Immobilized Enzymes as On-line Probes in Biochemistry and New Drug Discovery: Biosynthesis of Catecholamines

by

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Abstract

The use of immobilized enzymes has steadily increased in recent years. Based upon the advantages that immobilized enzymes possess over soluble enzymes, numerous applications have emerged in medical and analytical fields. This work demonstrates the applicability of a liquid chromatographic system based upon coupled on-line immobilized enzyme reactors (IMERs) to organic synthesis, biochemistry and pharmacology. It is envisioned that the model system will grow into a modular process where synthetic chemists can add or subtract the enzymes necessary for their particular synthetic goal. The system allows for on-line chromatographic purification and structural identification of products and could greatly reduce time required to discover new synthetic pathways. In addition, the construction of a coupled enzyme system provides a number of approaches to basic research into synthetic and metabolic pathways as well as a rapid method for the discovery of new pharmaceutical substances.

A coupled system using extremely different enzymes with incompatible cofactors and reaction conditions has been constructed. The significance of the proposed project not only lies in the development of the liquid chromatographic on-line enzyme cascade but also in the biosynthetic pathway chosen for this study. The biosynthetic pathway involving dopamine beta-hydroxylase and phenylethanolamine N-methyltransferase encompass the synthesis of the key transmitters, norepinephrine and epinephrine. The results demonstrate for the first time the immobilization of dopamine beta-hydroxylase and phenylethanolamine N-methyltransferase. The IMERs are active and can be used in a liquid chromatographic format for qualitative and quantitative determinations. Studies with the IMER-HPLC systems have also shown that the activity of the immobilized enzymes reflects the non-immobilized enzymes. Thus, the IMER-HPLC system can be used to carry out standard Michaelis-Menten enzyme kinetic studies and to quantitatively determine enzyme kinetic constants, identify specific enzyme inhibitors, provide information regarding the mode of inhibition and the inhibitor constants (K_i). The immobilized enzyme reactors used independently or as a combination will provide a unique opportunity to explore the

interrelationships between these enzymes, to investigate the source of catecholamine-related disorders and to design new drug entities for identified clinical syndromes.

Résumé

Au cours des dernières années, l'utilisation d'enzymes immobilisées connaît une croissance exponentielle. Fort des avantages qu'elles présentent par rapport aux enzymes solubles, les enzymes immobilisées se sont trouvées de nombreuses applications médicales et analytiques. Ce travail démontre l'applicabilité, pour la synthèse organique, la biochimie ou la pharmacologie, d'un model fait d'un système chromatographique en phase liquide directement couplé avec des réacteurs renfermants des enzymes immobilisées. Il est envisageable que ce model donnera lieu à un processus modulaire où le chimiste de synthèse peut ajouter de nouvelles enzymes au système ou bien retirer des enzmyes non-désirées pour atteindre son objective de synthèse. Le système devrait permet une purification chromatographique directe et une identification structurale des produits et, par conséquent, peut réduire significativement le temps nécessaire pour découvrir de nouvelles voies de synthèse. De plus, la construction de systèmes couplants l'activité de plusieurs enzymes offre de nombreuses approches pour la découverte de nouvelles substances pharmaceutiques.

Un système couplant des enzymes extrêmement différentes nécessitants une variante de cofacteurs et des conditions de réaction différentes a été construit. La signification du projet proposé n'est pas seulement liée au développement d'une cascade de réaction enzymatique directement au sein d'un système de chromatographie liquide mais elle est également liée à l'importance de la voie de biosynthèse choisie. Les voies de biosynthèses impliquants la dopamine beta-hydroxylase et la phenylethanolamine N-methyltransferase sont responsables de deux neurotransmetteurs clés, la norépinephrine et l'épinephrine. Les résultats montrent pour la première fois l'immobilsation de la dopamine beta-hydroxylase et la phenylethanolamine N-methyltransférase. Le réacteur refermant les enzymes immobilisées est actif et peut être utilisé dans un format de chromatographie liquide pour des mesures qualitatives et quantitatives. Les études avec les systèmes IMER-HPLC ont également montré que les activités des enzymes immobilisées reflètent bien celles des enzymes non- immobilisées.

Le système IMER-HPLC peut être utilisé pour mener des études pharmacocinétiques standards de Michaelis–Menten et déterminer quantitativement les paramètres cinétiques de l'enzyme en question, indentifier les inhibiteurs spécifiques, obtenir des informations concernant le type d'inhibition et les valeurs des Ki respectifs. Les réacteurs refermants des enzymes immobilisés utilisés indépendamment ou combinés devraient fournir une opportunité unique pour explorer les relations entres ces enzymes, pour explorer les pathologies catécholamines dépendantes et pour développer de nouvelles entités médicamenteuses pour des syndromes cliniques identifiés.

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Foreword

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Candidates have the option of including, as part of the thesis, the text of one or more papers submitted, for publication, or the clearly duplicated text of one or more published papers. These texts must be bound together as an integral part of the thesis.

If this option is chosen, connecting texts that provide logical bridges between the different papers are mandatory. The thesis must be written such a way that is more than a collection of manuscripts; in other words, results of a series of papers must be integrated.

The thesis must conform to all other requirements of the "Guidelines for Thesis Preparation" in addition to the manuscripts. The thesis must include: a table of contents, an abstract in English and French, an introduction which clearly states the rational and objectives of the study, a review of the literature, a final conclusion and summary, and a thorough bibliography or reference list.

