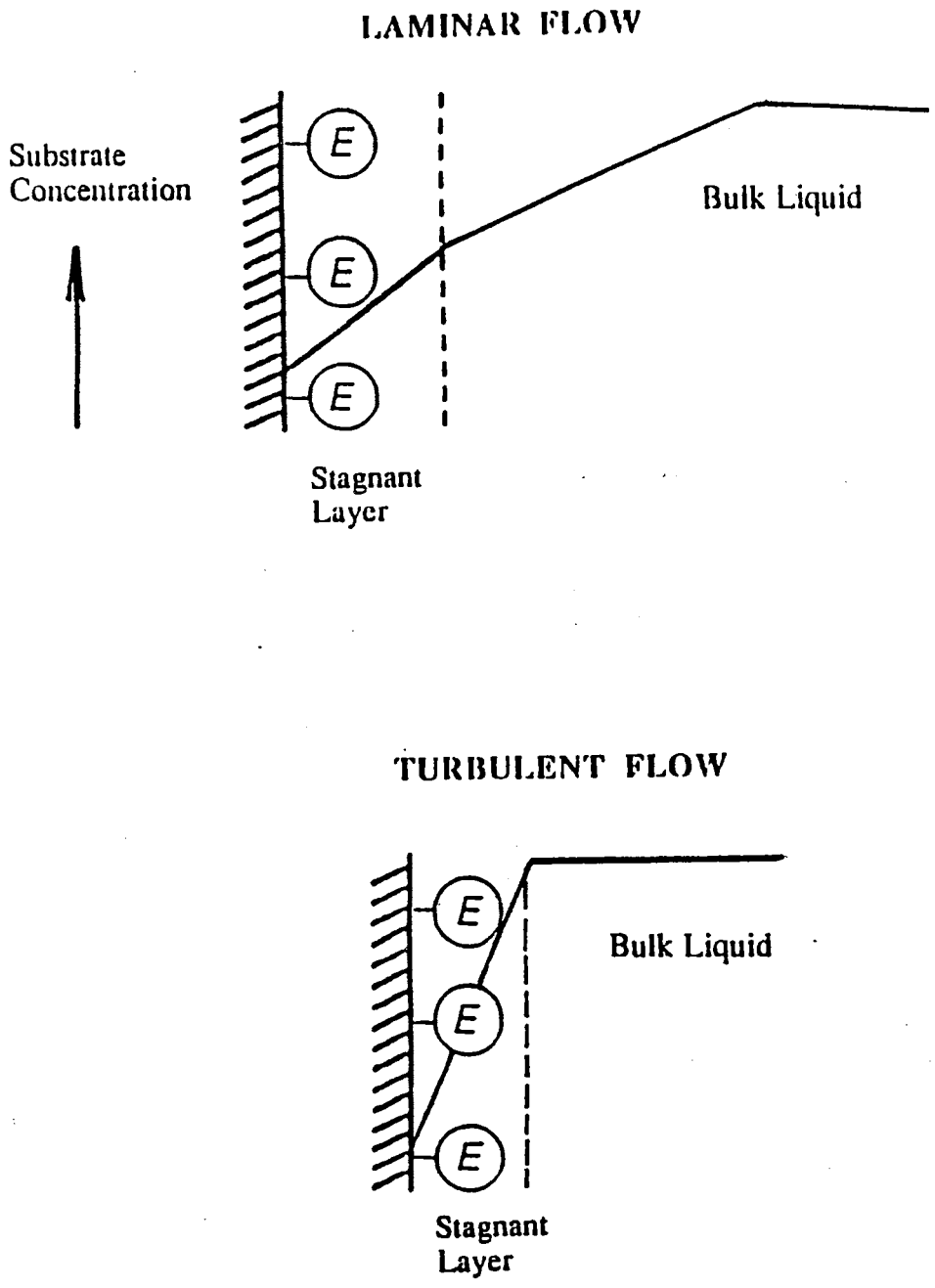

Figure 9. Concentration Profile in the Proximity of an Immobilized Enzyme Surface Under Laminar and Turbulent Flow Conditions.

Also the particles used for support may not be ideally smooth. Local turbulence at cylindrical/spherical bodies, protruding surface irregularities in packed-bed reactor develops at Reynolds numbers between 1 and 100. It has been estimated from the available data that the local Reynolds number, when using packed columns of 1 to 2 mm i. d. and flow rates of about 1.0 mL/min^{-1}, would be ≤ 1. But Reynolds number of at least 20 has been quoted necessary for turbulence to develop around the axis perpendicular to the flow, or at protruding surface irregularities. Therefore local turbulence, if present, will not be conducive in improving the diffusional constraints of mass transfer when packed-column reactors are used.

Chemical Kinetics. The rate at which the substrates are converted to products and removed from the active site of the enzyme is also important. If a Michaelis-Menten behavior is assumed, the initial rate of disappearance of substrate at the immobilized enzyme is given by

$$d[S_s] / dt = ((IR_{max} [S_s])) / (K'_M + [S_s]) \quad (20)$$

where IR_{max} is the maximum initial rate when all the enzyme is in the [ES] complex form. Hence, $IR_{max} = k_{cat} [E_{0,s}]$, in which k_{cat} is the rate coefficient for the conversion of the enzyme-substrate complex to products. The utilization of all active sites of the immobilized enzyme is given by $[E_{0,s}] \approx [ES_s]$.

It has been documented that the rotation of the platform supporting the immobilized enzyme reduces the value of K'_M [134, 135]. Under mass transfer control , $K'_M >> [S_s]$, and can be expected that the rate of conversion is:

$$d[S_s] / dt = (k_{cat} [ES_s] [S_s] / K'_M \quad (21)$$

Since the rotation decreases the value of K'_M the ratio k_{cat} / K'_M increases. The higher this ratio, the higher the sensitivity, and the greater the utilization of the active sites. A comparison of the performance of packed bed reactors and rotating reactors has been discussed in detail in reference 132.

Moreover the packed-bed reactors require heavy enzyme loading or a long residence time for an effective use , which is detrimental for effective determination of products in a system where the detecting unit is located downstream. Packed-bed reactors can be prone to self compression, and fouling by particulate materials in the substrate stream [136]. Also, these column reactors are less advantageous than a well-mixed reactor as substrate inhibition and/or product activation can occur more readily. Besides diffusional restrictions on mass transfer to immobilized biocatalysts, heat and gas transfer limitations are also part of these packed-column reactors.

When used in a continuous-flow system, the composition of the reactants varies along the length of the packed-column reactor, and these concentration profiles may affect both the activity and the stability of the immobilized enzyme. The proposed rotating reactor approach addresses all the problems discussed above. Since the diffusional constraints are reduced by increasing the mass transfer with the aid of convection, the products are removed faster, and the wear and tear on the enzyme are much less than in packed-column reactors.

The studies of variables like applied potential, pH, stop time, enzyme position, concentration of hexacyanoferrate(III), interference from other amino acids affecting the

analytical signal are discussed below.

Applied Voltage

For the determination of glutamate it is necessary to find the optimum electrode potential at which the response of the system is high with least interference from the background signal (best signal-to-noise ratio). This was estimated by measuring the current produced at different applied voltages (range 0.360 V to 0.600 V) both for the system and for the background(NADH). The cyclic voltammograms of the mediator (hexacyanoferrate(III)) and that of the system are shown in Figures 10a and 10b. The range was chosen based on the cyclic voltammograms. The result is presented graphically in Figure 11. The response of the system increases at an applied potential of 0.420 V and almost remains the same until 0.460 V and then increases. The response of the background does not change much between 0.420 V and 0.460 V, but increases sharply after 0.460 V. From this it is safe to assume as optimum applied potential a value of 0.420 V since the signal produced by the analyte is larger compared to that of the background. The continuous increase in the background signal at higher applied potentials may be due to the direct oxidation of NADH at the platinum electrode.

Effect of pH

Since enzymes are proteins, the catalytic activity is markedly affected by the environmental conditions, especially the pH of the media. Thus, information on the changes in pH-activity behavior caused by the immobilization of enzymes are essential for the useful operation of the system. The pH dependence of the two enzyme system

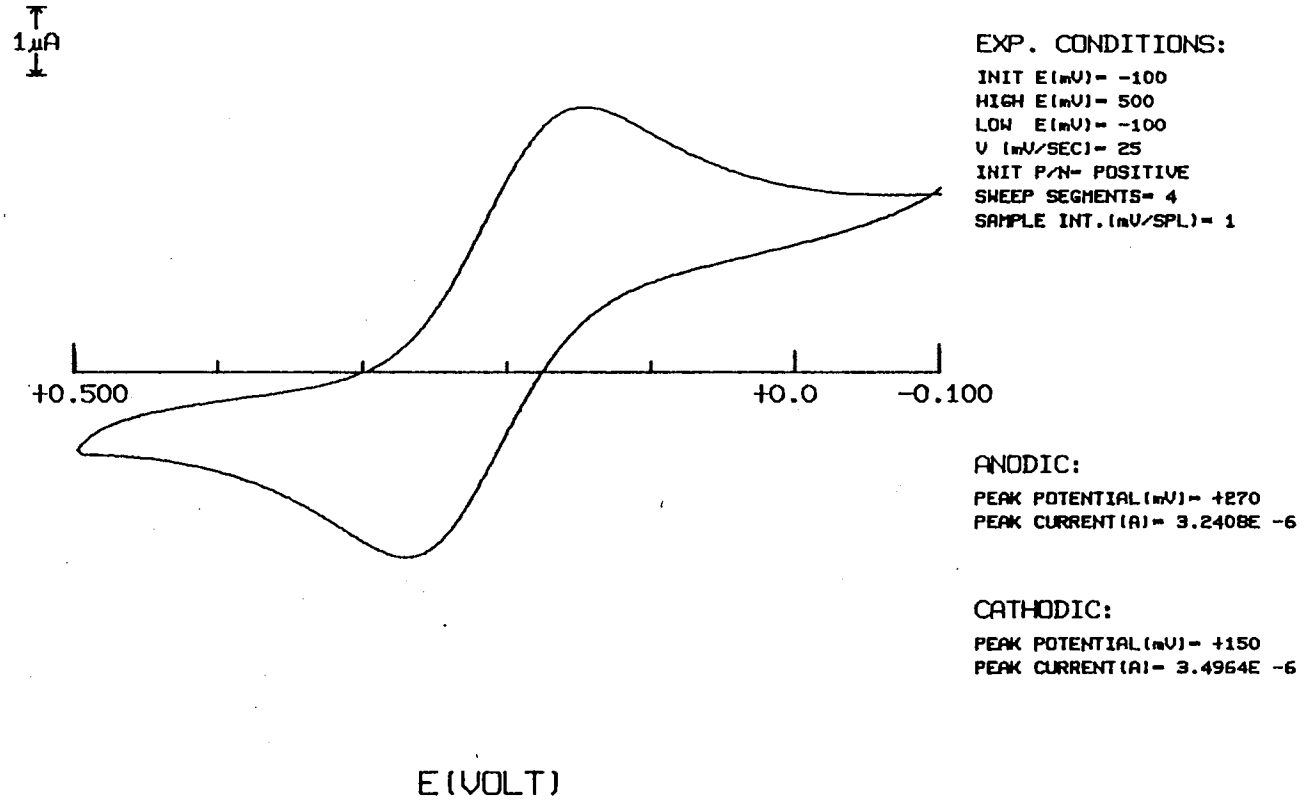

Figure 10a. Cyclic Voltammogram of Hexacyanoferrate(III/II) Couple with 2.0mM of Hexacyanoferrate(III) in 0.10 M Phosphate Buffer pH 7.80.

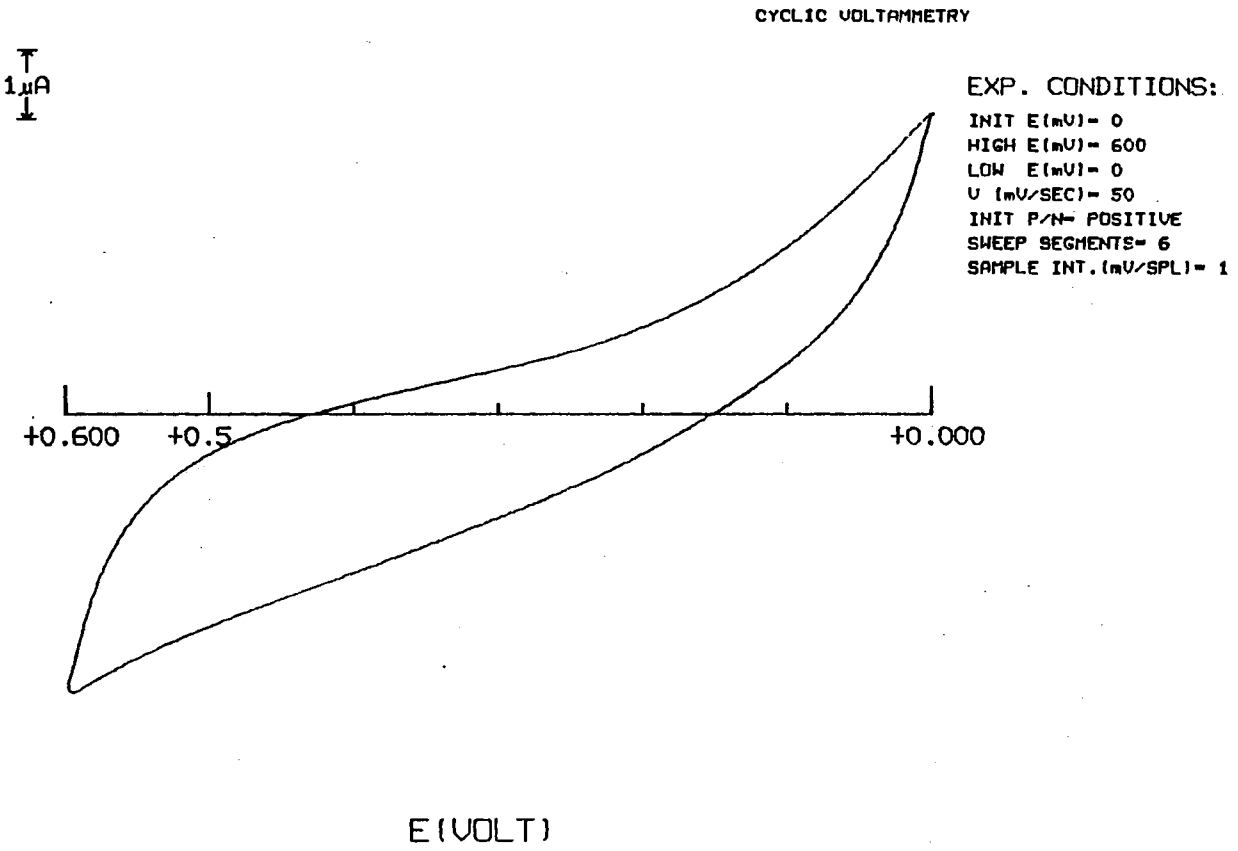

Figure 10b. Cyclic Voltammogram of the Glutamate System with 0.10 mMof Glutamate, Reagent, and the Enzymes in 0.10 M Phosphate Buffer pH 7.80.

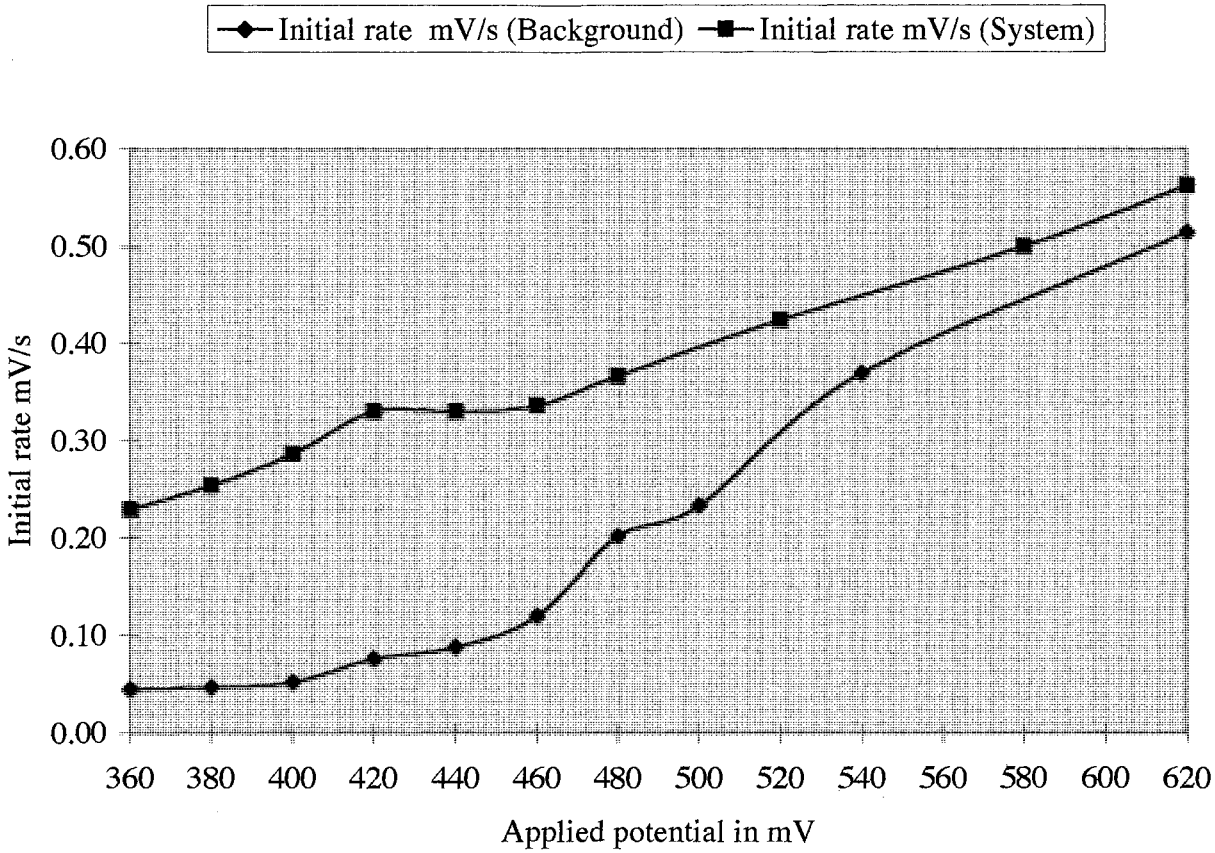

Figure 11. Effect of Applied Potential on the Initial Rate of the GIDH/DIA System

was investigated between pH 7.0 and pH 8.1 with phosphate, triethanolamine-HCl, and tris-NaOH buffers. This range was chosen as the initial rate of oxidation of glutamate at pH 9.0 is lower than at pH 7.0 [137] and the response for the diaphorase catalyzed reaction was found to increase between pH 7.0 and pH 8.5 [138]. Phosphate buffer (0.10M) provided the highest response and pH constancy. The result of this study is shown graphically in Figure 12. Since the difference in response between pH 7.8 and pH 8.0 is about the same, and since hydrolysis can affect the silicate structure of the CPG carrier, a pH of 7.80 is considered to be optimum in this case. Here the optimum pH is a compromise between the optimum pH for each enzyme.