Additional material must be provided (e.g. in appendices) in sufficient detail to allow a clear and precise judgment to be made of the importance and originality of the research reported in the thesis.

In the case of manuscripts co-authored by candidate and others, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. The supervisor must attest to the accuracy of this statement at the doctoral oral defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to clearly specify the responsibilities of all the authors of the co-authored papers.

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List of Abbreviations

AADC	Aromatic amino acid decarboxylase
ACHT	α- chymotrypsin
ATP	Adenosine triphosphate
CN	Cyano
COMT	Catechol-O-methyltransferase
DA	Dopamine
DBH	Dopamine beta-hydroxylase
DDC	Dopa-decarboxylase
DOPA	dihydroxyphenylalanine
E	enzyme
EP	Epinephrine
ES	Enzyme-substrate complex
Glut-P	Glutaraldehyde-P
HLADH	Horse liver alcohol dehydrogenase
HPLC	High performance liquid chromatography
HVA	Homovanillic acid
IAM	Immobilized Artificial Membrane
IMER	Immobilized Enzyme Reactor
K_i	Inhibitor constant
K_m	Michaelis constant
M	Metanephrine
MAO	Monamine oxidase

mDBH	membrane bound dopamine beta-hydroxylase
NE	Norepinephrine
NM	Normetanephrine
OCT	Octopamine
ODS	Octadecyl
P	Product
PNMT	Phenylethanolamine N-methyltransferase
S	Substrate
SAM	S-adenosyl-L-methionine
SAH	S-adenosyl-L-homocysteine
sDBH	soluble dopamine beta-hydroxylase
SP	Stationary phase
SYN	Synephrine
TH	Tyrosine Hydroxylase
TYR	Tyramine
V_{max}	maximal rate
VMATs	Vesicular monoamine transporters

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Chapter 1

INTRODUCTION

1.1. Preface

The therapeutic and toxic effects of drugs are governed by the interactions of these molecules with biopolymers such as proteins, receptors and enzymes (Katzung, 1995). The biopolymer-drug interactions define a drugs' pharmacological fate. As such, there have been interdisciplinary efforts amongst fields such as medicine, pharmacology and biochemistry to develop methods for the identification and characterization of these interactions.

Recent years have demonstrated a profound development within the study of enzyme-drug interactions. The understanding of how enzymes react with drugs and bring about chemical changes *in vivo* is a key factor for the determination of drug pharmacodynamics and pharmacokinetics, and is also important in the development of new therapeutic agents. The conventional uses of enzymes within many fields have been based upon enzymes in their soluble forms (Dixon et al., 1979). *In vivo* most enzymes are naturally found within a cellular matrix and can be membrane bound. Consequently, many previously reported *in vitro* assays that utilize solubilized enzymes are not a true reflection of what is occurring *in vivo*. This has naturally led to the development of immobilized enzymes.

Although many methods for assaying enzymes have been developed, High Performance Liquid Chromatography (HPLC) has become an increasingly popular method of choice due to its ability to accommodate the need for increased sensitivity and versatility (Lough et al., 1995). In particular, biochromatography is a unique method in that it utilizes immobilized biopolymers and HPLC techniques in order to study drug-biopolymer interactions. It has been shown that on-line chromatography

applying an immobilized enzyme reactor coupled to an analytical column allows for an ideal reflection of biological processes and quantitation of enzyme/substrate interactions (Alebic-Kolbah and Wainer, 1993a).

Many research groups have demonstrated the uses of immobilized enzyme reactors to follow and characterize metabolic and biosynthetic processes. The various methods of immobilization and their applications will be discussed in this review. The focus of this thesis is on the two catecholamine-system enzymes, dopamine beta-hydroxylase and phenylethanolamine N-methyltransferase and the importance of developing IMERs based upon these enzymes in order to characterize and follow the synthesis of catecholamines.

1.2. Catecholamines

Catecholamines are naturally occurring compounds that act as both hormones and neurotransmitters. The principal catecholamines, are dopamine (DA), norepinephrine (NE) and epinephrine (EP). They are highly polar compounds that resemble each other chemically by the presence of an amino and a catechol group. These compounds possess a wide range of biological activities with vital roles in numerous physiological processes. The differences in their activities are attributed to the physico-chemical properties of their side chains (Schusler-Van Hees et al., 1980).

Catecholamines are located in cells in the adrenal medulla and in the central and sympathetic nervous system localized in distinct regions of the brain and ganglion (Kobayashi et al., 1992). The adrenal medulla is a gland containing chromaffin cells, which are specialized cells that manufacture, store and secrete NE

or EP. It is controlled by nerves in the spinal cord and releases the catecholamines directly into the bloodstream eliciting a widespread response within the body (Carmichael et al., 1985).

Based upon the wide distribution of catecholamines, researchers have demonstrated their involvement in normal functions including heart stimulation, motor control, gastric motility and blood flow (Elenkov et al., 2000; Tani et al., 1982). These compounds are also involved in the stimulation of the autonomic nervous system in preparation of the bodies' "fight or flight" response to colds, fatigue and shock. Numerous neurological diseases (Alzheimer's disease, Schizophrenia, Manic depressive illnesses, and Parkinson's disease) are also associated with improper catecholamine regulation (Elenkov et al., 2000; Friedman et al., 1999).

Wide ranges of drugs that alter catecholamine function are used in various clinical settings. Knowledge of the catecholamine mechanisms of action is of fundamental importance in the development of new therapeutic agents for the various disease states. Many studies in the literature have examined the potential sites for drug intervention in catecholamine synthesis, storage, release and metabolism.