Effect of Stopped Flow Time

The initial rate (IR) of the system was measured from the slope of the signal and therefore it is important to investigate the effect of the stop time. This was done by recording the response for different stopped-flow times. The results are tabulated in Table V; T2 and T3 (see Table IV for the meaning of these times) were kept constant at 30 s and 25 s, respectively. A stoptime of 75 s was chosen for use because the difference in response between 75 s and 90 s is not large enough to justify longer stop times. Also, at a relatively short stop time more samples can be determined in a given unit of time.

Effect of Enzyme Position on the Response of the Reactor

Since two enzymes are used here, it is important to place the enzymes in such a way as to obtain as high a biocatalytic effect as possible. To find this, first the enzyme GlDH was pasted on the rotating reactor and Diaphorase on the stationary top half of the

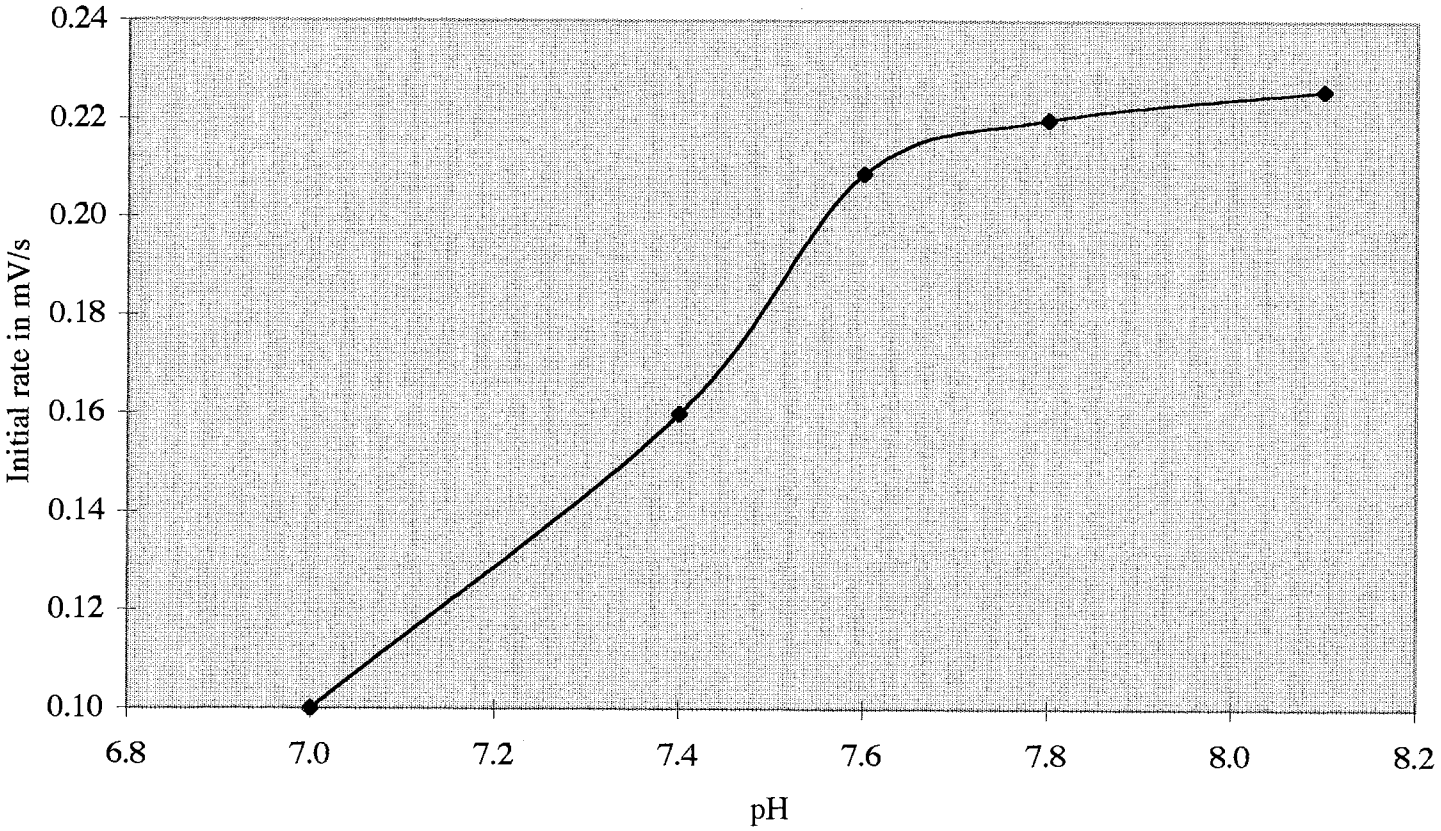

Figure 12. Effect of pH on the Initial Rate of the GlDH/DIA System with 0.10mM Glutamate Solution

TABLE V

EFFECT OF STOP TIME ON THE INITIAL RATE OF THE SYSTEM WITH 2.0 MM OF HEXACYANOFERRATE(III), 1.20 MM OF NAD^+ AND 1.0 MM OF GLUTAMATE.

T1 (flushing time in s)	T2 (stopped flow-time in s)	Initial rate (mV/s)
70	75	0.382
75	80	0.387
80	80	0.423
90	90	0.425
110	110	0.424
120	120	0.424

cell. The response was found to be 1.4 times higher than when diaphorase was placed on the rotating reactor. According to [139] the apparent Michaelis-Menten constant for reaction (1) is 1.8×10^{-3} M and for reaction (2) it is 2.7×10^{-4} M, using a soluble enzyme system. The relationship between initial rate (IR) and apparent Michaelis-Menten constant (K'_M) is as shown below [140],

$$IR = (IR)_{max} [S] / K'_M \qquad (22)$$

Assuming that the K'_M values after immobilization retain the ratio of the K'_M values in solution, it is apparent from equation (19) that the initial rate of the system will be higher if the enzyme GlDH is pasted on the rotating reactor, as this position will lower the K'_M of reaction (1).

The apparent Michaelis-Menten constant, K'_M was determined by stopping the flow for 75s and monitoring the initial rate of the reaction at four different rotation speeds. The relationship between the voltage settings and the revolutions per minute of the reactor was determined by filming the rotation of the reactor with time and by playing back at slow motion [134]. The value of the apparent constant was obtained from the slope and intercept of plots of 1/IR vs. 1/[S] and the values are tabulated in Table VI. As expected the K'_M decreased with an increase in rotation velocity.

Effect of Concentration of Hexacyanoferrate(III)

Since the response of the system is recorded as the current produced by the re-oxidation of the hexacyanoferrate(III), it is necessary to find the effect of different concentrations of hexacyanoferrate(III) on the system and to find the optimum

TABLE VI

VALUES OF THE APPARENT MICHAELIS-MENTEN CONSTANT FOR THE TWO ENZYME (GLDH/DIA) SYSTEM

Rotation velocity (rpm)	K'_M (mM)[a]	Linear regression Std. Deviation
258 ± 12	0.57	(±) 0.08
452 ± 25	0.36	(±) 0.09
664 ± 21	0.35	(±) 0.07
874 ± 26	0.33	(±) 0.07

[a]Each value of K'_M is based on triplicate of 5 different substrate concentrations.

concentration needed. The results from this study are presented in Figure 13. A concentration of 2.0 mM was chosen for further work because at higher concentrations the response decreases. At concentration less than 2.0 mM it was hard to differentiate between the signals from analyte and the background.

Calibration Plots

The range of determination for this method was characterized by determining glutamate over a broad range. The resultant calibration curves for the concentration range 0.01 mM to 0.10 mM (calibration I, Figure 14) and for the range 0.10 mM to 1.0 mM (calibration II, Figure 15) are included. These calibration curves show that the response of the system to glutamate concentrations extends over two orders of magnitude and this is of interest in food analysis because mostly the glutamate content falls in this range. The calibration has good linearity and the equations are given by

Initial rate of response (mV/s) = 0.609 ($C_{glutamate}$, mM) + 0.062 (for 10 μM-100 μM)

Initial rate of response (mV/s) = 0.778 ($C_{glutamate}$, mM) + 0.114(for 0.10 mM-1.0 mM)

with linear regression coefficients of 0.994 and 0.999 respectively.

The concentration of NAD^+ was changed to 1.20 mM for this purpose. This change was made to increase the sensitivity of the system for concentrations of glutamate lower than 0.10 mM.

Selectivity

The selectivity of this system was studied by using different amino acids as well

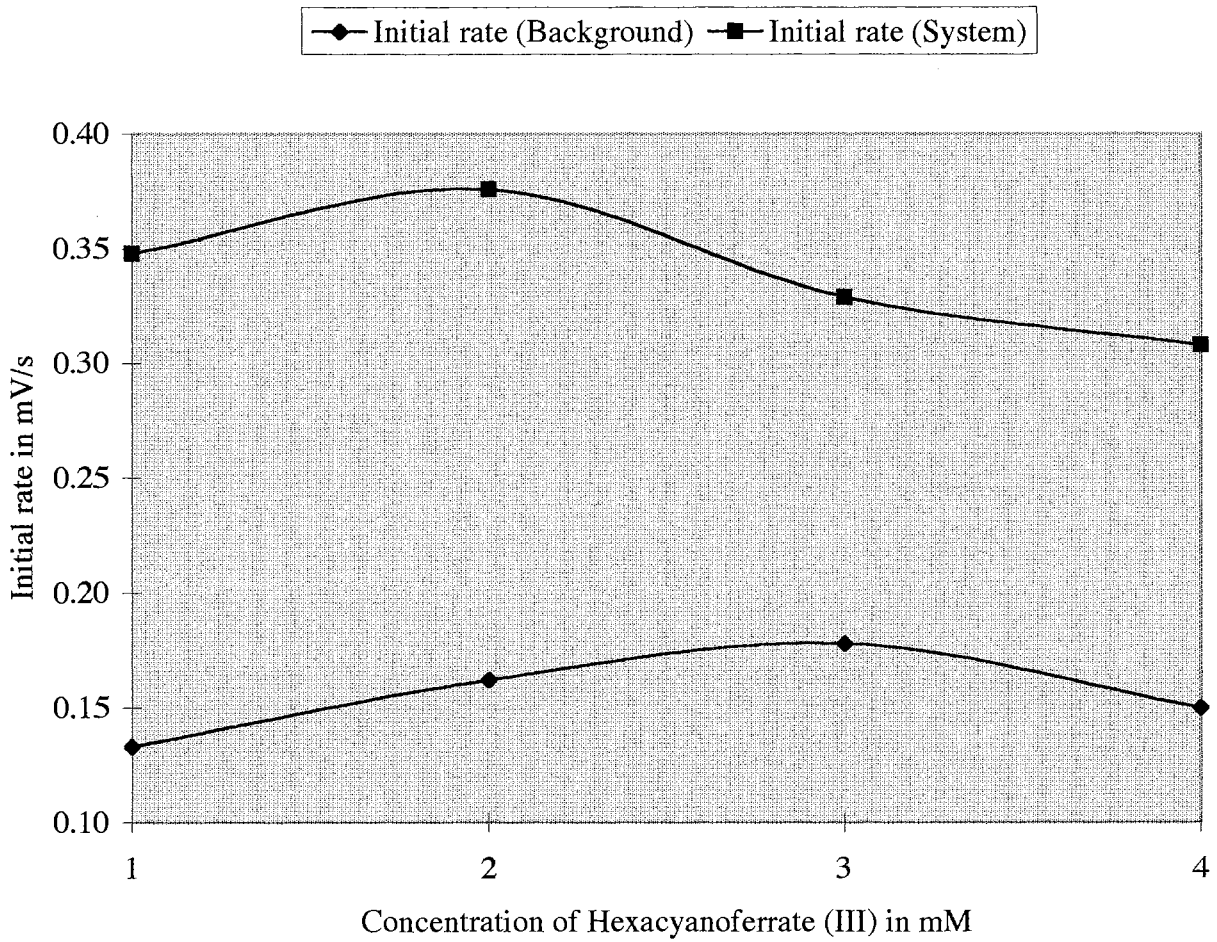

Figure 13. Effect of Hexacyanoferrate(III) Concentration on the Initial Rate of the GlDH/DIA System with the Concentration of Glutamate at 0.10 mM

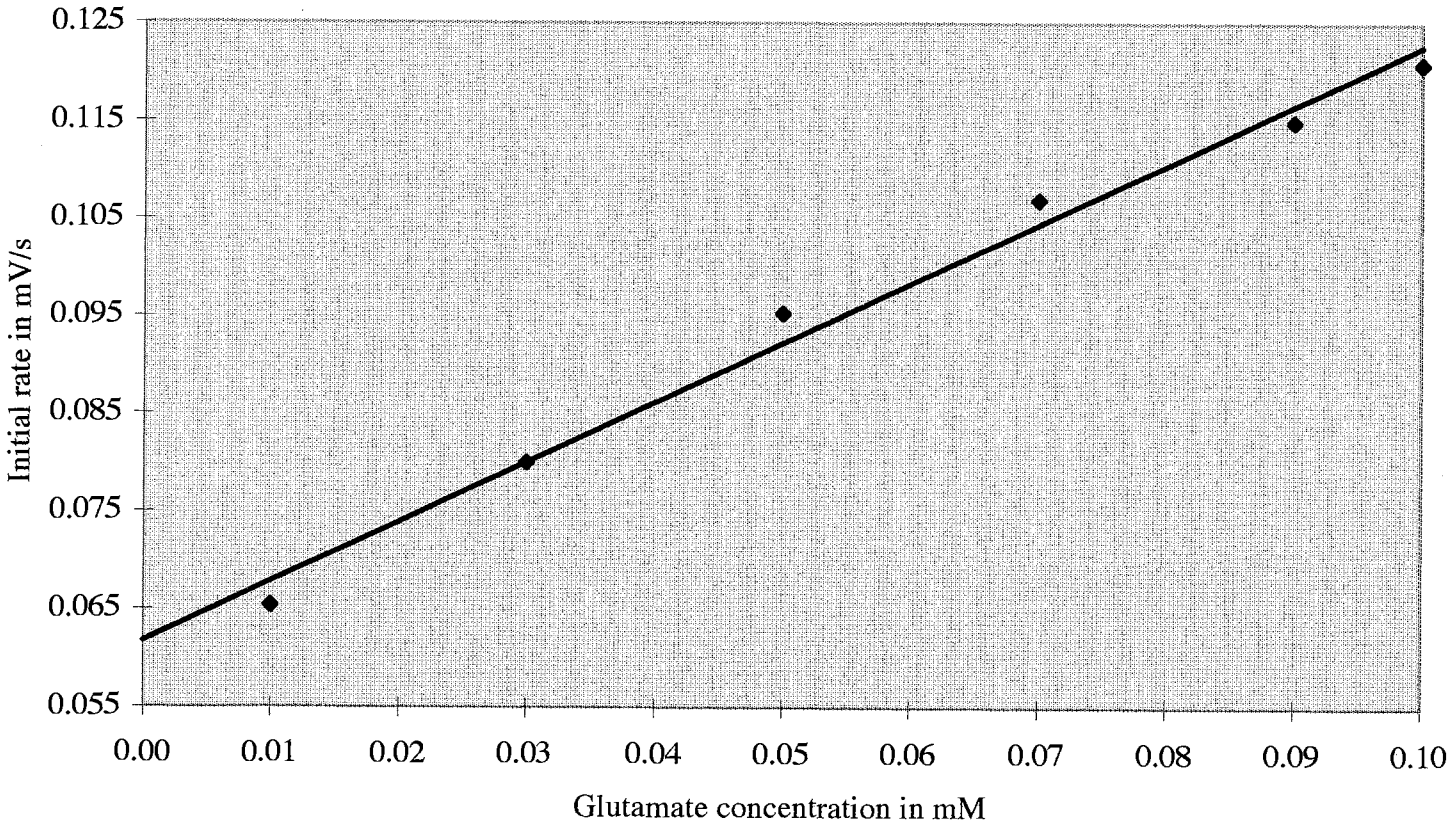

Figure 14. Calibration Curve I with the Immobilized GlDH/DIA System

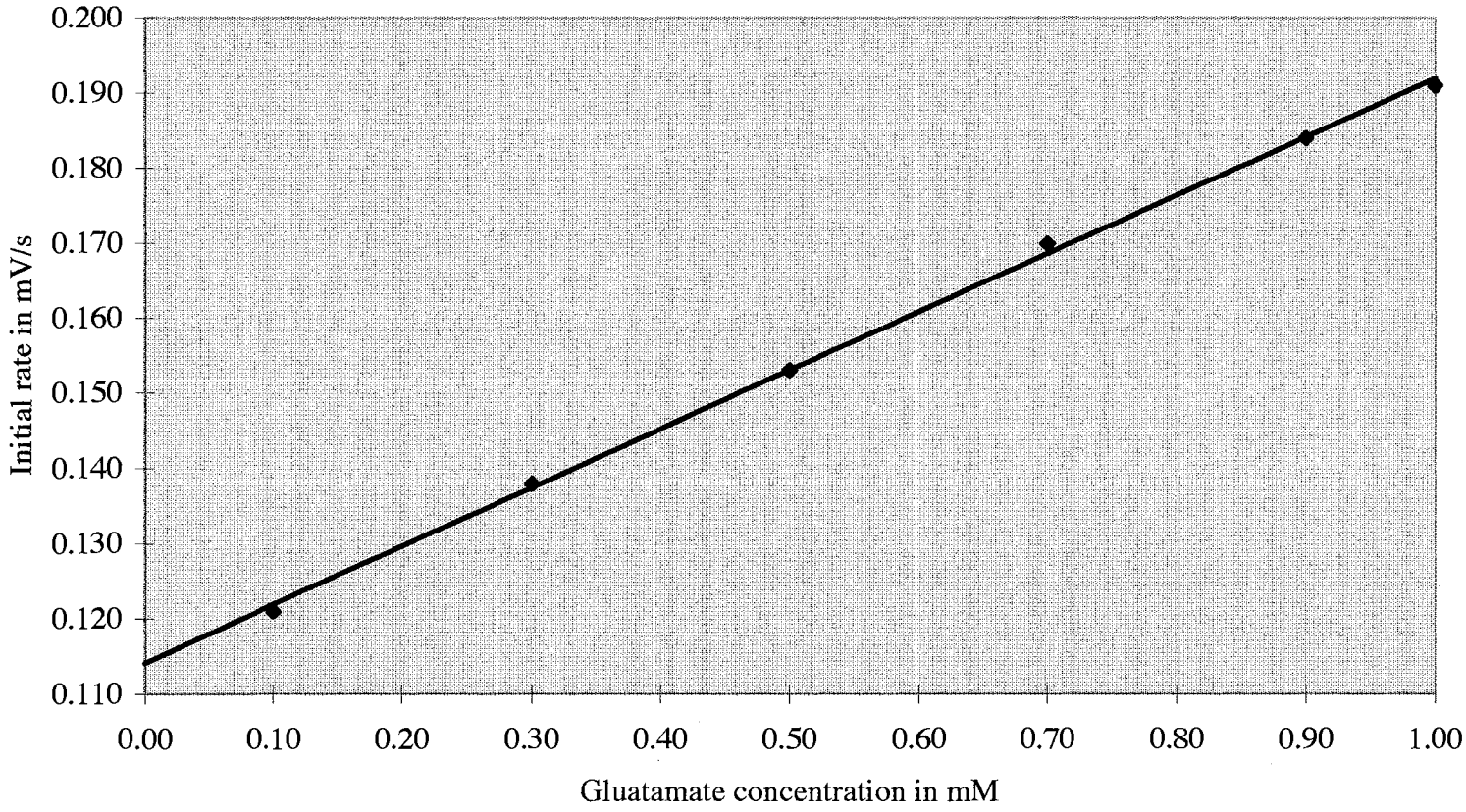

Figure 15. Calibration Curve II with the Immobilized GlDH/DIA System

as ascorbic acid as these are bound to be present in the targeted real samples. Test solutions were prepared that contained 0.10 mM of glutamate and 1.0 mM of the interfering compound in a 0.10 M phosphate buffer of pH 7.80. The results are presented in Table VII. This study showed that ascorbic acid and glutathione interfere. The ascorbic acid interference may be due to the detection system as ascorbic acid is electrochemically active in the voltage window used in this work. As far as glutathione is concerned, it may be because the enzyme diaphorase can oxidize NADH in presence of sulfur groups. From this study we can say that none of the compounds listed in the table, except as already noted, will interfere if present at the same concentration of glutamate.

Application to Food Samples

The L-glutamate content of some food samples purchased from a regular grocery store was measured using this system. Depending on the glutamate content the samples were diluted with the phosphate buffer (pH 7.80). To avoid matrix effect the standard addition (see appendix) method was used. A known amount of the sample was added to standards with three different concentrations and the initial rate was measured. The results are tabulated in Table VIII. It can be seen that the glutamate content of the tested products is within the normally accepted (available) values.

TABLE VII

SELECTIVITY STUDY: RELATIVE RESPONSES FOR POTENTIAL INTERFERENCES

COMPOUND[a]	RELATIVE RESPONSE[b]	STD. DEVIATION
None	100.0	(±) 0.002
Alanine	104.0	(±) 0.004
Arginine	101.0	(±) 0.002
Asparagine	103.0	(±) 0.003
Aspartic acid	97.0	(±) 0.002
Cystine	98.0	(±) 0.002
Glutamine	99.0	(±) 0.003
Glucose	102.0	(±) 0.004
Glycine	100.0	(±) 0.002
Isoleucine	98.0	(±) 0.002
Leucine	101.0	(±) 0.002
Lysine	101.0	(±) 0.002
Methionine	100.0	(±) 0.003
Phenylalanine	95.0	(±) 0.002
Proline	101.0	(±) 0.002
Serine	102.0	(±) 0.002
Threonine	103.0	(±) 0.002
Tryptophan	99.0	(±) 0.004
Valine	102.0	(±) 0.002
Tyrosine	97.0	(±) 0.002

[a]Solutions containing 0.10 mM glutamate and 1.0 mM of the potentially interfering compound.

[b]Each value based on triplicate measurements.

TABLE VIII

DETERMINATION OF GLUTAMATE CONTENT OF FOOD PRODUCTS USING THE GIDH/DIA SYSTEM

PRODUCT	EXPERIMENTAL VALUES[a] (w/w %)	NORMALLY ACCEPTED VALUES FOR PRODUCTS (w/w %)
Season all salt	5.40 ± 0.20[c]	5.80[b] - 17.2[161]
Beef bouillon cube	4.26 ± 0.30[c]	3.00[b] -6.80[161]
Chicken broth	1.22 ± 0.01[c]	1.27[163] - 1.38[164]
Soy sauce (Brand A)	2.34 ± 0.02[c]	1.09[13] - 15.8[162]
Soy sauce (Brand B)	2.58 ± 0.01[c]	1.09[13] - 15.8[162]
Soy sauce (Brand C)	2.20 ± 0.02[c]	1.09 [13]- 15.8[162]

[a]Values represent the average of three determinations.

[b] Manufacturer's specification

[c] Relative std. deviations

CHAPTER IV

DETERMINATION OF GLUTAMATE USING GLUTAMATE DEHYDROGENASE AND DIAPHORASE CO-IMMOBILIZED ON THE ROTATING REACTOR

The objective of this chapter is to re-investigate the effects of various parameters like applied potential, mediator, coenzyme, pH, reactor rotation and determination of glutamate when glutamate dehydrogenase and the diaphorase are co-immobilized onto the rotating reactor described in chapter III. The experimental setup and the reaction scheme used for the determination of glutamate are the same as discussed in chapter III. The only modification here is the co-immobilization of both the enzymes. The enzyme glutamate dehydrogenase was used in the form of lyophilized powder here, so that both enzymes are dissolved in the same phosphate buffer (pH 7.00) and can be co-immobilized on the support. The various chemicals and reagents used for this part of the studies, the procedure for enzyme immobilization, as well as the initial rate measurements are briefly described first.

EXPERIMENTAL

Chemicals

Diaphorase (EC 1.8.1.4; from *Clostridium Kluyveri*) was bought from Calbiochem (LaJolla, CA), glutamic dehydrogenase (EC 1.4.1.3; from bovine liver),

aminopropyl-CPG (700Å), methionine, isoleucine, tryptophan, histidine, and ß-nicotinamide adenine dinucleotide were bought from Sigma (St. Louis, MO). Glutamic acid and glutathione were obtained from Fisher Scientific (Springfield, NJ). The rest of the amino acids were purchased from Nutritional Biochemicals Corporation (Cleveland, OH). Dibasic sodium phosphate was from (CMS, Houston, TX), monobasic sodium phosphate and potassium hexacyanoferrate(III) were from Mallinckrodt (St. Louis, MO), and glutaraldehyde (25% w/w aqueous solution) was from Aldrich (Milwaukee, WI). Seasonings, sauces, and other food products were purchased from a local grocery store.

Reagents

All the solutions were prepared in deionized distilled water. Buffer solutions were prepared by dissolving the appropriate amounts of monobasic and dibasic phosphate in de-ionized-distilled water. The reagent consisting of NAD^+ (1.0 mM) and hexacyanoferrate(III), (1.0 mM) was prepared in the working phosphate buffer solution (pH 7.80).

Enzyme Immobilization

Aminopropyl-CPG (700Å) was spread on the double-sided tape pasted on the rotating disk. The CPG was allowed to react with a 5% glutaraldehyde solution (pH 8.50, carbonate buffer 0.10 M) for 2 h. After this, the CPG was washed thoroughly with pH 7.00 phosphate buffer and was reacted with 4 mg of glutamate dehydrogenase and 2 mg of diaphorase dissolved in 150 µl of phosphate buffer (pH 7.00) overnight and at 4°C. Then the reactor was washed well with phosphate buffer (pH 7.00) to remove the

unbound enzymes and stored at 4°C when not in use. The reaction scheme is similar to the one depicted in chapter III (Figure 7). This enzyme preparation was found to retain its activity for about a month.

Measurement of Initial Rate

For the measurements of the initial rate, a continuous-flow/stopped-flow/ continuous-flow approach was used. In the continuous-flow step the carrier solution (0.10 M phosphate buffer, pH 7.80) was pumped through the system. During this time the sample and the reagent were loaded, mixed, and carried to the optimum position into the cell. During the stopped-flow step, the pump was stopped for a programmed interval of time allowing the reaction to take place. Then the products were flushed out of the cell with the buffer solution and the cycle repeated. The different time intervals used in this work are tabulated in Table IX. The resulting signal produced by the oxidation of the hexacyanoferrate(II) formed during the reaction was recorded on a chart paper and the rate was determined by measuring the slope of the initial part of the peak (Figure 16). It was found that, for this system the maximum response takes 6 minutes. So the rate measured during the first 30s can be used as the initial rate of the main reaction as this will fall within 10% of the reaction.

TABLE IX

TIME INTERVALS USED IN THE CONTINUOUS-FLOW/STOPPED-FLOW/CONTINUOUS-FLOW OPERATION AS PROGRAMMED WITH THE SHS-200 UNIT FOR DETERMINATION OF GLUTAMATE USING CO-IMMOBILIZED GlDH AND DIAPHORASE.

TIME INTERVAL (s)	OPERATING CONDITIONS	PUMP
90 (T1)	Carrier passed through the system till sample introduction	ON
25 (T2)	sample loading	ON
20 (T3)	reagent loading; both sample and reagent transported by carrier to the cell	ON
90 (T4)	stopped flow Cycle repeated for introduction of next sample	OFF

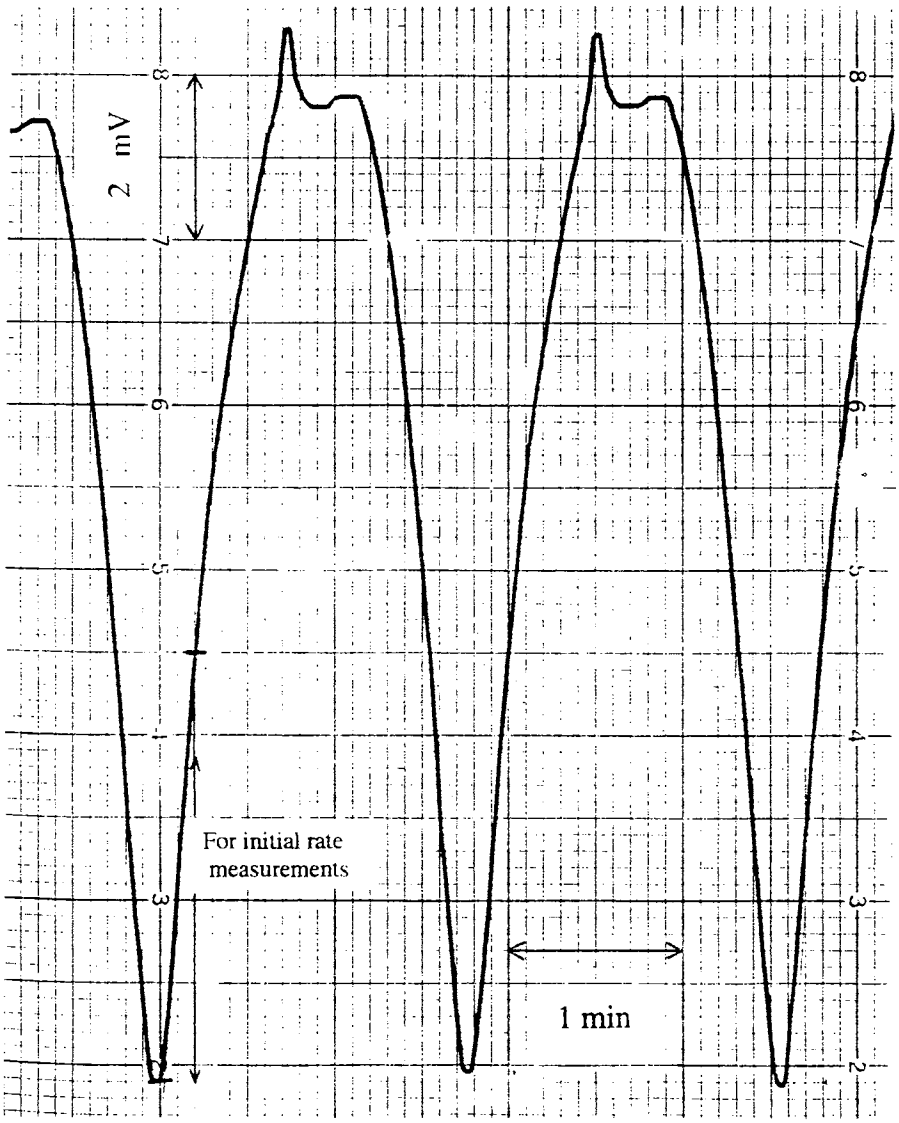

Figure 16. Typical Response of the Co-immobilized GlDH/DIA System

RESULTS AND DISCUSSION

Hydrodynamic Voltammogram

Cyclic voltammetry was used to choose the potential window for this system. The cyclic voltammetry of both the hexacyanoferrate(II/III) couple (1.0 mM in pH 7.80 phosphate buffer) and the system (1.0 mM glutamate, 1.20 mM NAD, 1.0 mM hexacyanoferrate(III), and the co-immobilized enzymes on the rotating reactor) was performed for this purpose. The working electrode was a platinum electrode, the reference electrode was Ag/AgCl, 3M NaCl and the auxiliary electrode consisted of a Pt wire. The cyclic voltammograms are shown in Figure 17a and 17b. Taking into account these voltammograms the range was chosen between 0.320V and 0.650 V.

To detect an optimum potential for this system the initial rate was plotted against the applied potential, both for the reagent and for the system. The result of this study is shown in Figure 18. Although the response of the system increased continually from 0.320 V, the response of the background remained almost the same in the range 0.400 V to 0.440 V, and then increased sharply. From this one can assume that the optimum potential is 0.420 V, since the signal from the system is larger compared to the background. Higher potentials are avoided because NADH can be directly oxidized at the platinum electrode. Moreover, other electrochemically active species can also interfere.

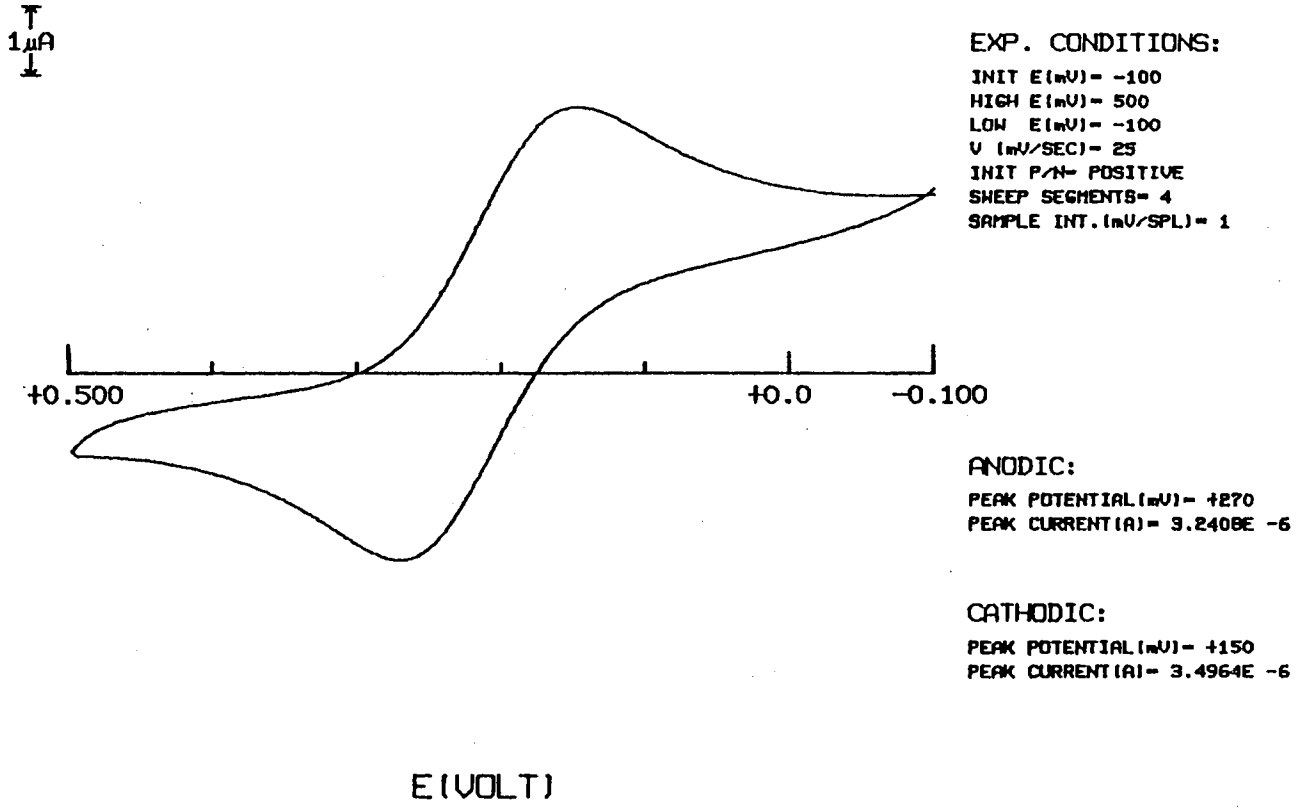

Figure 17a. Cyclic Voltammogram of 0.10 mM Hexacyanoferrate(III/II) Couple in 1.0 M Phosphate Buffer, pH 7.80

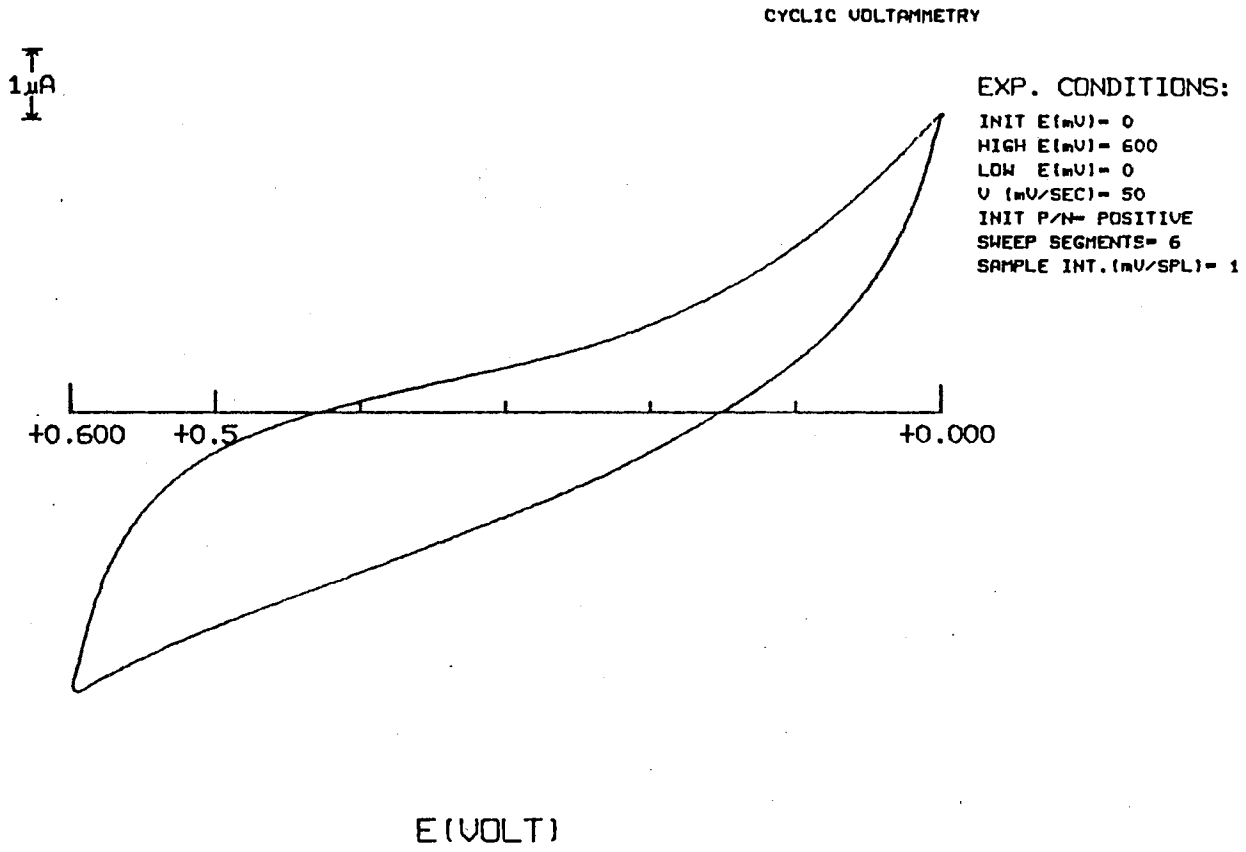

Figure 17b. Cyclic Voltammagram of the System Containing 0.10 mM Glutamate, 1.0 mM NAD^+, 1.0 mM Hexacyanoferrate(III) and the Enzymes