1.2.1. Biosynthesis

The catecholamines, DA, NE and EP share a common synthetic pathway (Figure 1.1). The pathway has been extensively studied and detailed kinetic analysis, substrate specificity, and cofactor requirements of the enzymes have been determined. Antibodies against the enzymes have allowed for the determination of their location

by immunohistochemical techniques (Liposits et al., 1986). Catecholamines are synthesized from the precursor amino acid, L-tyrosine. L-tyrosine is taken up from the circulation into catecholamine secreting neurons and adrenal medullary cells by an active transport mechanism. It then undergoes a series of chemical transformations resulting in the formation of DA, NE and EP.

Tyrosine hydroxylase (TH) is the first enzyme in the pathway, which catalyzes the formation of 3,4 dihydroxyphenylalanine (DOPA), from L-tyrosine (Nagatsu et al., 1964). TH is present in the adrenal medulla, sympathetically innervated tissues and in all catecholaminergic neurons. TH is stereospecific such that the enzyme oxidizes L-tyrosine and L-phenylalanine whereas D-tyrosine does not serve as a substrate. TH requires molecular oxygen, ferrous iron atom and tetrahydropteridin as a cofactor (Almas et al., 1996). It has been shown to display a high degree of substrate specificity and catalyzes the rate-limiting step of catecholamine biosynthesis (Nagatsu, 1995). Inhibitors of TH include amino acid analogues catechol derivatives, tropolones and iron chelators (Cooper et al., 1996).

The TH human gene has been cloned and the single gene shown to encode multiple mRNAs (Grima et al., 1987; Nagatsu, 1991). TH is under the control of numerous and complex mechanisms (Nagatsu, 1991). Pharmacological intervention of this rate-limiting step has proven to result in the decrease in the endogenous levels of dopamine and norepinephrine in various tissues. Regulation of TH activity can be achieved by long-term and short-term regulation mechanisms. Short-term regulation can result from the activation of TH by feedback inhibition or by reversible phospho-

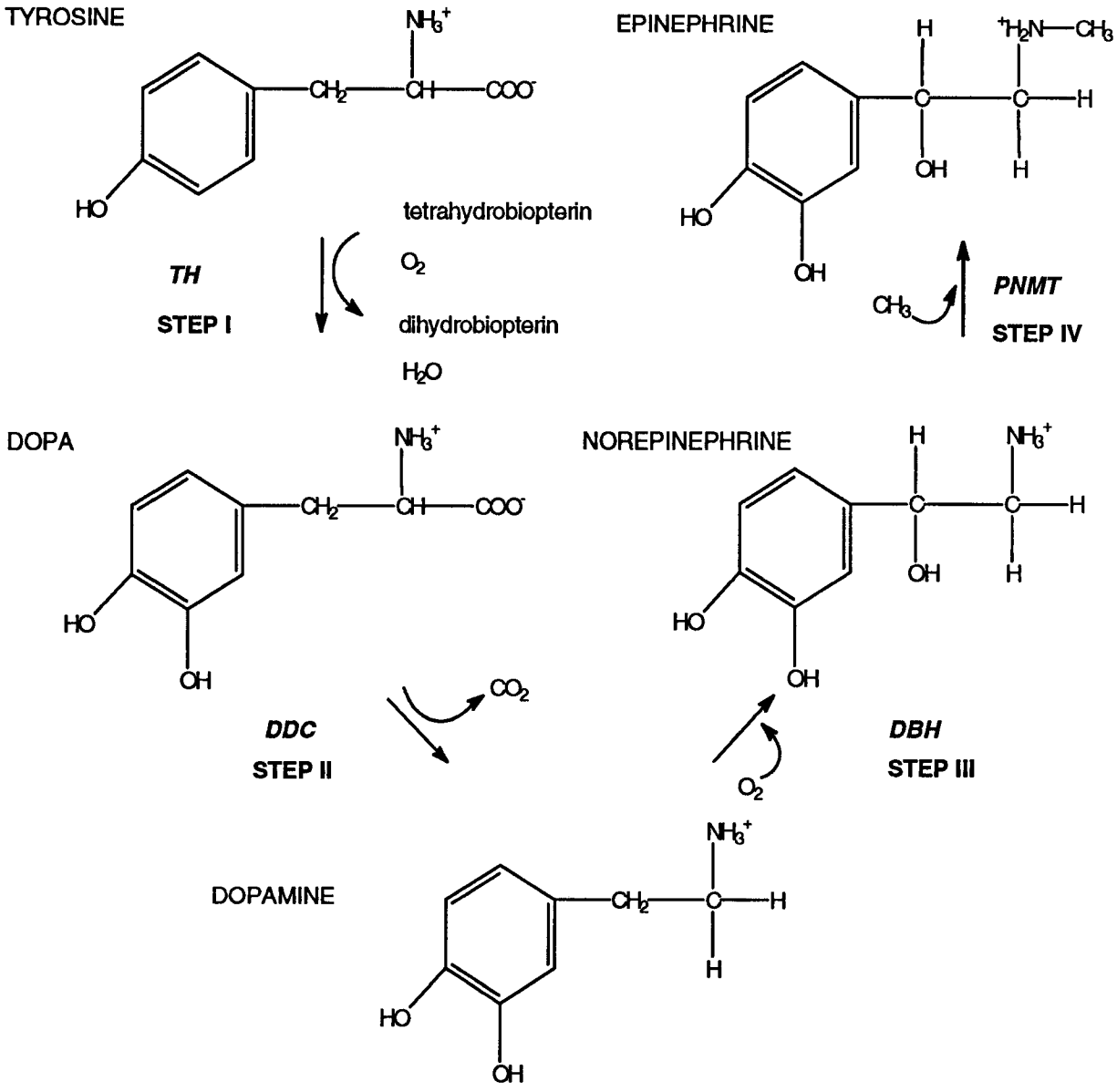

Figure 1.1: Pathway of the biosynthesis of catecholamines.

rylation of serine residues (Meloni et al., 1998; Ramsey et al., 1998). Long-term regulation involves alterations in the rate of TH synthesis and TH degradation. For instance, TH activity is increased several folds following prolonged stress or drug treatment (Gilad and McCarty, 1981).

The second step in the pathway involves the decarboxylation of DOPA to dopamine. Dopa decarboxylase, which is located in the cytoplasm of cells, is responsible for dopamine synthesis. The enzyme is found in the adrenal medulla, catecholaminergic neurons and in tissues such as liver, kidney and gastrointestinal tract (Cooper et al., 1996). Relative to the other catecholamine enzymes dopa decarboxylase is present in excess and requires pyridoxal phosphate (vitamin B6) as a cofactor. The enzyme displays broad substrate specificity. Based upon the enzyme's ability to catalyze the various L-amino acids such as DOPA, histidine and tryptophan, dopa decarboxylase was appropriately renamed to L-amino acid decarboxylase (AADC) (Voltattorni et al., 1983). The D-isomers have been shown to bind the active site and inhibit the decarboxylation (Voltattorni et al., 1983).

Aromatic amino acid decarboxylase has been purified from various tissues such as pig, rat, bovine adrenals and human pheochromocytoma (Moore et al., 1996; Boomsma et al., 1986; Ichinose et al., 1985). Some therapeutic regimens have proven successful due to the pharmacological intervention of AADC. For instance, the slow degeneration of dopaminergic neurons results in the movement disorder, Parkinson's disease. The clinical features of this disorder are alleviated and dopaminergic activity restored by the administration of L-dopa and dopamine agonists (Katzung, 1995).

NE is synthesized in the vesicles and granules by dopamine β-hydroxylase (DBH) and then released by exocytosis. In nerve cells, NE is released during nerve stimulation. NE is then methylated by phenylethanolamine N-methyltransferase, PNMT, the final enzyme in the pathway. This thesis focuses on the final two enzymes in the pathway, dopamine β-hydroxylase (DBH) and phenylethanolamine N-methyltransferase (PNMT).

1.2.2. Metabolism

Similar pathways metabolize the catecholamines NE, DA and EP. The main metabolizing enzymes are monoamine oxidase (MAO) and catechol-O-methyltransferase (COMT). MAO exists in two forms MAO-A and MAO-B. These isoenzymes display differences in substrate and inhibitor specificities (Geha et al., 2001). MAO-A is located throughout the body and predominantly in the gastrointestinal tract. This enzyme is specific for norepinephrine and serotonin whereas the isoenzyme MAO-B displays broad specificity acting on numerous phenylethylamines (Sablin et al., 1998). COMT, the second metabolizing enzyme is relatively non-specific (Mannisto et al., 1989; Percy et al., 1999). The enzyme is located in the cytoplasm and is abundant in the liver and kidney. The enzyme subjects the deaminated metabolites produced by MAO and any surrounding catecholamines to O-methylation (Percy et al., 1999).

MAO transforms NE, EP and DA into their corresponding aldehydes while COMT catalyzes the transfer of methyl groups from S-adenosyl-L-methionine to the hydroxyl group of the catecholamines. For example, within dopaminergic cells DA is

metabolized by MAO to produce 3,4 dihydroxyphenylacetic acid (DOPAC). DOPAC is then released from the cells and transformed by COMT to homovanillic acid (HVA) (Mannisto et al., 1992). Similarly any DA that has been released upon synthesis into the cytosol can be O-methylated by COMT followed by oxidation by MAO to yield HVA (Kuczensky and Segal, 1992).

1.3. Dopamine β-hydroxylase

Dopamine β-hydroxylase (DBH) catalyzes the third step in the catecholamine biosynthetic pathway. DBH is a copper-containing protein present in higher eukaryotes and is responsible for the production of NE. DA, which is achiral, is converted to R-NE. L-NE is also designated R-NE using Cahn-Ingold-Prelog configurations. The enzyme's stereospecificity is demonstrated through the removal of the pro-R benzylic hydrogen (DeWolf et al., 1989; Wimalasena et al., 1999) (Figure 1.2).

DBH is a mixed function oxidase requiring ascorbic acid and molecular oxygen for activity. It reduces one oxygen atom to water and inserts another into its substrate (Robertson et al., 1990). The enzyme is situated within catecholamine-containing granules and vesicles in contrast to the other enzymes, which are present in the cytoplasm (Wong et al., 1990; Wimalasena et al., 1991). DBH activity is highest in the adrenal medulla and in tissues such as the heart and the spleen reflecting peripheral sympathetic activity. The enzyme also appears in cerebrospinal fluid from the brain.