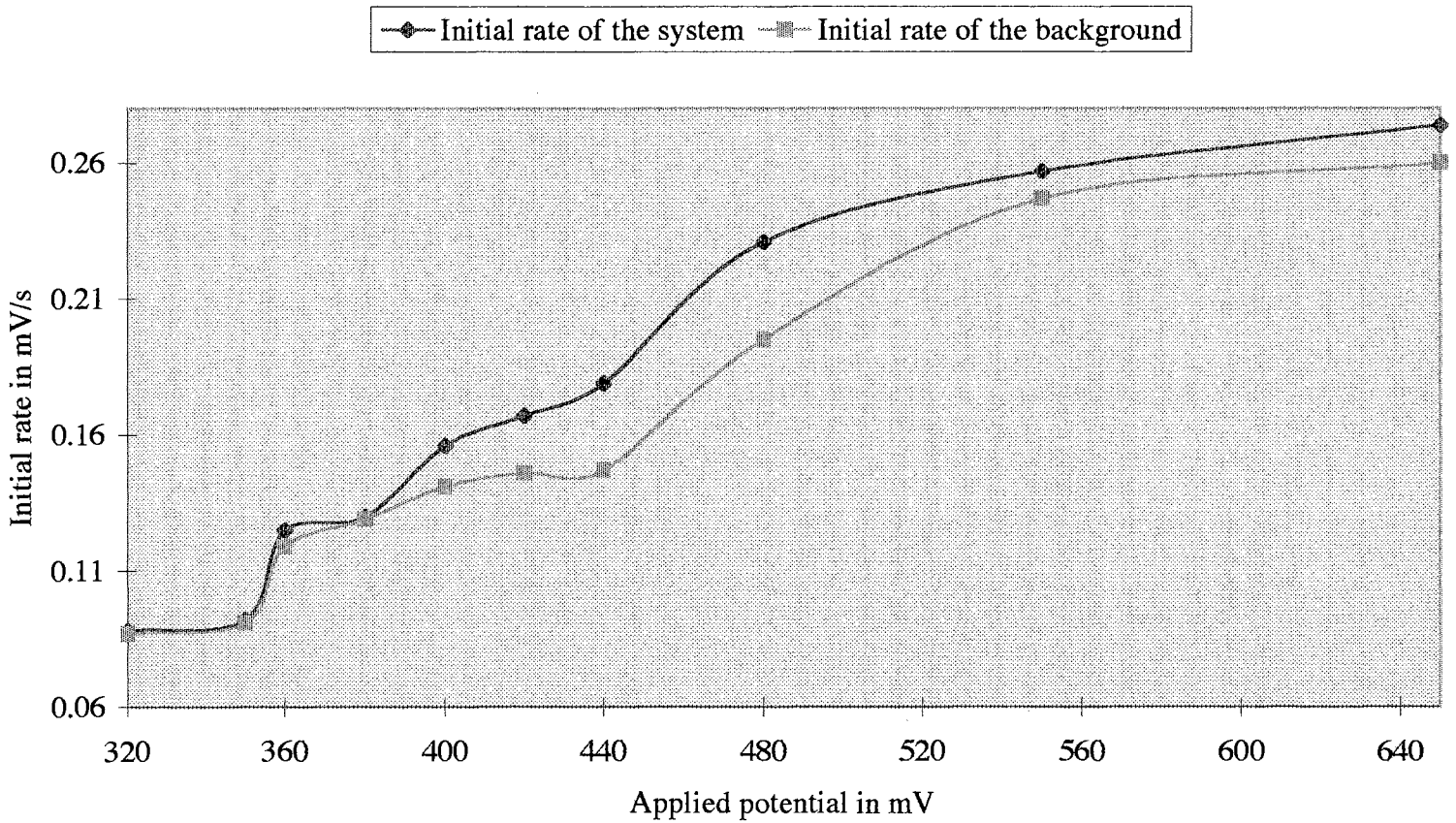

Figure 18. Effect of Applied Potential on the Initial Rate of the Co-immobilized Enzyme System

Effect of pH

The pH has a marked effect on the activity of enzymes and hence on the rate of the enzyme-catalyzed reaction. The marked effect is due to the acidic or basic groups that are generally present in the active sites of enzymes. Besides the acid/base groups that are directly involved in catalysis, those that are responsible for maintaining the active conformation of the enzyme are also important [141]. Usually the rates of the enzyme-catalyzed reactions pass through a maximum as the pH is varied. The pH at which the maximum response is obtained is termed as optimum pH and this is of interest to the analytical chemist.

To study the effect of pH on the activity of the co-immobilized enzymes, the manifold described in chapter III was used. A 1.0 mM glutamate solution (0.10 mM phosphate buffer, pH 7.80), and the reagent were passed through the electrochemical reactor and the resulting signal was recorded. A plot of initial rate versus pH is shown in Figure 19. The pH increases sharply from pH 7.00 to pH 7.80. The increase between pH 7.80 and pH 8.00 is almost insignificant. Consequently, the optimum pH for this system was fixed at pH 7.80. It should be noted that this pH is the same for the enzymes immobilized separately (chapter III). A higher pH was avoided to prevent hydrolyzation of the support (CPG), which may occur after a pH= 8.00. The reason for selecting this range of pH has been explained in chapter III.

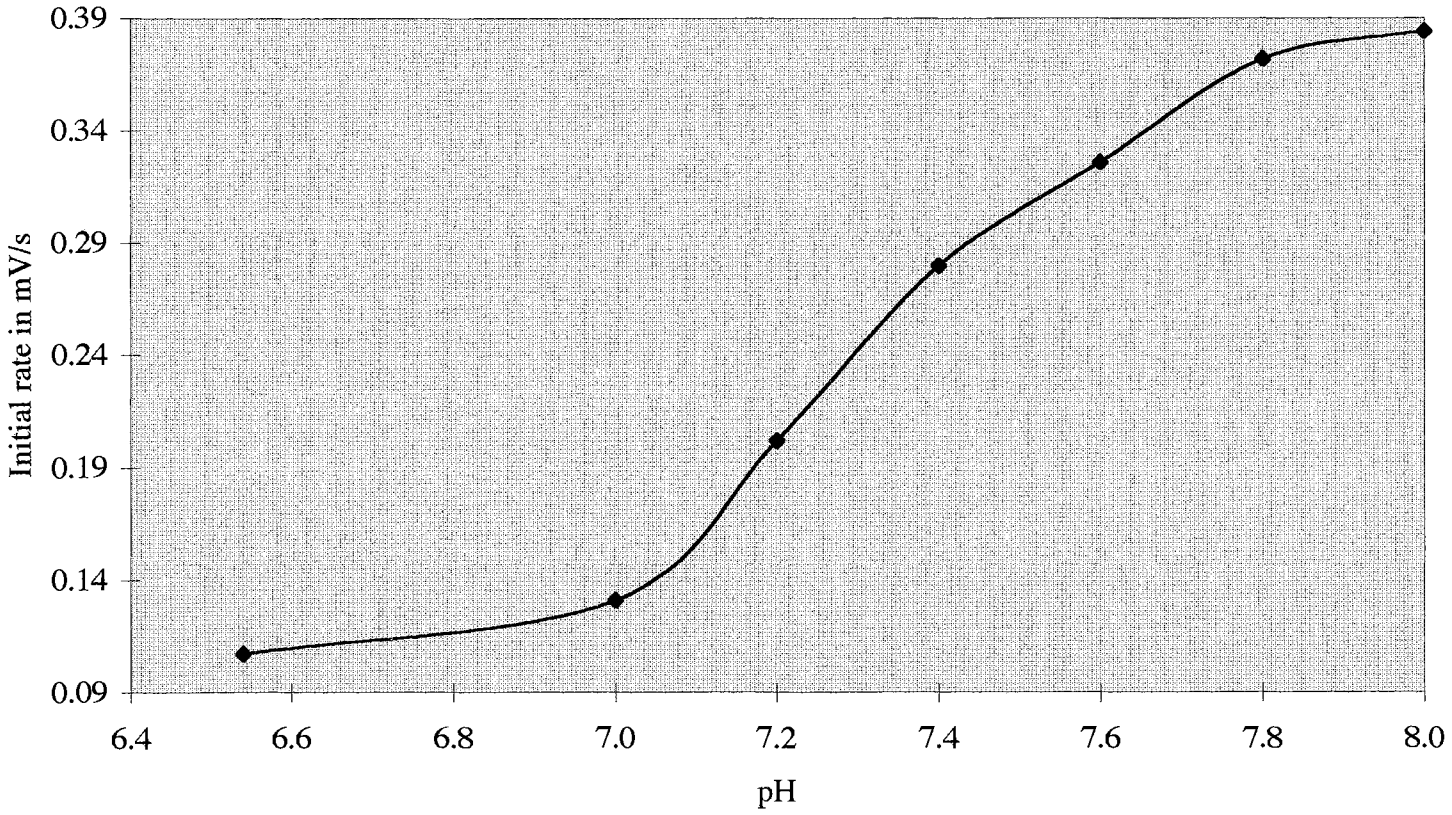

Figure 19. Effect of pH on the Initial Rate of the Co-immobilized Enzyme System

Effect of Hexacyanoferrate(III)

Since hexacyanoferrate(III) acts as the mediator for the reaction used in glutamate determination the effect of its concentration on the initial rate of the system is an important factor to consider. To examine this factor, the concentration of hexacyanoferrate(III) alone was varied in the reagent mixture. The concentration of NAD^+ was maintained at 1.20 mM. The concentration of glutamate used for this purpose was then 1.0 mM. A plot of initial rate versus concentration of hexacyanoferrate(III) is shown in Figure 20. For concentrations smaller than 1.0 mM the response of the system using these two enzymes were found to be negligible [142]. The response was largest at 1.0 mM and decreased at higher concentrations. This could be because diaphorase is also immobilized on the rotating reactor so that even small concentrations of mediator was enough to produce a satisfactory result.

Effect of NAD^+

Since NAD^+ acts as a cofactor for the glutamate dehydrogenase catalyzed reaction, the effect of its concentration on the initial rate of the system needed to be investigated. This study was performed by fixing the concentration of the mediator as 1.0 mM, glutamate as 1.0 mM, and varying the concentration of NAD^+ from 1.0 mM to 2.0 mM (0.10 M phosphate buffer, pH 7.80). The result of this study is shown in Figure 21. When the concentration of NAD^+ was increased from 1.0 mM to 1.20 mM the magnitude of response of the system increased to a maximum and remained almost the same for further increase in concentrations. Hence, a concentration of 1.20 mM NAD^+

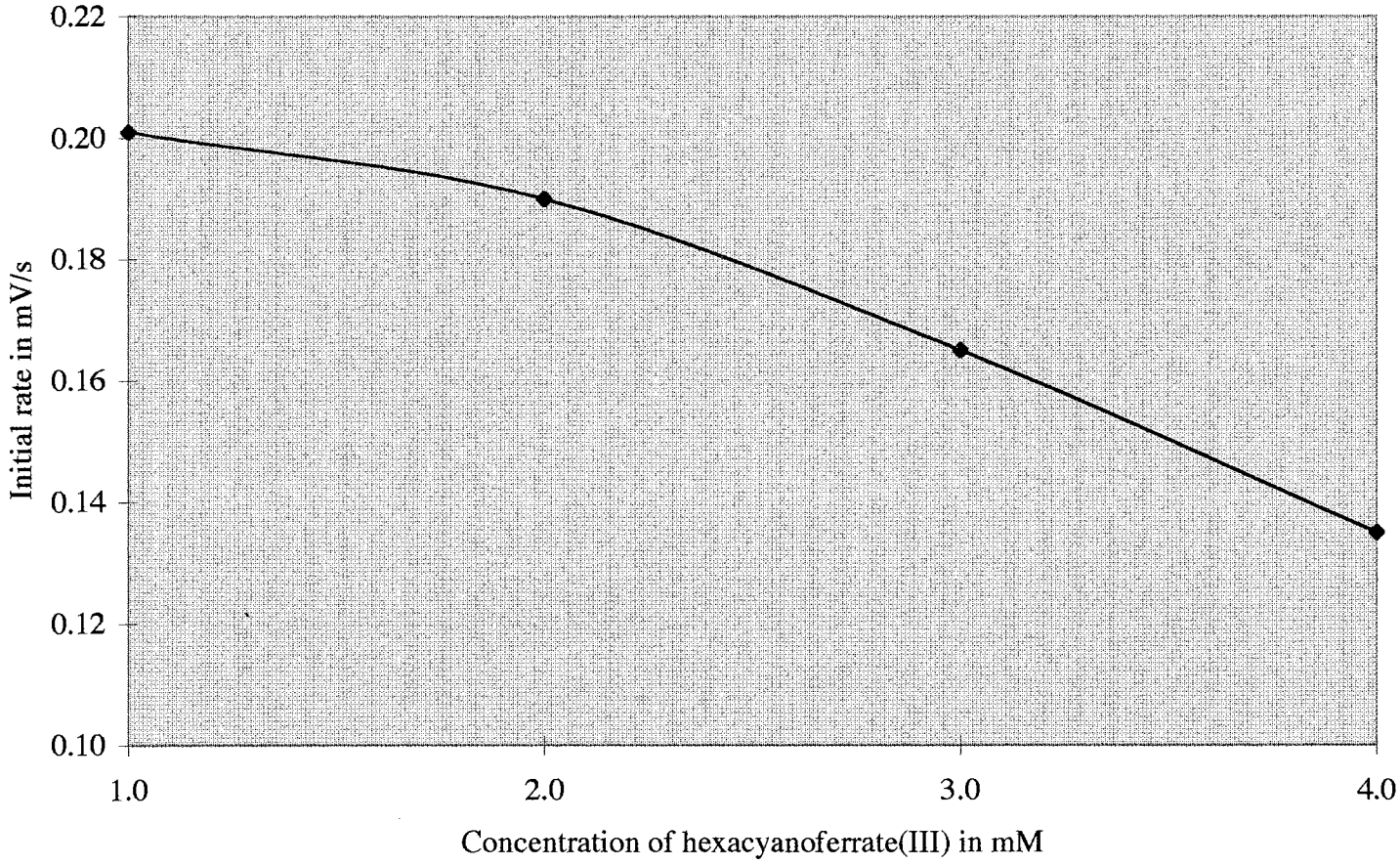

Figure 20. Effect of Concentration of Hexacyanoferrate(III) on the Initial Rate of the Co-immobilized Enzyme System

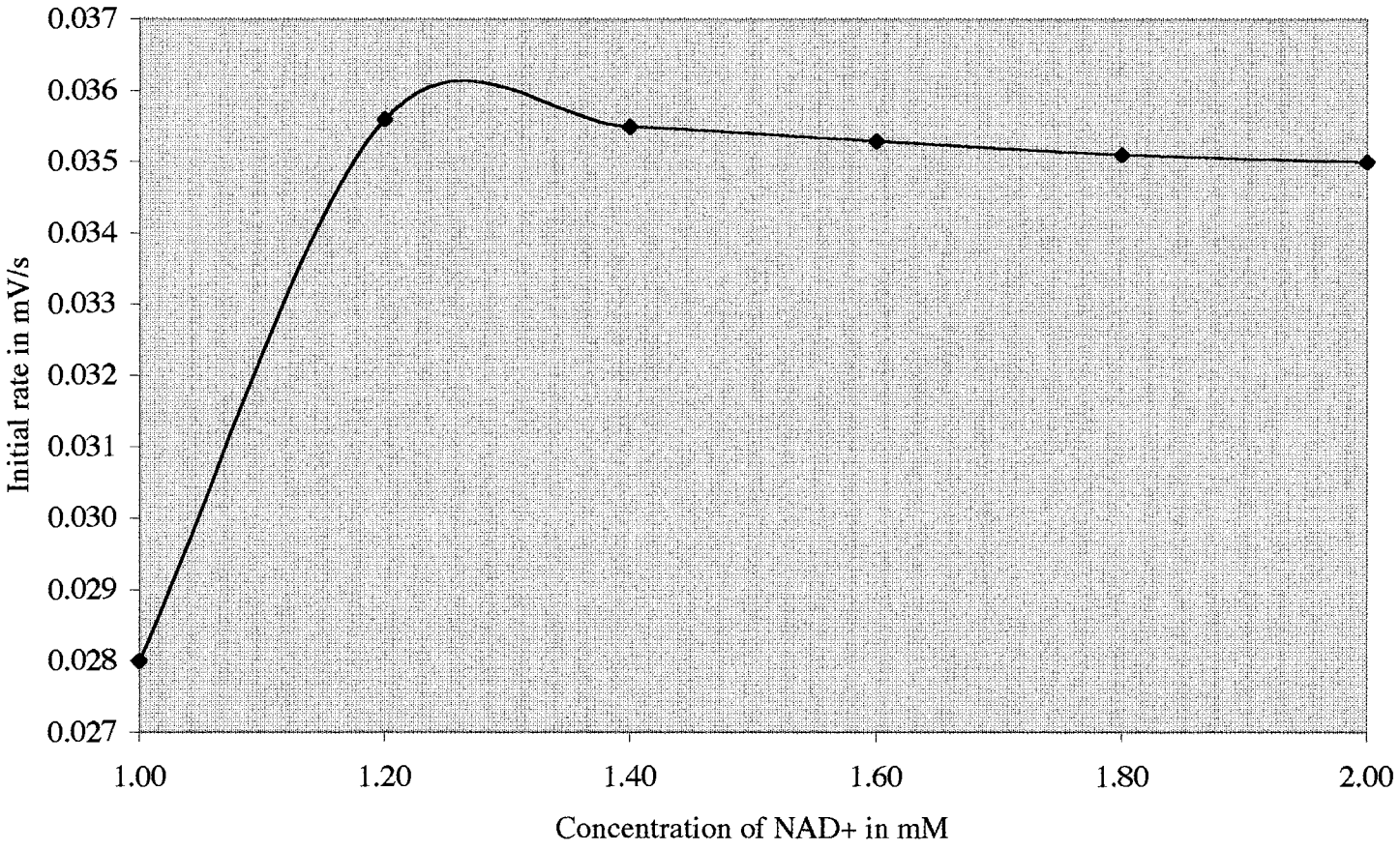

Figure 21. Effect of NAD+ Concentration on the Initial Rate of the Co-immobilized Enzyme System

was used in subsequent experiments.

Effect of Bioreactor Rotation on the Michaelis-Menten Constant

One of the main factors dictating the value of K'_M is the diffusional constraint for the substrate to approach the enzyme site and for the products to exit it, especially when the enzyme is immobilized. Moreover, the coupled reaction should take place in the same defined space to enhance the response of the system.

As documented earlier, the rotation of the reactor holding the immobilized enzymes is expected to decrease the value of the Michaelis-Menten constant K'_M, with a concomitant increase in initial rate. This makes possible sensitive determinations with relatively small amounts of catalyst. The value of K'_M was obtained at three different rotation velocities of the reactor and by stopping the flow for 90 seconds. The calculation of K'_M was performed by using an adaptation of the Lineweaver-Burk plot [134]. The results are tabulated in Table X. As expected, the value of K'_M decreases as the velocity of rotation increases, and this confirms the minimization of diffusional constraints. However, at rotation velocities higher than 874 rpm, vibration of the reactor was observed, and, therefore, the rotation speed of the reactor was maintained at 874 rpm in subsequent studies.

Calibration Curve with the Co-immobilized Enzyme System

One of the analytical usefulness of a method is defined by its linear range of response. The dynamic range of this system was determined using 1.0 mM of hexacyanoferrate(III) and 1.20 mM of NAD^+ in phosphate buffer (pH 7.80). The

TABLE X

VALUES OF THE APPARENT MICHAELIS-MENTEN CONSTANT FOR THE CO-IMMOBILIZED ENZYME SYSTEM

Rotation velocity (rpm)	K'_M[a] (mM)	Linear regression Std. Deviation
452 ± 25	0.91	(±) 0.04
664 ± 21	0.51	(±) 0.07
874 ± 26	0.21	(±) 0.04

[a]Each value of K'_M is based on triplicates of 5 different substrate concentrations.

rotation velocity of the reactor was 874 rpm. A broad calibration range was first studied to determine the working ranges of this method by using glutamate standards ranging from 10^{-6} to 10^{-3} M. The calibration curves obtained are shown in Figures 22 and 23. These graphs indicate that the method responds over two orders of magnitude of concentration, from 10 μM to 1.0 mM. The upper limit of the response may be limited by the amount of the co-immobilized enzymes. Likewise the lower limit of response may be limited by the background response. The equations for the curves are

$$\text{Initial rate (mV/s)} = 1.77\ (C_{\text{glutamate}}, \text{mM}) + 0.009 \quad (10\ \mu\text{M to } 100\ \mu\text{M})$$

$$\text{Initial rate (mV/s)} = 0.079\ (C_{\text{glutamate}}, \text{mM}) + 0.164 \quad (0.10\ \text{mM to } 1.0\ \text{mM})$$

with 0.997 and 0.995 as correlation coefficients, respectively. The detection limit (estimated from three standard deviations of the blank) was 7.89 μM and the sensitivity (slope of the calibration curve) was 0.379 mV/s mM.

Selectivity

The interference study was performed to ensure that the co-immobilization of the enzymes did not affect the selectivity of this system. The experiment conducted was similar to the one described in Chapter III except that here both the enzymes were immobilized on the same platform. The results reflected that the selectivity was not affected by this modification.

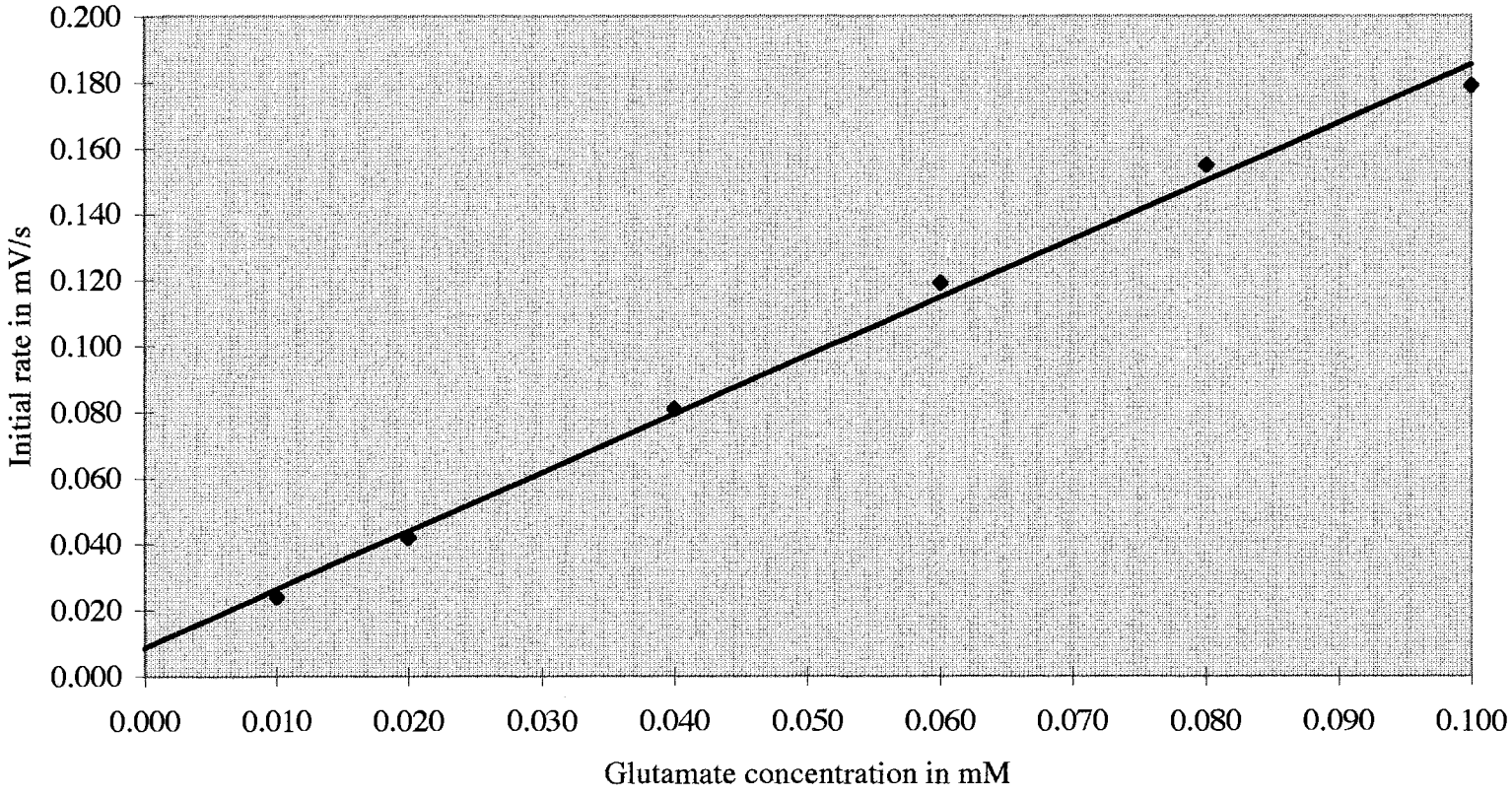

Figure 22. Calibration Curve I of Glutamate Using the Co-immobilized Enzyme System

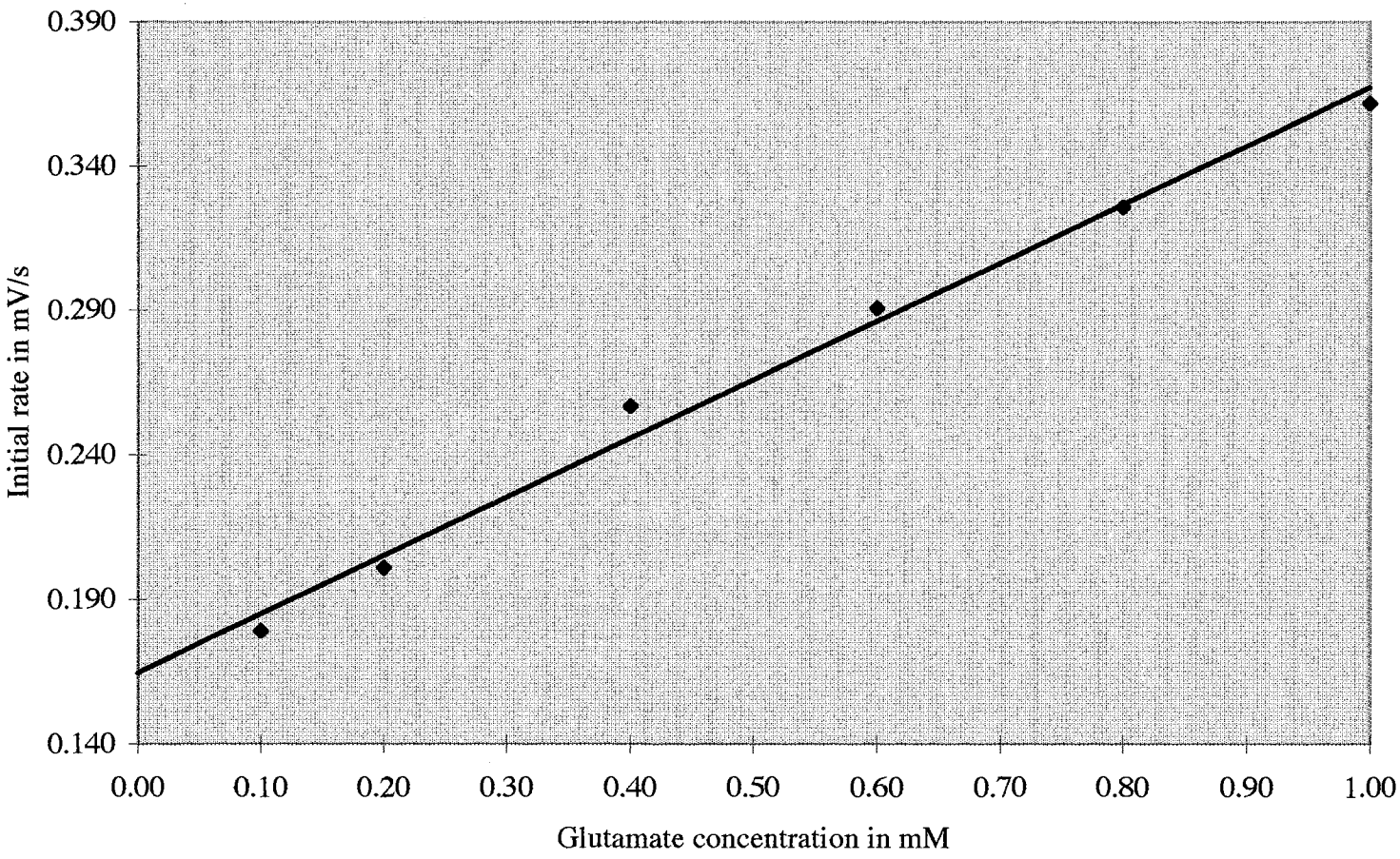

Figure 23. Calibration Curve II of Glutamate Using the Co-immobilized Enzyme System

Determination of Glutamate in Food Samples

Glutamate present in different food samples was determined using this method and the results are presented in Table XI. A standard addition method was used for this purpose and it is described in the Appendix. The concentration of the standards used for this was chosen based on the value of Michaelis-Menten constant. The samples were diluted with the working buffer (phosphate buffer, pH 7.80) so that the glutamate content could be determined with the method described in this chapter, and according to the procedure discussed in Appendix.

In almost all the samples analyzed, the glutamate content was well within the accepted values. The results show that this method can be used for the glutamate determination even in the presence of other amino acids.

Comparisons and Conclusion

The enzymes glutamate dehydrogenase and diaphorase were used for the determination of glutamate here and in the previous experimental approach. The methods will be referred to as the separate enzyme system (Chapter III) and co-immobilized enzyme system (current chapter) for easy understanding. The aim of this discussion is to compare the methods (systems) and evaluate their usefulness.

From the results presented in Chapter III and here, it can be seen that both systems offer good sensitivity and detection limits. Both systems are selective to glutamate over most of the amino acids expected in food samples, and the interference is only from glutathione and ascorbic acid. The glutamate content can be determined

TABLE XI

DETERMINATION OF GLUTAMATE IN FOOD PRODUCTS

PRODUCT	EXPERIMENTAL VALUES[a] (w/w %)	NORMALLY ACCEPTED VALUES (w/w %)
Season all salt	5.44 ± 0.12[c]	5.80[b] - 17.2[161]
Beef bouillon cube	4.64 ± 0.11[c]	3.00[b] - 6.80[161]
Chicken bouillon cube	5.00 ± 0.10[c]	4.89[b] - 8.70[161]
Soy sauce	2.16 ± 0.02[c]	1.09[13] - 15.8[162]

[a]Average of three runs

[b]Manufacturer's specification

[c]Relative std. deviations

with reasonable accuracy using both the methods.

On the other hand, the concentration of the mediator and the Michaelis-Menten constant in the co-immobilized enzyme system was lower compared to the separate enzyme system. Moreover the co-immobilized enzyme system was found to be more sensitive with lower detection limits than the separate enzyme system. The above results are summarized in Table XII. The explanation for these variations could be due to the alteration in the initial rates of the reaction effected by the rotation of the platform containing enzymes implying that the active sites are utilized better. The K'_M value for reaction (5) is 1.8 mM and for reaction (6) is 0.274 mM. As explained in chapter III the rotation of the platform containing the immobilized enzymes decreases the K'_M value. This could explain the overall K'_M being low here since both the enzymes are on the rotating disk. A smaller K'_M value indicates faster reaction and better utilization of the active sites of the enzymes. This may account for the increased response with less number of active sites compared to the situation where only one enzyme was immobilized on the rotating disk.

In conclusion, though both methods can be used for the determination of glutamate in food products the co-immobilized enzyme system is more sensitive, stable and uses lower concentration of the mediator.

TABLE XII

COMPARATIVE RESULTS FOR THE SEPARATE ENZYMES AND CO-IMMOBILIZED ENZYME SYSTEMS FOR GLUTAMATE DETERMINATIONS

	Separate enzymes	Co-immobilized enzymes
Concentration of mediator (mM) used to obtain the signal	2.000	1.000
Apparent Michaelis-Menten constant (mM) at 874 rpm	0.330	0.210
Sensitivity {mV/(s mM)}	0.104	0.379
Detection limit (μM)	14.420	7.890
Correlation coefficient of the linear regression line	0.994	0.997

CHAPTER V

DETERMINATION OF GLUTAMATE USING THE ENZYME GLUTAMATE OXIDASE (EC 1.4.3.11)

The aim of the study presented in this Chapter was to develop a simpler method for the determination of glutamate in food samples. This method is based on a reaction catalyzed by the enzyme L-glutamate oxidase (Glu.oxi). This enzyme was first purified from an aqueous extract of a wheat bran culture of *Streptomyces* Sp. X119-6 by Kuskabe et al. [143] and it was found to react solely with L-glutamate with few inhibitors.

Unlike glutamate dehydrogenase, this enzyme is a flavoprotein, that is, the co-factor is an integral part of the enzyme. Therefore the reducing equivalents from the amino acid are received by the tightly bound FAD prosthetic group. The prosthetic groups remain reduced until an oxidizing substrate (in this case oxygen) appears. The FAD is then reoxidised, and remains bound, as before, while the H_2O_2 produced departs. In fact, enzymes with prosthetic groups may be thought of as two enzymes rolled into one, with the added sophistication that the "middle" substrate never comes off [137]. Glutamate oxidase catalyzes the reaction of glutamate according to:

$$\text{L-glutamate} + O_2 + H_2O \xrightarrow{\text{Glu. Oxi}} \alpha\text{–ketoglutarate} + NH_4^+ + H_2O_2 \qquad (1)$$

Studies have shown [143] that 1 mol of glutamate is converted to 1 mol of α–ketoglutarate, ammonium ions, and H_2O_2 with the consumption of 1 mol of oxygen.

L-aspartate was found to be also oxidized by this enzyme but only to an extent of 0.6% in comparison to glutamate.

Mode of Detection

As shown in reaction (1) glutamate can be determined by observing the production of hydrogen peroxide. In the present work, the reaction was followed by monitoring the current produced by the anodic decomposition of the H_2O_2 at a platinum ring electrode located in the electrochemical cell that contains the rotating reactor. The method, as previous ones, uses a continuous-flow/stopped-flow operation. The advantage of using a rotating reactor over a packed column has been described earlier along with the instrumentation used for this method (Chapter III).

EXPERIMENTAL

Chemicals

Glutamate oxidase (*Streptomyces* sp. X119-6, 6.8 units/ mg) was obtained from Yamasa Corporation (Choshi, Japan). Ascorbate oxidase (*Cucurbita* sp., 300 units/ mg) was purchased from Calbiochem (LaJolla, CA). Aminopropyl-CPG (700Å), methionine, isoleucine, tryptophan, and histidine were bought from Sigma (St. Louis, MO). Glutamic acid and glutathione were obtained from Fisher Scientific (Springfield, NJ). The rest of the amino acids and ascorbic acid were from Nutritional Biochemicals Corporation (Cleveland, OH). Dibasic sodium phosphate was from CMS (Houston, TX), monobasic sodium phosphate was from Mallinckrodt (St. Louis, MO), and glutaraldehyde (25% w/w

aqueous solution) was from Aldrich (Milwakee, WI). Seasonings, sauces, and other food products were purchased from a local grocery store.

Solutions

All the solutions were prepared with the deionized distilled water, as described in Chapter III. Buffer solutions were prepared by dissolving the appropriate amounts of monobasic and dibasic phosphate in deionized distilled water. All the samples and the standards were prepared using the working buffer (phosphate (0.10 M) solution, pH 7.4)

Enzyme Immobilization

Glutamate oxidase. Aminopropyl-CPG (700Å) was spread on a double-sided tape pasted onto the rotating reactor. The CPG was allowed to react with a 5% glutaraldehyde solution (pH 8.50, carbonate buffer 0.10 M) for 2 hrs. After this the CPG was washed thoroughly with pH 7.00 phosphate buffer and was reacted with 1.5 mg of the enzyme dissolved in 125 μl of phosphate buffer (pH 7.00) overnight at 4°C. Then the reactor was washed well to remove the unbound enzyme and stored at 4°C when not in use. The reaction scheme for immobilization is similar to the one depicted in Chapter III. This enzyme was stable and retains its activity for more than two months.

Ascorbate oxidase. The same procedure of glutaraldehyde coupling used for glutamate oxidase was used in the immobilization of ascorbate oxidase. The enzyme immobilized on CPG was packed in a column of Tygon tubing (5.5 cm in length, 0.2 cm i. d.), which was stored in the refrigerator between uses.

Measurement of Initial Rate

For the measurement of initial rate of the reaction, the continuous-flow/stopped-flow approach described earlier was used. In the continuous-flow step the carrier (0.10 M phosphate buffer, pH 7.40) was flushed through the system. During this time the analyte was loaded and transported by the carrier to the cell. In the stopped-flow step, the pump was stopped for a given time interval so that the reaction could take place. The current produced during this step was recorded. The typical time intervals used for this system are listed in Table XIII. The flow rate provided by this unit was 1.0 ml/min.

Typical shapes of the signals are shown in Figure 24. The initial rate was measured from the slope of the signal produced (shown by the arrow marks on Figure 24) due to the current change with respect to time. The completion of the reaction took about five minutes, so that the rate measured during the first 30 s is within the 10% of substrate conversion, validating an assumed "initial rate".