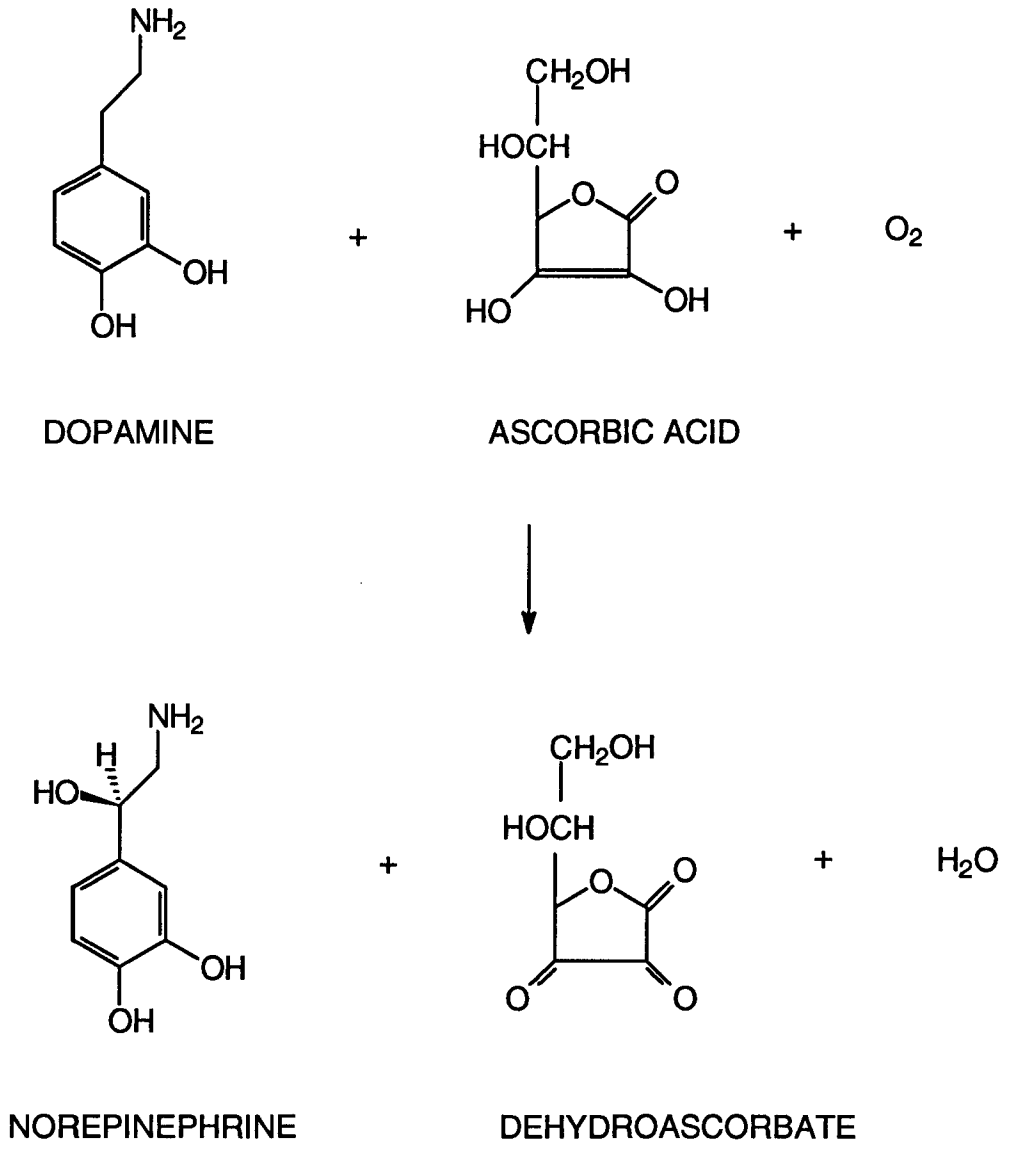

Figure 1.2: Stereospecific reaction of dopamine beta-hydroxylase (DBH).

Dopamine and ascorbic acid are required *in vivo* for activity. The enzyme however does not display a high degree of substrate specificity, converting any phenylethylamine to the corresponding phenylethanolamine *in vitro* (Van der Schoot and Creveling, 1965; Farrington et al., 1990). Although, ascorbic acid is the physiological reductant of the reaction, other reducing agents such as ferrocyanide and N-substituted phenylenediamines have served as ideal reductants in many assays. Benzylhydrazine, benzyloxyamine, and a variety of chelating agents such as tropolone and disulfiram are known inhibitors of DBH. Assaying DBH activity in tissues proves to be difficult at times due to the presence of endogenous inhibitors in the enzyme preparation (Molinoff and Axelrod, 1971). Inhibition of DBH by sulfhydryl compounds such as cysteine and glutathione is due to the chelation of the copper atom located in the enzymes' active site (Nagatsu et al., 1967). The effect of these inhibitors can be minimized by the addition of sulfhydryl reagents such as N-ethylmaleimide to assay mixtures. Numerous assays have been reported for assessing DBH activity. Extensive studies have been carried out in order to describe the physical and chemical characteristics of DBH.

1.3.1. Structure

DBH is a copper-dependent glycoprotein with a molecular weight of 290 kDa (Ishii et al., 1991). The enzyme consists of four subunits. Disulfide bonds join two of the monomers and the resulting dimers are noncovalently attached to one another to form a tetramer (Robertson et al., 1994). Analyses of disulfide bonds in bovine DBH have revealed fourteen cysteine residues and seven disulfides per

monomer. Molecular mass spectrometric studies have confirmed one intermolecular and six intramolecular disulfide linkages for each monomer (Robertson et al., 1994). Five of the intramolecular disulfide linkages display high densities of histidine residues. Numerous studies have been carried out to investigate the four subunits of DBH. Molecular weights ranging from 66 to 77 kDa have been reported for the individual subunits (Wong et al., 1990; Sabban et al., 1983). Studies on bovine adrenal medullary DBH demonstrate the presence of three subunits: alpha, beta and gamma that combine in a 1:2:1 ratio to form the tetramer.

Copper ions have been shown to bind to DBH and be essential for catalytic activity. The copper is not tightly bound as in other metalloenzymes (Skotland and Flatmark, 1979). It can be easily removed and then reintroduced allowing for the recovery of activity. Binding studies have displayed the presence of 8 Cu^{2+} per tetramer (Robertson et al., 1990; Ash et al., 1984). However, the amount of copper necessary for catalysis remains a matter of ongoing discussion (Abudu et al., 1998). The literature has focused on whether 2 Cu/subunit or 1 Cu/subunit is involved in the catalytic process. Spectroscopic analysis has demonstrated 3-4 histidines to be coordinated to each copper in DBH (Hasnain et al., 1984; Blackburn et al., 1991). The histidine rich areas comprise the enzyme's catalytic site, which is situated in the interior surface of the membrane (Grouselle et al., 1982).

DBH exists in two forms: a membrane-bound form (mDBH) that is reinternalized upon exocytosis and a soluble form (sDBH) that is stored in the granule and secreted (Ledbetter et al., 1981; Helle et al., 1984). Both forms of the enzyme have been purified and the human, bovine, rat and mouse genes have been cloned

(Kobayashi et al., 1989; McMahon et al., 1990; Nakano et al., 1992; Wang et al., 1990). The two forms display differences in pH stabilities and substrate affinities. Amino acid analysis of bovine and human DBH displays high portions of glutamic acid, aspartic acid, asparginase, glycine and leucine (Lamoureux et al., 1987; Wong et al., 1990). Sequence analysis of human DBH has revealed four consensus sequences for glycosylation (Robertson et al., 1990; Wong et al., 1990). mDBH is ampiphilic and sDBH is hydrophilic in nature. mDBH in contrast to sDBH, consists of a portion of hydrophobic amino acids which act as an anchor in the chromaffin granule. The anchor traverses the membrane with the remainder of the enzyme located in the outer surface of the membrane (Blakeborough et al., 1981).

1.3.2. Mechanism of action

Kinetic isotope effects and steady state kinetic studies of DBH have proven to be useful in eliciting the enzymes' mechanism of action. DBH proceeds by a ping-pong mechanism and is highly stereospecific (Njus et al., 1987). The conversion of dopamine to epinephrine has been shown to be copper, ascorbate and oxygen dependent. Ascorbate acts as the reductant for DBH and releases a free radical prior to substrate and dioxygen binding. Ascorbate can lose two electrons however DBH utilizes it as a one-electron donor resulting in the formation of the free radical semidehydroascorbate (Diliberto & Allen, 1981; Njus et al., 1987; Skotland and Ljones, 1980). Ascorbate and semidehydroascorbate are anionic and do not readily diffuse across the membrane. Loss of both electrons would result in the production of dehydroascorbate. Dehydroascorbate however is neutral, diffuses easily across the

membrane and can be hydrolyzed. Overall, production of dehydroascorbate would be wasteful for organelles.

Secretory vesicles and chromaffin granules possess an advantageous mechanism for the regeneration of internal ascorbic acid and the ability to maintain redox equilibrium between cytosolic and intravesicular ascorbic acid. Many researchers have demonstrated that a membrane bound protein cytochrome b_{561} acts as a transmembrane electron donor (Flatmark and Gronberg, 1981; Harnadek et al., 1985; Njus et al., 1983). Cytochrome b_{561} has been identified in synaptic vesicles from rat tissue (Srivastava, 1998) and in bovine splenic nerves (Flatmark and Gronberg, 1981). It is hydrophobic consisting of 273 amino acids which span the membrane six times (Perin et al., 1988). Cytochrome b_{561} contains two heme prosthetic groups that are located on both sides of the membrane and in close proximity (Kobayashi et al., 1998). Its oxidation-reduction cycle does not involve interaction with any other proteins. Instead, semihydroascorbate acts as an electron acceptor inside the vesicles or granules and ascorbate serves as an electron donor in the cytosol. Cytochrome b_{561} transfers electrons from the cytosol to regenerate internal ascorbic acid (Harnadek et al., 1985).

ATP in the cytosol is broken down by ATPase into ADP and inorganic phosphate resulting in the release of energy and the pumping of protons across the membrane of the chromaffin vesicle. A proton gradient drives the transport of dopamine into the vesicle to be acted upon by DBH. Ascorbic acid loses an electron becoming semidehydroascorbate and reduces the enzyme. Cytochrome b_{561} transfers electrons into the vesicles from the cytosol and renews ascorbic acid and allows for

NE synthesis to proceed (Figure 1.3). A copper-peroxo species, Cu(II)-O-O-H, has been shown to be responsible for the abstraction of the H atom from the benzylic position of DA. Instantaneously, the O-O bond is cleaved giving rise to Cu(II)-O· species, water and a substrate-radical species (Ahn et al., 1983; Miller and Klinman, 1985). The Cu-O· and the DA-radical species combine quickly to form an alkoxide that is copper bound. Hydrolysis generates the release of the product from the active site. The pro-R benzylic hydrogen of the substrate has been shown to be accessible to the copper-oxygen species in the active site whereas the pro-S hydrogen is not accessible. The enzymes absolute stereospecificity is due to the accessibility to the benzylic hydrogen (Taylor, 1974).