RESULTS AND DISCUSSION

Applied Potential

For amperometric detection an optimum potential is necessary for the determination of the species in question. For this study, the applied potential was varied and the response of the system was recorded in the form of initial rate. A concentration of 0.050 mM glutamic acid at pH 7.40 was used for this purpose. The rotation speed of the reactor was 874 rpm, and the potential was scanned from 0.500 V to 0.650 V

TABLE XIII

TIME INTERVALS USED IN THE CONTINUOUS-FLOW/STOPPED-FLOW/CONTINUOUS-FLOW OPERATION AS PROGRAMMED IN THE SHS-200 FOR GLUTAMATE DETERMINATION USING GLUTAMATE OXIDASE

TIME INTERVAL	OPERATING CONDITIONS	PUMP
120 (T1)	carrier passed through unit till sample introduction	ON
20 (T2)	sample loading (sample transported by carrier to cell)	ON
100 (T3)	stopped flow cycle repeated for introduction of next sample	OFF

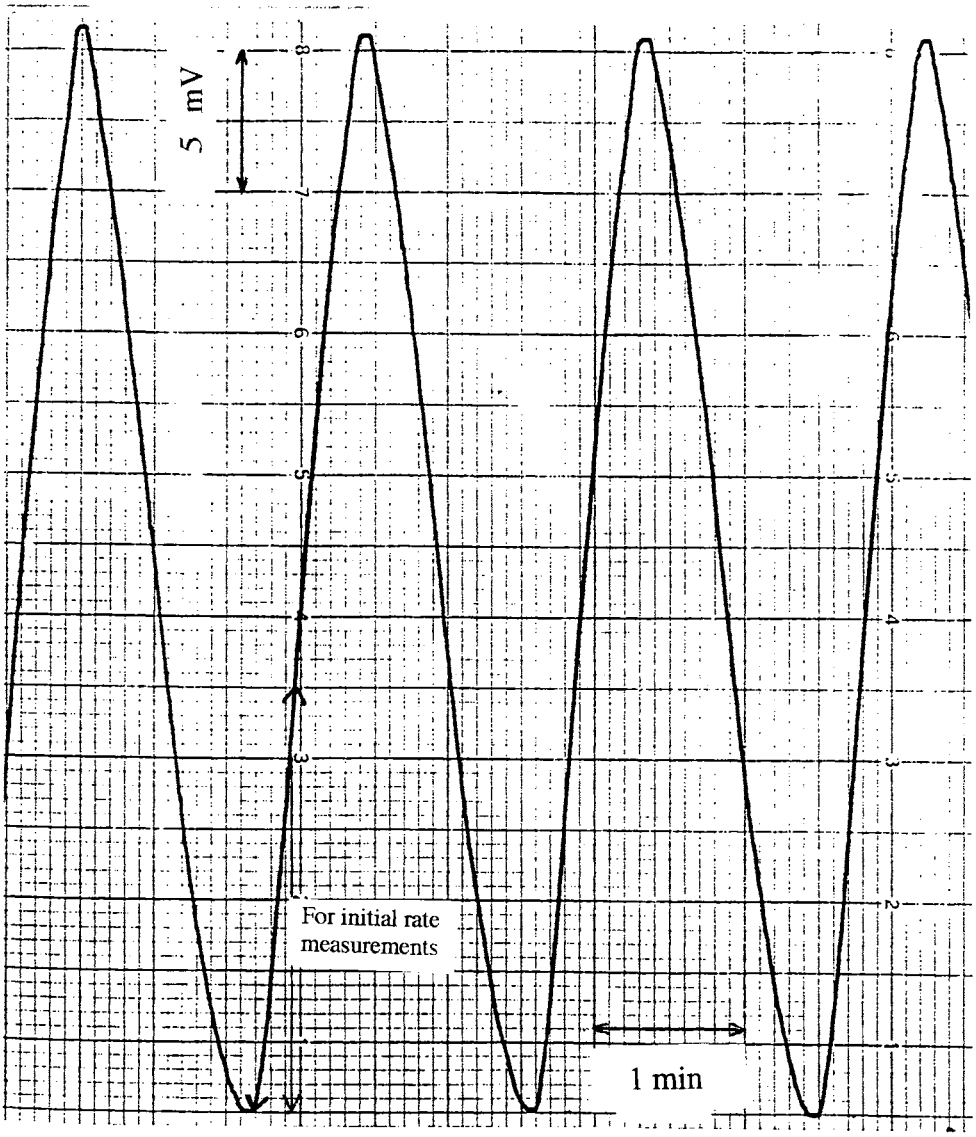

Figure 24. Typical Response of the Glutamate Oxidase System

vs. Ag/AgCl (3M NaCl).

As shown in Figure 25, the initial rate of the system increased slightly with potential. The optimum potential for this system was fixed at 0.650 V. The scan was stopped at 0.650 V because at higher potentials molecular oxygen and other electroactive species may interfere.

Effect of pH

pH has a considerable influence on the activity of the enzyme. The rate of an enzyme catalyzed reaction commonly passes through a maximum as a function of pH. At extreme pH, the enzyme may undergo irreversible denaturation. Of great interest is the reversible behavior exhibited in the general area of optimum pH [15]. Also the pH change may alter the binding of the substrate, and the catalytic activity of the groups in the active site of the enzyme.

The optimum range for this enzyme was found to be between pH 7.00 - 8.00 [143]. Therefore the effect of pH on this system was observed for this range with 0.10 M phosphate buffer. Figure 26 shows the result of this study with the bell shaped curve characteristic of enzyme-catalyzed reaction. This shape is due to the amphoteric nature of the amino acids that make up the active site of the enzyme, specifically those affecting the binding of the substrate. A pH of 7.40 was chosen as the response of the system decreased above this pH.

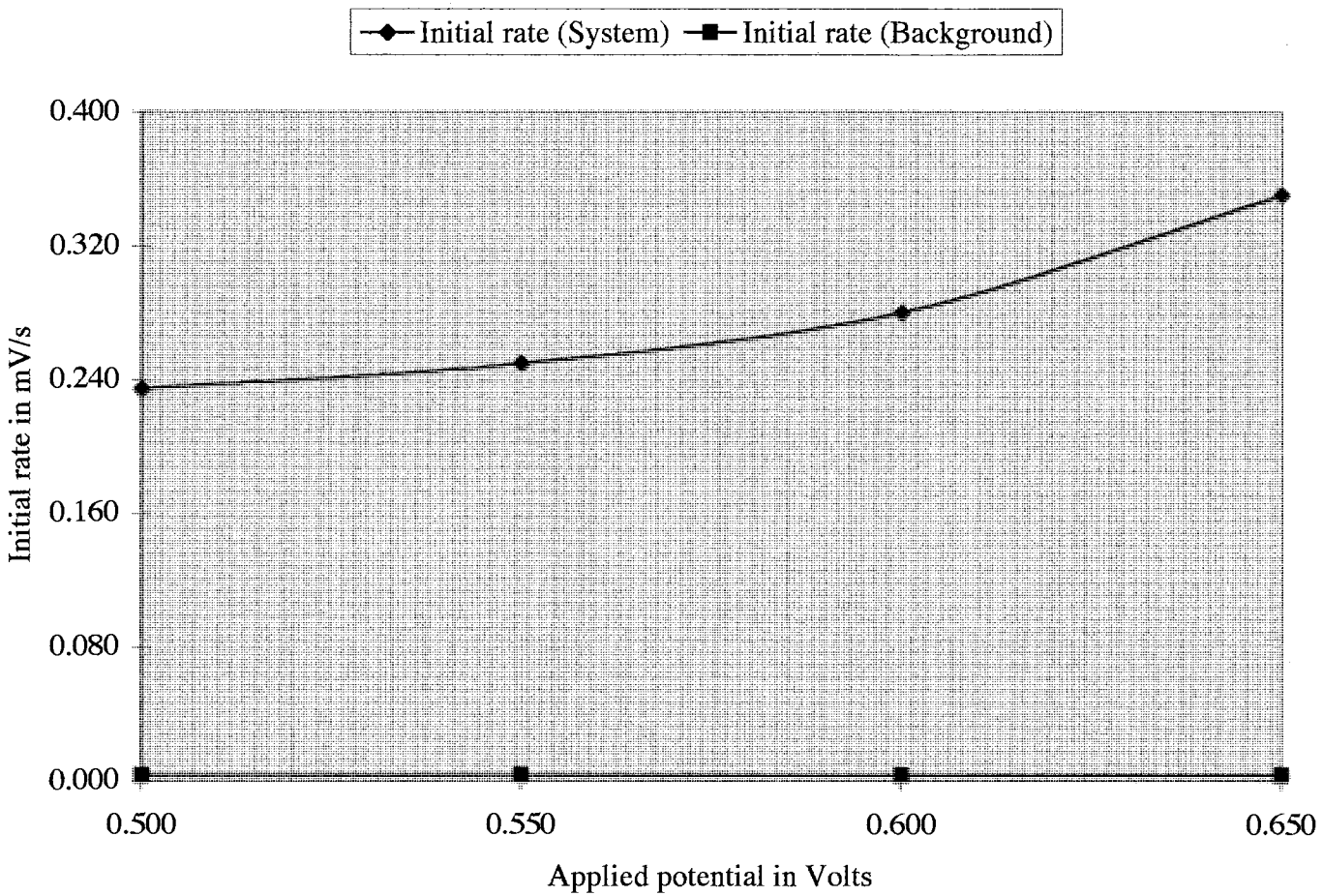

Figure 25. Effect of Applied Potential on the Initial Rate of the Glutamate Oxidase System

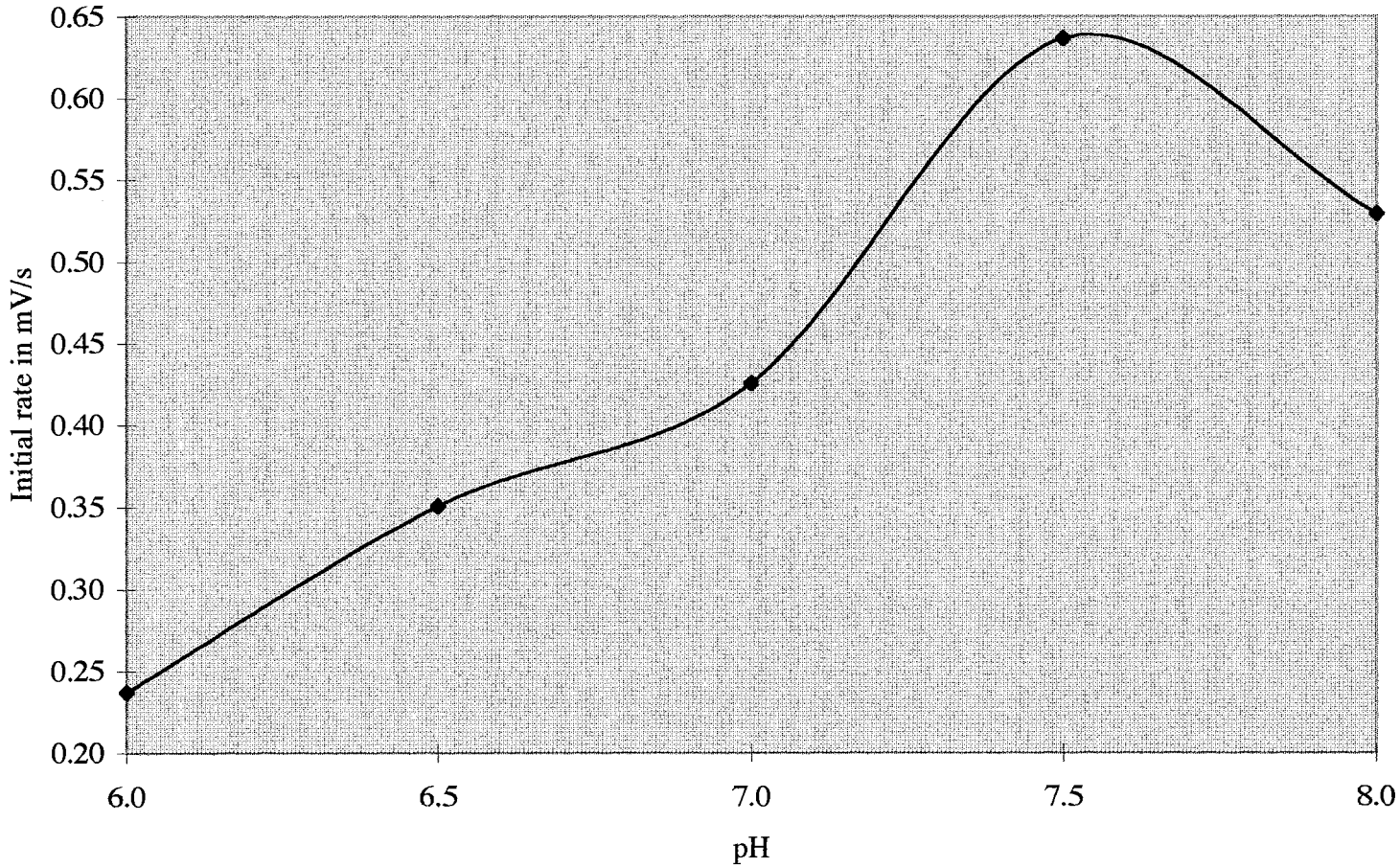

Figure 26. Effect of pH on the Initial Rate of the Glutamate Oxidase System

Effect of Rotation on Michaelis-Menten Constant

As explained in Chapter III the rotation of the platform containing the immobilized enzyme is expected to decrease the value of the apparent Michaelis-Menten constant, $K'_{M,}$ and thereby increase the ratio $k_{cat}/ K'_{M.}$ This corresponds to a better utilization of the active sites of the immobilized enzyme. To verify this, the value of the Michaelis-Menten constant for immobilized glutamate oxidase was obtained at different rotation speeds by plotting 1/IR vs. 1/[S] at different concentrations of the substrate. The results are tabulated in Table XIV. As expected the values in Table XIV confirm the trend of decreasing K'_M values with increasing rotation and justify the technique used in this work. A rotation speed of 874 rpm was used since the K'_M was found to be the lowest at this speed and higher velocities were avoided because of the vibration of the reactor.

Calibration Curve

The working calibration curve was traced using the optimum conditions determined before. A linear relationship was observed between the initial rate of the reaction and the substrate concentration in the range 1 μM to 100 μM at 874 rpm. For convenience the graphs are shown separately in Figures 27 and 28, respectively. The equations for the lines are

$$\text{Initial rate (mV/s)} = -0.001 + 9.48\ (C_{glutamate}, \text{mM})\ (1\ \mu M \text{ to } 10\ \mu M)$$

$$\text{Initial rate (mV/s)} = 0.072 + 7.82\ (C_{glutamate}, \text{mM})\ (10\ \mu M \text{ to } 100\ \mu M)$$

TABLE XIV

VALUES OF APPARENT MICHAELIS-MENTEN CONSTANT FOR THE GLUTAMATE OXIDASE SYSTEM

Rotation velocity (rpm)	K'_M [a](mM)	Linear regression Std. Deviation
452 ± 25	0.22	(±) 0.08
664 ± 21	0.19	(±) 0.07
874 ± 26	0.18	(±) 0.08

[a]Each value of K'_M is based on triplicate of 5 different substrate concentrations.

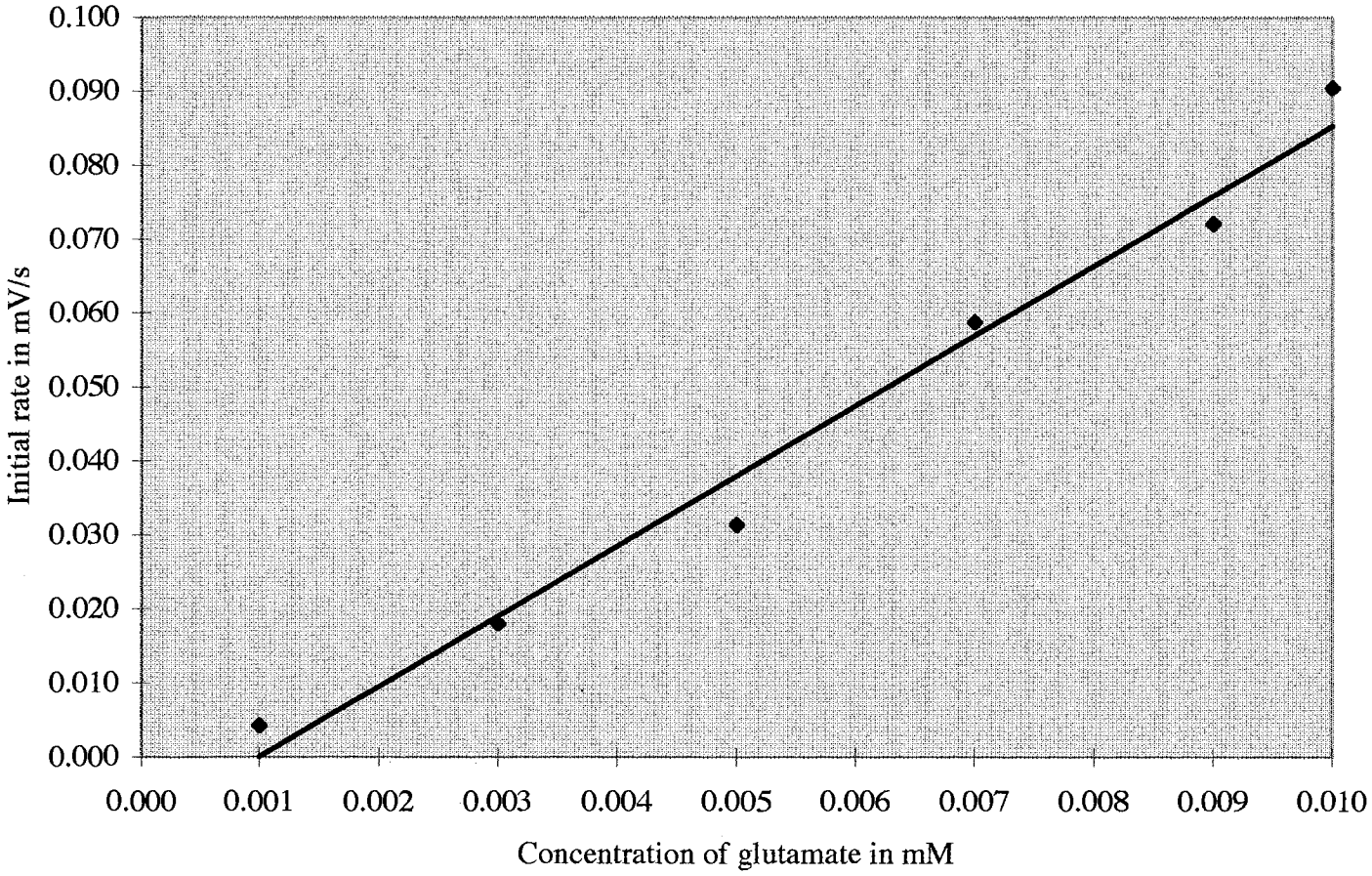

Figure 27. Glutamate Calibration Curve I of the Glutamate Oxidase System

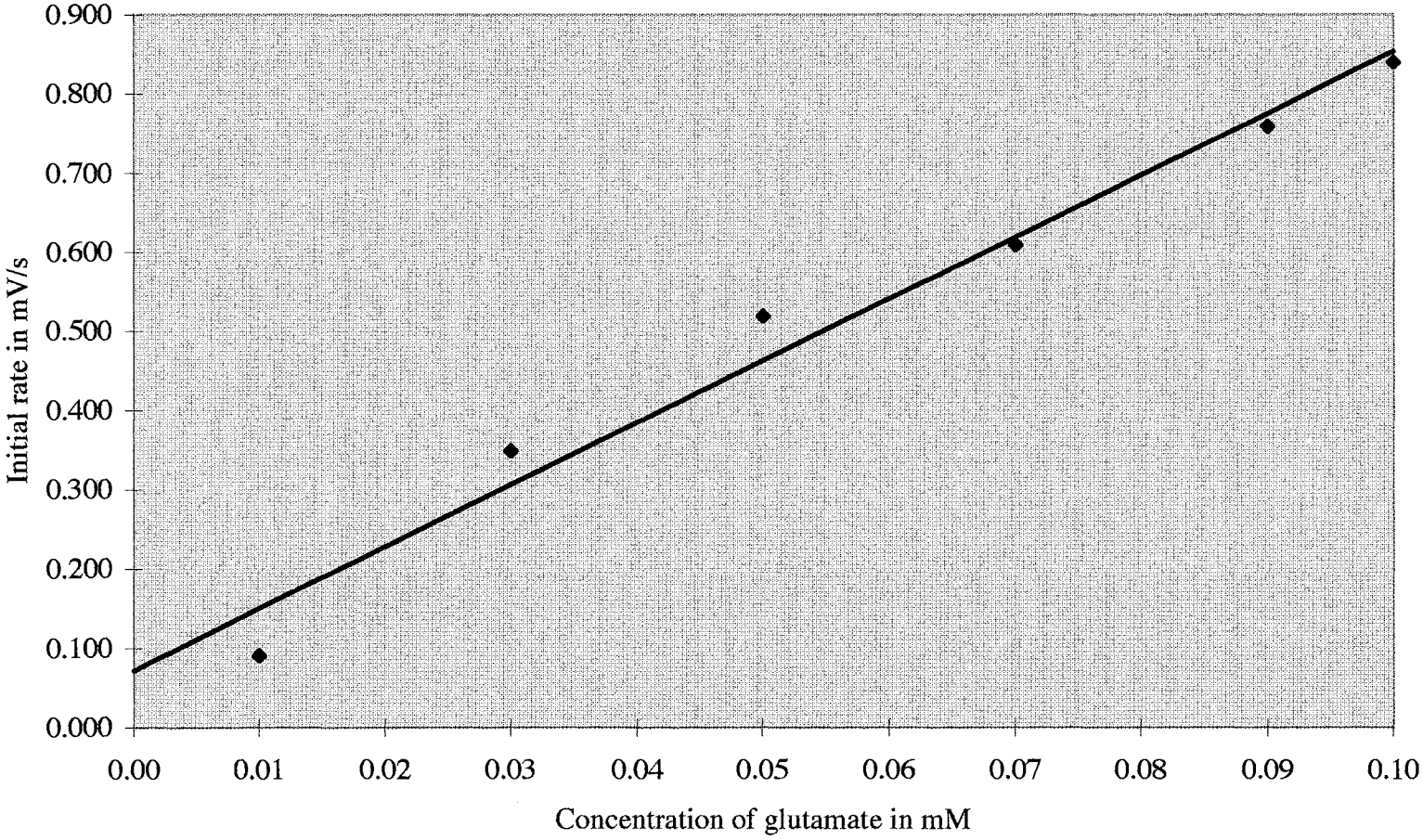

Figure 28. Glutamate Calibration Curve II of the Glutamate Oxidase System

with linear regression coefficients of 0.991 and 0.988 respectively. The limit of detection (estimated from 3 standard deviations of the lowest concentration of glutamate calibrated) is 0.35 μM with a sensitivity (slope of the calibration graph) 8.6 mV/(s mM). This curve indicates that the method responds over at least 2 orders of magnitude from 1.0 μM to 0.10 mM.