1.3.3. Regulation

The human DBH gene has been mapped to chromosome 9q34 (Craig et al., 1988). It is encoded by a single gene copy consisting of twelve exons and with an approximate length of 23 kb. cDNAs of DBH have been cloned from human pheochromocytoma (Kobayashi et al., 1989), rat pheochromocytoma (McMahon et al., 1992) and bovine adrenal glands (Lewis et al., 1990). Both *in vivo* and *in vitro* studies have been carried out in order to identify and characterize the regulatory mechanisms of DBH gene expression (Kim et al., 2001). Hormones, stress and growth factors have been shown *in vivo* to elevate DBH expression (Sabban et al., 1995; Sabban et al., 1998). Similarly, in cultured cells elevations of DBH expression

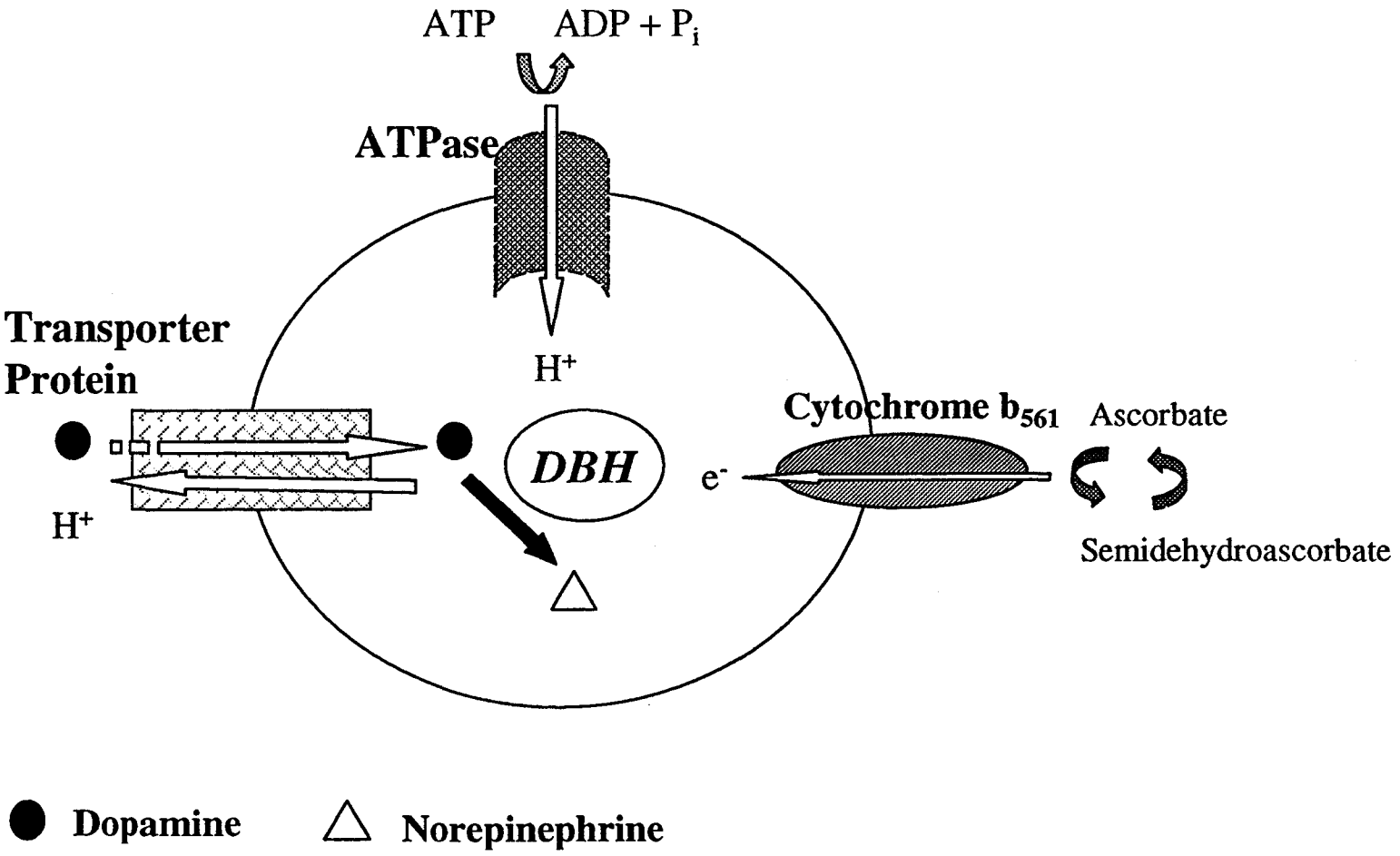

Figure 1.3: Mechanism of action of dopamine beta-hydroxylase within a chromaffin cell.

have been viewed upon treatment with nicotine, NGF, bradykinin and glucocorticoids (Acheson et al., 1984; Gebreyesus et al., 1993; Hwang et al., 1993).