Selectivity

To establish the selectivity of this system to glutamate, the response in the presence of different amino acids as well as ascorbic acid was tested as before. Test solutions were prepared containing 10 μM glutamate and 100 μM of the potentially interfering compound in 0.10 M phosphate buffer, pH 7.40. The magnitude of the responses was compared by averaging the initial rates of each test solution (containing glutamate and the interfering compound) to the average initial rate of glutamate (alone) and converting to %. The results are listed in Table XV.

Kusakabe and co-workers [143] report that the enzyme glutamate oxidase exclusively catalyses glutamate with only 0.6 % interference from aspartate. This study supports it. The responses generated by other amino acids was not significantly different from the response generated by glutamate alone. Ascorbic acid being an electroactive compound was found to interfere with this determination. A procedure for the elimination of ascorbic acid is described later in this Chapter. As shown in this study this system works selectively for glutamate determination.

TABLE XV

SELECTIVITY STUDY: RELATIVE RESPONSES FOR POTENTIAL INTERFERENCE

COMPOUND[a]	RESPONSE[b]	STD. DEVIATION
None	100.0	(±) 0.002
Alanine	99.0	(±) 0.001
Arginine	99.0	(±) 0.001
Asparagine	100.0	(±) 0.001
Aspartic acid	99.0	(±) 0.002
Cystine	98.0	(±) 0.002
Glutamine	101.0	(±) 0.001
Glutathione	98.0	(±) 0.001
Glucose	99.0	(±) 0.001
Glycine	96.0	(±) 0.003
Isoleucine	98.0	(±) 0.002
Leucine	95.0	(±) 0.003
Lysine	101.0	(±) 0.002
Methionine	103.0	(±) 0.003
Phenylalanine	99.0	(±) 0.002
Proline	100.0	(±) 0.002
Serine	99.0	(±) 0.003
Threonine	96.0	(±) 0.003
Tryptophan	96.0	(±) 0.003
Valine	97.0	(±) 0.002
Tyrosine	97.0	(±) 0.002

[a] Solution contain 0.010 mM glutamate and 0.10 mM of potentially interfering compound

[b] each value based on at least triplicate measurements

Determination of Glutamate in Food Samples

This method using glutamate oxidase was applied for the determination of glutamate in some food products. Appropriate dilutions were made to the sample with the working buffer (phosphate, pH 7.40). A standard addition (see Appendix) method was used to determine glutamate in order to avoid matrix effects. Standard glutamate solutions of three concentrations were added to the sample and the initial rate was measured. The results are tabulated in Table XVI. The glutamate levels in the chicken bouillon cube and seasoning salt are closer to the manufacturer's specification than the beef bouillon cube but well within the normally accepted values.

Elimination of Ascorbic acid

Ascorbic acid is added to some foods to act as stabilizers (or) preservative and added in most drinks for its nutritional value. It is soluble in water and has both acidic and strong reducing properties [144].

Ascorbic acid is one of the major interferents encountered in electrochemical detection. Different approaches have been used to circumvent this problem. Masoom and Townshed [145] incorporated a dialyzer (or) a column of copper (II) diethyl dithiocarbomate on controlled porosity glass, and Yao et al. [146] placed an electrolytic column before electrode to destroy the ascorbic acid. Guilbault et al. [147] modified a platinum electrode by coating it with a layer of a lipid mixture (asolectin) to measure glucose in the presence of interferents, including ascorbate.

Conventional enzyme electrodes employ discrete macroscopic membranes to

TABLE XVI

DETERMINATION OF GLUTAMATE IN FOOD PRODUCTS USING THE ENZYME GLUTAMATE OXIDASE

PRODUCT	EXPERIMENTAL VALUES[a] (w/w %)	NORMALLY ACCEPTED VALUES FOR PRODUCTS (w/w %)
Season all salt	5.39 ± 0.14[c]	5.80[b] - 17.2[161]
Beef bouillon cube	4.46 ± 0.10[c]	3.00[b] - 6.80[161]
Chicken bouillon cube	5.04 ± 0.12[c]	4.89[b] - 8.70[161]
Soy sauce	2.16 ± 0.12[c]	1.09[13] - 15.8[162]
Milk (skim)	0.014 ± 0.001[c]	0.015[13]

[a]Average of three runs.

[b]Manufacturer's specification.

[c] Relative std. deviations

overcome this problem. Membranes such as cellulose acetate has been developed as permeability barriers to physically exclude the interferents, while permitting the product of the enzyme-catalyzed reaction to reach the electrode surface [148, 149]. Polymer films have been cast on an electrode surface either by radio frequency plasma treatment [150], or by electropolymerization [151, 152, 153]. Sasso et al. [154], for example, have used a diamino benzene coating to exclude ascorbate. There are certain limitations when membrane coatings are used. Depending on the thickness of the membrane, the diffusion paths of the reactants reaching the enzyme and product reaching the electrode were found to be slow and complex. This affects the response time and sampling rate. The methods mentioned above work on the principle of permeation, so when food samples are analyzed, any particle present may block pores in the membranes thereby preventing the product from reaching the electrode surface.

Another approach to eliminate ascorbic acid is to use the enzyme ascorbate oxidase (EC 1. 10. 3. 3). This method is discussed in [155]. Here the ascorbate is eliminated before reaching the detection chamber, by passing the sample through a column containing the immobilized ascorbate oxidase. This method of elimination was adopted in this work as this was found to be more efficient than the other methods discussed above. The column is placed just before the electrochemical cell such as the sample passes through the column first and eliminates the interferent species. This column eliminated successfully up to 0.10 mM of ascorbic acid. The glutamate in two fruit juices and in tomato juice was determined with this arrangement. The results are listed in Table XVII. It was found that even a low concentration of glutamate can be determined reliably in the presence of ascorbic acid using this scheme.

TABLE XVII

RESULTS OF GLUTAMATE CONTENT AFTER ASCORBATE ELIMINATION

PRODUCT	GLUTAMATE CONTENT[a] (w/w %)	NORMALLY ACCEPTED VALUES FOR THE PRODUCTS (w/w %)
Apple juice	0.0119 ± 0.0001^{c}	0.0117^{13} - 0.0240^{165}
Orange juice	0.022 ± 0.001^{c}	0.012 - 0.027^{13}
Tomato juice	0.109 ± 0.002^{c}	0.109^{13}

[a]Average of three runs.

[c] Relative std. deviations

CONCLUSION

Glutamate is an important amino acid due to its role as a flavor agent in food industry and in metabolic processes. The work described in this thesis demonstrates the use of two enzymes (glutamate dehydrogenase and glutamate oxidase) to catalyze the oxidation of glutamic acid and its subsequent determination in a continuous-flow/stopped-flow system by electrochemical monitoring of the oxidation product.

The use of packed column reactors (with immobilized enzymes) in conjunction with peristaltic pumping are unsatisfactory due to the back pressure problems associated with it. The rotating reactor described and used in this work eliminates this problem. Also, the design allows the use of small amount of enzymes by fully using the available active sights efficiently.

It has been documented before that the use of rotating reactors reduces the diffusional constraints when immobilized enzymes are used. This is proven here by the lowering of the apparent Michaelis-Menten constant value with increasing rotation speed of the reactor.

Practically no sample preparation is needed here unlike other methods like HPLC. Moreover, since electrochemical detection is used even turbid and colored samples can be analyzed successfully with this setup. The flow injection manifold is very simple and can be assembled in any laboratory with minimum cost.

Of the two methods described here the results indicate that the approach that uses

the enzyme glutamate oxidase is easier to handle, direct, sensitive, and cost efficient. Ascorbic acid was found to be an interference in samples analyzed in this work. A strategy to eliminate this interference is also discussed. As far as selectively of glutamate in the presence of other amino acids are concerned, both of the methods offer excellent selectivity.

To summarize the use of glutamate oxidase in continuous-flow system combines the selectivity of the enzyme with the speed of flow analysis. The immobilized enzyme has been found to retain its activity for about two months. As the enzyme is reused several times during this, the cost per determination is reduced considerably.

REFERENCES

1. R. H. Cagan, In "*The Chemical Senses and Nutrition*"; M. R. Kare and O. Maller, Eds.; Academic Press: New York, **1977**, pp 343 - 359.

2. D. Voet and J. G. Voet, In "*Biochemistry*", John Wiley and Sons: New York, **1990**, p. 62.

3. G. Schwartz, In "*Bad Taste: The MSG Syndrome*", Signet Books: New York, **1988**.

4. D. Allen, J. Delohery, and G. Baker, *J. Allergy Clin. Immunol.*, **1987**, *80*, 530.

5. P. Morselli, and S. Garattini, *Nature*, **1979**, *227*, 611.

6. L. R. Hac, M. L. Long, and M. J. Blish, *Food Techol.*, **1949**, *3*, 351.

7. S. Maeda, S. Eguchi, and H. Sasaki, *J. Home Econ. Jpn.*, **1958**, *9*, 163.

8. R. Powell, In "*Monosodium Glutamate and Glutamic Acid*", Noye Development Corporation: New Jersey, **1968**, p. 1.

9. P. Talioferro, *J. Environ. Health*, **1995**, *57*, 8.

10. S. Sdrauling, L. J. Rogers, and A. L. A. Boura, *Physiol. Behav.*, **1980**, *24*, 493.

11. S. P. Bullock, and L. J. Rogers, *Pharmol. Biochem. Behav.*, **1986**, *24*, 549.

12. *Monosodium Glutamate (MSG)*, Federation of American Societies for Experimental Biology A Report, FDA home page at www., **1995**, p. 1.

13. R. S. Skurray and N. Pucar, *Food Chemistry*, **1988**, *27*, 179.

14. FAO/WHO, "*Guide to the safe use of food additives*", FAO/WHO of the United Nations, Rome, Italy, **1979**.

15. D. H. Allen, and G. J. Baker, *New Engl. J. Med.*, **1981**, *305*, 56.

16. H. H. Schaumberg, R. Byck, R. Gerstyle, and J. H. Masham, *Science*, **1969**, *163*, 826.

17. *An Introduction to Citizen Petition # 94P-0444,* P. O. Box 2532, Darien, Il, Sept 15, **1995**.

18. J. Ruzicka, and E. H. Hansen, "*Flow Injection Analysis*", John Wiley: New York, **1988**.

19. P. S. J. Cheetham, In "*Handbook of Enzyme Biotechnology*", A. Wiseman, Ed.; John Wiley: New York, **1985**, p. 60.

20. M. H. Ho, In "*Methods in Enzymology*": K. Mosbach, Ed.; Academic Press: New York, **1988**, Chapter 24.

21. D. Confer, B. Logan, B. Aiken, and D. Kirchman, *Water Environ. Res.*, **1995**, *67*, 118.

22. H. Brueckner, S. Hassmann, M. Langer, T. Westhauser, R. Wittner, and H. Godel, *J. Chromatogr.*, **1994**, *666*, 259.

23. H. Brueckner, P. Jack, M. Langer, and H. Godel, *Amino Acids*, **1992**, *2*, 271.

24. R. Soto-Otero, E. Mendez-Alvarez, J. Galan-Valiente, E. Aguilar-Veiga, and, G. Sierra-Marcuno, *Biomed. Chromatogr.*, **1994**, *8*, 114.

25. M. Zhao and J. Bada, *J. Chromatogr. A.*, **1995**, *690*, 55.

26. D. Thomas and K. Parkin, *J. Agri. Food Chem.*, **1994**, *42*, 1632.

27. J. Kirschbaum, B.Luckas, and W. Beinert, *J. Chromatogr.*, **1994**, *661*, 193.

28. S. R. Hagen, B. Frost, and J. Augustin, *J. Assoc. Off. Anal. Chem.*, **1989**, *72*, 912.

29. B. A. Bidlingmeyer, S. A. Cohen, T. L. Tarvin, and B. Frost, *J. Assoc. Off. Anal. Chem.*, **1987**, *70*, 241.

30. J. D. H. Cooper, D. Turnell, B. Green, D. Demarais, and P. Rasquin, *Proc. Chromatogr. Soc. Int. Symp.*, **1994**, *87*.

31. L. Greig, J. Kibby, and J. Redmond, *J. Chromatogr.*, **1991**, *588*, 107.

32. M. Aminuddin and J. Miller, *J. Chem. Soc. Pak.*, **1994**, *16*, 17.

33. S. S. Yang and I. Smetena, *Chromatographia*, **1993**, *37*, 593.

34. A. T. Rhyswillams and S. A. Winfield, *Analyst*, **1982**,*107*, 1092.

35. R. L. Heinrikson and S. C. Meredith, *Anal. Biochem.*, **1980**, *104*, 254.

36. J. Khan, Y. H. Kuo, N. Kebede, and F. Lambein, *J. Chromatogr. A.*, **1994**, *687*, 113.

37. E. Tatar, M. Khalisa, G. Zaray, and I. Molnarperl, *J. Chromatogr.* A, **1994**, *672*, 109.

38. Y. Yang, Q. Qin, and W. Guo, *Sepu*, **1994**, *12*, 295; Chem. Abstr. **1994**, *121*, 194596j.

39. M. Senden, A. Vander-Meer, J. Limborgh, and H. Wolterbeek, *Plant Soil*, 1992, *142*, 81.

40. E. Monastero, W. Deutsch, and M. Ellero, *Ind. Aliment.*, **1991**, *30*, 817; Chem. Abstr. **1992**, *116*, 3987o.

41. M. Aristoy and F. Toldra, *J. Agri. Food Chem.*, **1991**, *39*, 1792.

42. L. Murthy and K. Iqbal, *Anal. Biochem.*, 1991, *193*, 299.

43. M. Puig-Deu, and S. Buxaderas, *J. Chromatogr. A.*, **1994**, *685*, 21.

44. B. Chang, H. Liu, H. Yan, F. Yu, and X. Liu, *Fenxi Huaxue*, **1995**, *23*, 100; Chem. Abstr. **1995**, *122*, 74049g.

45. H. Liu, *J. Chromatogr. A.*, **1994**, *670*, 59.

46. M. Vandyck, B. Rollmann, and B. Tilquin, *J. Pharm. Belg.*, **1993**, *48*, 53.

47. H. L. Rowley, K. F. Martin, and C. A. Marsden, *J. Neurosci. Methods*, **1995**, *57*, 93.

48. R. Sherwood, *J. Neurosci. Methods*, **1990**, *34*, 17.

49. J. Benson, and P. Hare, *Proc. Natl. Acad. Sci. U.S.A.*, **1975**, *72*, 619.

50. S. Simons, and D. Johnson, *J. Org. Chem.*, **1978**, *43*, 2886.

51. M. Aristoy, and F. Toldra, *J. Agri. Food Chem.*, **1991**, *39*, 1792.

52. H. B. Conacher, and J. R. Iyengar, *J. Assoc. Off. Anal. Chem.*, **1979**, *62*, 604.

53. R. Corradetti, G. Monetti, F. Moroni. G. Pepeu, and A. Wieraszko, *J. Neurochem.*, **1983**, *41*, 1518.

54. L. Bertilsson, and E. Costa, *J. Chromatogr.*, **1984**, *42*, 1624.

55. S. Mackenzie, and D. Tenaschuk, *J. Chromatogr.*, **1985**, *322*, 228.

56. P. L. Wood, and D. L. Cheney, In "*Neuromethods 3: Amino Acids*", A. A. Boulton, G. B. Baker, and J. D. Wood, Ed.; Humana Press: New Jersey, **1985**, Chapter 3.

57. P. Husek, *J. Chromatogr.*, **1991**, *552*, 289.

58. P. Simek, A. Jegorov, and A. Heydova, *J. High Resolut. Chromatogr.*, **1994**, *17*, 145.

59. K. Kim, J. Kim, C. H. Oh, and T. Mabry, *J. Chromatogr.*, **1992**, *605*, 241.

60. M. Wolfensberger, In "*Measurement of Neurotransmitter Release In Vivo*", C. A. Marsden, Ed.; John Wiley: New York, **1984**, Chapter 2.

61. J. A. Prieto, C. Collar, and C. Bendito de-barber, *J. Chromatogr. Sci.*, **1990**, *28*, 572.

62. S. F. Y. Li, *Capillary Electrophoresis*, Elsevier: New York, **1992**, p 6.

63. J. W. Jorgenson and K. D. Lukacs, *Anal. Chem.*, **1981**, *53*, 1298.

64. J. Mattusch and K. Dittrich, *J. Chromatogr.*, **1994**, *680*, 279.

65. S. Wu and N. Dovichi, *Talanta*, **1992**, *39*, 173.

66. J. Zhao, J. Labbe, and N. Dovichi, *J. Microcolumn Sep.*, **1993**, *5*, 331.

67. P. Camilleri and G. Okafo, *J. Chromatogr.*, **1991**, *541*, 489.

68. J. Liu, K. Cobb, and M. Novotny, *J. Chromatogr.*, **1988**, *468*, 55.

69. M. Albin, R. Weinberger, E. Sapp, and S. Morig., *Anal. Chem.*, **1991**, *63*, 417.

70. P. Oefner, *Electrophoresis*, **1995**, *16*, 46.

71. J. Zhou, and S. Lunte, *Electrophoresis*, **1995**, *16*, 498.

72. S. Kelly and H. Swift, *Amer. J. Epidem.*, **1967**, *85*, 250

73. M. L. Efron, D. Young, H. W. Moser, and R. A. MacCready, *New Engl. J. Med.*, **1964**, *270*, 1378.

74. D. Liu, Y. Lai, L. Nie, and S. Yao, *Anal. Chim. Acta*, **1995**, *313*, 229.

75. K. Gu, "*Important Organic Reactions*", Shanghai Publishing House of Science and Technology: Shanghai, **1983**, p 266.

76. H. Chung, M. Arnold, M. Rhiel, and D. Murhammer, *Appl. Spectrosc.*, **1996**, *50*, 270.

77. *Chinese Pharmacopoeia, Vol. II*, **1990**, p. 247.

78. J. Song, X. Wang, F. Xu, and L. Jiangyan, *Huaxue Fence*, **1991**, *27*, 225.

79. G. Guilbault, "*Analytical Uses of Immobilized Enzymes*", Marcel Dekker: New York, **1984**, pp 2 and 77.

80. B. W. Mathews, H. Nicholson, and W. J. Becktel, *Proc. Natl. Acad. Sci. USA*, **1987**, *84*, 6663.

81. M. Matsumura and S. Aiba, *J. Biol. Chem.*, **1985**, *260*, 15298.

82. J. F. Kennedy, In "*Handbook of Enzyme Biotechnology*", A. Wiseman, Ed.; John Wiley: New York, **1985**, pp 147 - 188, 380 - 411.

83. O. R. Zaborsky, "*Immobilized Enzymes*", CRC Press: Ohio, **1973**.

84. R. Epton, J. V. McLaren, and T. H. Thomas, *Carbohydr. Res.*, **1972**, *22*, 301.

85. E. Brown and A. Racois, *Tetrahedron Lett.*, **1972**, *28*, 5077.

86. I. Ugi, *Angew. Chem. Int. Ed.*, **1962**, 1, 8.

87. V. C. Borlazza, N. W. H. Cheetham, and P. T. Sowthwell-Kelly, *Carbohydr. Res.*, **1980**, *79*, 125.

88. F. A. Quiocho and F. M. Richards, *Proc. Nat. Acad. Sci. USA*, **1964**, *52*, 833.

89. G. B. Broun, In "*Methods in Enzymology*", K. Mosbach, Ed.; Academic Press: New York, **1976**; Vol. 44, pp 263 - 280.

90. G. V. Diaz, L. H. El-issa, M. A. Arnold, and R. F. Miller, *J. Neurosci. Meth.*, **1988**, *23*, 63.

91. B. Dremel, R. Schmid, and O. Wolfbeis, *Anal. Chim. Acta*, **1991**, *248*, 351.

92. R. L. Villarta, D. D. Cunningham, and G. G. Guilbault, *Talanta*, **1991**, *38*, 49.

93. C. Y. Chen and C. S. Yuan, *Anal. Chim. Acta*, **1991**, *243*, 9.

94. M. A. Arnold and T. F. Ostler, *Anal. Chem.*, **1968**, *58*, 1137.

95. F. D. Rhines and M. A. Arnold, *Anal. Chem.*, **1988**, *60*, 76.

96. S. Kar and M. A. Arnold, *Anal. Chem.*, **1992**, *64*, 2438.

97. E. Zilkha, T. P. Obrenovitch, A. Koshy, H. Kusakabe, and H. P. Bennetto, *J. Neurosc. Methods.*, **1995**, *60*, 1.

98. J. H. T. Luong, A. Mulchandani, and K. B. Male, *Enzyme Microb. Technol.*, **1991**, *13*, 116.

99. G. Palleschi, M. G. Lavagnini, D. Comagnone, and P. Bertocchi, *Electroanalysis*, **1992**, *4*, 851.

100. T. Yao, N. Kobayashi, and W. Tamotsu, *Electroanalysis,* **1990**, *2*, 563.

101. F. Botre, C. Botre, G. Lorenti, F. Mazzei, F. Porcelli, and G. Scibona, *J. Pharm. Biomed. Anal.,* **1993**, *11*, 679.

102. E. Tamiya, Y. Sugiura, T. Takeuchi, M. Suzuki, I. Karube, and A. Akiyama, *Sensors & Actuators, B*, **1993**, *10*, 179.

103. M. O. M. Berners, M. G. Boutelle, and M. Fillenz, *Anal. Chem.*, **1994**, *66*, 2017.

104. S. Yoshida, H. Kanno, and T. Watanabe, *Anal. Sci.*, **1995**, *11*, 251.

105. W. Vahjen, J. Bradley, V. Brilitewski, and R. Schmid, *Anal. Lett.*, **1991**, *24*, 1445.

106. C. Y. Bang, Q. S. Li, Y. R. Li, Y. B. Li, and J. T. Yu, *J. Biotech.*, **1995**, *42*, 45.

107. T. Yao, N. Kobayashi, and T. Wasa, *Anal. Chim. Acta*, **1990**, *231*, 121.

108. U. Wollenberger, F. W. Scheller, A. Bohmer, M. Pussarge, and H. G. Muller, *Biosensors*, **1989**, *4*, 381.

109. K. Hajizadeh, H. B. Halsall, and W. R. Heineman, *Talanta*, **1991**, *38*, 37.

110. N. F. Almeida and A. Mulchandain, *Anal. Chim Acta*, **1993**, *282*, 353.

111. P. D. Hale, H. S. Lee, Y. Okamota, and T. A. Skotheim, *Anal. Lett.*, **1991**, *24*, 345.

112. G. Blankenstein, F. Preuschoff, U. Spohn, K. H. Mohr, and M. R. Kula, *Anal. Chim Acta.*, **1993**, *271*, 231.

113. M. Kaltenbach, Ph.D. Dissertation, University of Iowa, Iowa, **1991.**

114. M. Ueda, Y. Kuraishi, K. Sugimoo, and M. Satoh, *Neurosci. Res.*, **1994**, *20*, 231.

115. N. Kiba, T. Moriya, and M. Furusawa, *Anal. Chim. Acta*, **1992**, *256*, 221.

116. C. D. Stalikas, M. I. Karayannis, and S. T. Karrayanni, *Talanta*, **1994**, *41*, 1561.

117. Ae-June Wang, Ph.D. Dissertation, University of Iowa, Iowa, **1992.**

118. Y. Tonogai, A. Kingkate, W. Thanissorn, and U. Punthanaprated, *J. Food Protec.*, **1983**, *46*, 522.

119. J. I. Korenbrot, R. Perry, and D. Copenhagen, *Anal. Biochem.*, **1987**, *161*, 187.

120. S. Girotti, S. Ghini, R. Budini, A. Pistillo, G. Carrea, R. Bovara, S. Piazzi, R. Merighi, and A. Rhoda, *Anal. Lett.*, **1992**, *25*, 637.

121. Z. Samec, and P. J. Elving, *J. Electroanal. Chem.*, **1983**, *144*, 217.

122. W. J. Blaedel, and R. A. Jenkins, *Anal. Chem.*,**1975**, *47*, 1337.

123. S. Yabuki, F. Mizutani, and M. Asai, *J. Electrochem. Soc. Japan*, **1990**, *58,* 1215.

124. L. Gorton, A. Torstensson, H. Jaegfeldt, and G. Johansson, *J. Electroanal. Chem.*, **1984**, *161*, 103.

125. L. G. Lee and G. M. Whitesides, *J. Am. Chem. Soc.*, **1985**, *107*, 6999.

126. B. Persson, H. L. Lan, L. Gorton, Y. Okamoto, P. D. Hale, L. I. Boguslavsky, and T. Skotheim, *Biosensors & Bioelectronics*, **1993**, *8*, 81.

127. F. Schubert, D. Kirstein, F. Scheller, I. Appelqvist, L. Gorton, and G. Johansson, *Anal. Lett.*, **1986**, *19*, 1273.

128. S. Yabuki, F. Mizutani, and M. Asai, *J. Electrochem. Soc. Japan*, **1990**, *58*, 1215.

129. H. Booker and J. Haslem, *Anal. Chem.*, **1974**, *46*, 1054.

130. R. D. Allison, and D. L. Purich, In "*Methods in Enzymology*", D. L. Purich, Ed.; Academic Press: New York,**1979**, Vol. 63, Sec. 1.

131. I. Chibata, *"Immobilized Enzymes"*, John Wiley: New York, **1978**, p 124.

132. O. Richter, B. L. Ruiz, M. Sanchez-Cabezuda, and H. A. Mottola, *Anal. Chem.*, **1996**, *68*, 1701.

133. B. L. Karger, L. R. Snyder, and C. Horvath, *"An Introduction To Separation Science"*, John Wiley: New York, **1973**, pp 83 - 92.

134. K. Matsumoto, J. J. Baeza Baeza, and H. A. Mottola, *Anal. Chem.*, **1993**, *65,* 636.

135. J. Raba and H. A. Mottola, *Anal. Chem.*,**1994**, *66*, 1485.

136. P. Cheetham, In *"Principles Of Industrial Enzymology"*, 2nd ed.; A.Wiseman., Ed.; John Wiley: NewYork, **1985,** Chapter 3.

137. P. C. Engel, *"Enzyme Kinetics"*, John Wiley: NewYork, **1977**, p 37.

138. K. Kronkvist, K. Wallentin, and G. Johansson, *Anal. Chim. Acta*, **1994**, *290*, 335.

139. H. Bergmeyer, *"Methods of Enzymology"*, Academic Press: New York, **1965,** pp. 448 and 461.

140. H. A. Mottola, *"Kinetic Aspects of Analytical Chemistry"*, John Wiley: New York, **1988,** p. 57.

141. J. J. Baeza Baeza, K. Matsumoto, and H. A Mottola, *Quimica Analitica*, **1993**, *62*, 12.

142. L. C. Thomas, and G. D. Christian, *Anal. Chim. Acta*, **1976**, *82*, 265.

143. H. Kusakabe, Y. Midorikawa, T. Fujishima, A. Kuninaka, and H. Yoshino, *Agri. Biol. Chem.*, **1983**, *47*, 1323.

144. S. R. Tannenbaum, V. R. Young, and M. C. Archer, In "*Food Chemistry*", O. R. Fennema, Ed.; Marcel Dekker: New York, **1985**, Chapter 7, p. 477.

145. M. Masoon and A. Townshed, *Anal. Chim. Acta,* **1984**, *166*, 111.

146. T. Yao, Y. Kobayashi, and M. Sato, *Anal. Chim. Acta*, **1983**, *153*, 337.

147. A. Amine, J. M. Kaufmann, C. J. Patriarche, and G. G. Guilbault, *Anal. Lett.*, **1989**, *22*, 2403.

148. W. H. Muller, F. H. Keedy, S. J. Churchouse, and P. M. Vadgamma, *Anal. Chim. Acta,* **1986**, *183*, 59.

149. Z. Koochaki, I. Christie, and P. Vadgama, *J. Membr. Sci.*, **1991**, *57*, 83.

150. R. Nowak, F. Schultz, M. Umana, H. Abruna, and R. W. Murray, *J. Electroanal. Chem.*, **1979**, *94*, 219.

151. W. R. Heinman, H. J. Wieck, and A. M. Yacynych, *Anal. Chem.*, **1980**, *52*, 345.

152. G. Cheek, C. P. Wales, and R. J. Nowak, *Anal. Chem.*, **1983**, *55*, 380.

153. A. M. Yacynych and H.B. Mark, *J. Electrochem. Soc.*, **1976**, *123*, 1346.

154. S. Saso, R. Pirece, R. Walla, and A. Yacynych, *Anal. Chem.*, **1990**, *62*, 1111.

155. M. O. Rezende and H. A. Mottola, *Analyst*, **1994**, *119*, 2093.

156. M. Bader, *J. Chem. Educ.*, **1980**, *57*, 703.

157. D. A. Skoog, "*Principles of Instrumental Analysis*", Saunders College Publishing: Florida, **1985**, pp 210 - 212.

158. N. R.Larsen and E. H. Hansen, *Anal. Chim. Acta.*, **1976**, *84*, 31.

159. N. R. Larsen, E. H. Hansen and G. G. Guilbault, *Anal. Chim. Acta.*, **1975**, *79*, 9.

160. E. H. Hansen and J. Ruzicka, *Anal. Chim. Acta.*, **1974**, *72*, 353.

161. Official Methods of Analysis of the Association of Official Analytical Chemist's, AOAC, Sec. 20.149 - 20.151, Washington, DC, 15th ed., **1975**.

162. A. Suleiman, R. L. Villarta, and G. G. Guilbault, *Bull. Electrochem.*, **1992**, *8*, 189.

163. D. Daniels, F. Joe, and G. Daichenko, *Food Additives and Contaminanats*, **1995**, *12*, 21.

164. E. Fernandez-Flores, A. Johnson, and V. Blomquist, *J. Assoc. Off. Anal. Chem.*, **1969**, *52*, 744.

165. D. W. Kunenan, J. K. Braddock, and L. McChesney, *J. Agri. Food Chem.*, **1988**, *36*, 6.

APPENDIX

STANDARD ADDITION METHOD FOR ENZYMATIC RATE DETERMINATIONS

Ideally, calibration standards should approximate the composition of the sample to be analyzed, not only concerning the analyte concentration but also with regard to the concentrations of the other species in the matrix. The reason behind this is to minimize the matrix effects. For example, the absorbance of many colored complexes of metal ions decreases in the presence of sulfate or phosphate ions, as these ions form colorless complexes with the metal ions. This matrix effect of sulfate and phosphate ions can often be counteracted by adding these ions to the standards. But reproducing matrix effects in real life samples is very difficult. In such cases, the standard addition method is helpful in countermanding these effects.

The standard addition method can be used in several ways [156]. The common method is the one used in spectrometric analysis. Here, one or more increments of the standards are added to the sample, diluted to a fixed volume, and the corresponding absorbance measured. For example, if to several aliquots (V_x) of the sample with concentration C_x, a variable volume (V_s) of the standard solution with concentration C_s is added and the absorbance is measured [157], then according to Beer's law,

$$A_s = \frac{\varepsilon_b V_x C_x}{V_t} + \frac{\varepsilon_b V_s C_s}{V_t} \qquad \text{(A-1)}$$

V_t is the total volume. If A_s is plotted vs. V_s, it will be a straight line with a slope $\varepsilon_b C_s/V_t$ and an intercept $\varepsilon_b V_x C_x/V_t$. By dividing the intercept by the slope we get,

$$C_x = \frac{\text{intercept } C_s}{\text{slope } V_x} \qquad \text{(A-2)}$$

This method is frequently employed for equilibrium measurements in thermodynamically stable systems.

For enzymatic reactions, with fixed enzyme activities the Michaelis - Menten rate expression should apply

$$IR = \frac{IR_{max}[S]}{K'_M + [S]} \qquad \text{(A-3)}$$

Where IR is the initial rate of the reaction, K'_M is the Michaelis constant, [S] is the initial substrate concentration, and IR_{max} is the maximum initial rate.

In kinetic determinations, initial rate measurements are generally evaluated at substrate concentration an order magnitude or smaller than K'_M since IR will be proportional to [S] under these conditions. However, this signifies that IR_{max} and K'_M maintain their values irrespective of whether a calibration or analysis is performed in the actual medium. This leads to two significant limitations:

- Presence of certain species (like metal ions) may have a big influence on the enzyme activity, thereby on IR_{max} and K'_M

- Highly sensitive methods are essential as substrate concentration would be very low ($S << 0.1\ K'_M$, where K'_M is typically in the order of mM).

These limitations can be overcome by following a method described by Larsen and Hansen [158]. The only prerequisite is that the Michaelis- Menten rate expression be satisfied. The theory behind their method is as follows. If the concentration of the substrate in equation (A-3) is replaced by $X + C_i$, where X is the concentration of the unknown and C_i is the concentration of the added standard, then

$$IR_i = \frac{IR_{max} + (X + C_i)}{K'_M + (X + C_i)} \tag{A-4}$$

Where IR_i signifies the initial rate of the i^{th} reaction. Since the equation contains three unknowns, three equations are needed for solving that one. The value of X can be determined by choosing three different standards and determining their rates.

$$X = \frac{IR_1IR_2C_3(C_1 - C_2) + IR_1IR_3C_2(C_3 - C_1) + IR_2IR_3C_1(C_2 - C_3)}{IR_1IR_2(C_2 - C_1) + IR_1IR_3(C_3 - C_1) + IR_2IR_3(C_3 - C_2)} \tag{A-5}$$

This expression can be simplified by fixing $C_1 = 0$, $C_3 = aC_2$; the only restriction being $C_2 > C_1$ and $a \neq 0$. Then

$$X = \frac{aC_2(IR_1)(IR_3 - IR_2)}{IR_1(IR_2 - aIR_3) + (a - 1)IR_2IR_3} \tag{A-6}$$

Highest accuracy can be obtained from this method when $(IR_2 - IR_1) = (IR_3 - IR_2)$. This method finds use mainly at $X > 0.1\ K'_M$, but equation A-3 may not be valid at higher substrate concentrations due to product inhibition. Therefore, for all practical

purposes, C_2 is chosen at the same magnitude as K'_M (of the chosen reaction) and 'a' at high value (low enough to avoid substrate inhibition). The authors applied this method for the determination of urea in serum at a constant activity of the enzyme urease, by monitoring the liberated ammonia with an air-gap ammonia sensitive electrode [159, 160]. Larsen and Hansen's method was used to determine glutamate in food products in the work presented in this thesis. As pointed out before, it is often advantageous to use the standard addition method in enzyme kinetic substrate determinations. In some cases, it may be the only applicable method if the activity of the enzyme is affected by the medium.

VITA

Chitra Janarthanan

Candidate for the Degree of

Doctor of Philosophy

Thesis: ENZYMATIC DETERMINATIONS USING A ROTATING BIOREACTOR AND CONTINUOUS-FLOW/STOPPED-FLOW PROCESSING DETERMINATION OF GLUTAMATE IN FOOD PRODUCTS.

Major Field: Chemistry

Biographical:

Personal data: Born in Madras, Tamil Nadu, India, February 4, 1964, the daughter of Janarthanan and Dakshayani.

Education: Graduated from Model Higher Secondary School, Madras, India in May 1982; received Bachelor of Science from Quaid-E-Millet Government College for Women, Madras, India in May 1985; received Master of Science from Alagappa College of Technology, Madras, India in May 1987. Completed the requirement for the Doctor of Philosophy at Oklahoma State University in December 1996.

Experience: Employed as Analyst in Bharat Petroleum Corporation, Madras, India from 1987 to 1988; Lecturer in Ethiraj College for Women from January 1989 to April 1989; Marketing Services Officer in Hindustan Petroleum Corporation, Madras, India from 1990 to 1991; as a graduate teaching assistant, Oklahoma State University, Department of Chemistry, August 1991 to present.

Professional Memberships: Phi Lambda Upsilon-Honorary Society member, American Chemical Society member