The rat and human DBH promoters have been characterized utilizing Dnase I footprinting and site-directed mutation analysis (Ishiguro et al., 1993; Yang et al., 1998; Sabban et al., 1998). Two cis-acting regulatory elements have been identified in noradrenergic cell lines. They are responsible for the cell-specific promoter activity of the DBH gene (Seo et al., 1996; Kim et al., 1998; Ishiguro et al., 1998). Several transcriptional regulatory elements have been identified in the DBH gene. They include the activator protein-2 (Greco et al., 1995), the cyclic AMP-responsive element (Ishiguro et al., 1995), glucocorticoid responsive element (Kobayashi et al., 1989), and insulin-like growth factor I (Hwang et al., 1995). Interestingly, the transcription factor AP-2 has been demonstrated to be essential in specifying and maintaining the differential phenotype of biosynthetic enzymes. For example AP-2, interacts specifically with the promoter elements of the TH and DBH genes in noradrenergic cell (Kim et al., 2001). Within the DBH gene, the 4kb 5'-flanking region is responsible for tissue specific expression. Multiple proteins bind to this area thus coordinating the activation of the specific cell-type transcription of the DBH gene (Seo et al., 1996).

A microsatellite repeat (GT)n, has been identified at the human DBH gene locus and five alleles have been identified (Porter et al., 1992). Investigations of the allelic fragments have revealed that the gene is controlled by a co-dominant mechanism associated with the repeat (Wei et al., 1998). A single amino acid change between Ala and Ser at nucleotide 910 (G to T) displays a decreased DBH activity

(Ishii et al., 1991). Extensive examination of the gene locus has lead to the identification of various disorders that are associated with DBH.

1.3.4. Disorders associated with DBH

A novel approach used to study the clinical significance of DBH has been through the use of genetically altered mice. Targeted disruption of the DBH gene has revealed the various roles the enzyme possesses *in vivo*. Thomas and Palmiter displayed the importance of DBH in reproduction (Thomas and Palmiter, 1998). Their investigations revealed that most of the DBH -/- mice died in utero (Thomas et al., 1995). The small proportion that survived was due to the presence of maternal catecholamines that can cross the placenta. DBH was also shown to be essential in the developmental stages as well as in the retention of certain behaviors (Nagatsu and Stjarne, 1998). For instance, changes in maternal behavior have been shown to be due to DBH deficiency. Deficient females abandon their litters whereas normal females retrieve their pups. Administration of dihydroxyphenylserine (DOPS), a precursor of NE independent of DBH, in the mothers' drinking water prior to and the day after birth resulted in the mothers' acceptance of her pups (Thomas and Palmiter, 1998).

NE is also important in energy balance, thermoregulation, immune regulation, and cardiovascular control (Alaniz et al., 1999; Thomas and Palmiter, 1997). DBH -/- mice were more susceptible to infections in comparison to normal mice (Alaniz et al., 1999). When mice were housed in pathogen-free environments they appeared normal. However, upon infection with *Mycobacterium tuberculosis* the DBH-/- mice displayed impaired T-cell function and became susceptible to infection (Alaniz et al.,

1999). Similarly, NE involvement in cold acclimatization was demonstrated with the mutants' inability to adapt to cold temperatures (Thomas and Palmiter, 1998).

DBH deficiency results in a decrease in NE and EP levels and an accumulation of DA and L-dopa in urine, plasma and CSF. Changes in DBH activity are also reflected in metabolite changes. HVA and 3-methoxytyramine displays increased levels whereas VMA decreases with decreased levels of NE. DBH deficiency is diagnosed through the measurement of NE/DA ratio. Normal patients display a ten-fold difference from patients with DBH deficiency. Patients with pheochromocytoma have displayed increased blood levels of DBH (Nagatsu, 1986). Interestingly upon removal of the tumor the DBH levels are shown to decrease. The main biochemical markers for Parkinson's disease have been decreases in TH and tetrahydrobiopterin concentrations however DBH has also been show to decrease in these patients (Nagatsu et al., 1984; Hurst et al., 1985).

Additional disorders that have been identified and correlated with DBH deficiency include: spontaneous abortions, hypoglycemia, hypotension, ptosis of eyelids and occasional syncope (Robertson et al., 1991). Appropriate treatment of DBH deficiency has been challenging. Treatments utilizing compounds such as phenylpropanolamine, tranylcypromine and metyrosine gave rise to adverse effects including paranoid thinking and increases in blood pressure reaching levels characteristic of hypertension (Robertson et al., 1991). Extensive understanding of NE pharmacology and DBH activity will allow for effective therapeutic intervention in many of the above mentioned disorders.

1.4. Phenylethanolamine N-methyltransferase

The terminal enzyme in the catecholamine biosynthetic pathway is phenylethanolamine N-methyltransferase (PNMT). PNMT catalyzes the transmethylation of NE requiring S-adenosyl-L-methionine (SAM) as a methyl donor (Pendleton and Snow, 1973). The reaction results in the production of epinephrine (EP) and S-adenosyl-L-homocysteine (SAH) (Grunewald et al., 1996) (Figure 1.4). The enzyme is primarily situated in chromaffin cells of the adrenal medulla as well as in discrete regions of the brain (Park et al., 1986). Depending upon its location, EP is synthesized to function as either a hormone or as a neurotransmitter. PNMT activity has been reported in the adrenal medulla of a rabbit, rat, monkey, cow, pig, frog, mouse, dog and snake (Park et al., 1986).

Its substrate specificity has been extensively investigated, and the enzyme has been shown to display poor substrate specificity. However, the enzyme requires that a hydroxyl group at the β position of the ethyl side chain be present on the substrate for reaction (Grunewald et al., 1996). Commonly used phenylethanolamine derivatives for assaying PNMT include norepinehrine, normetanephrine, synephrine, octopamine, metanephrine and epinephrine. PNMT is inhibited by its own substrates and products *in vitro* (Borchardt et al., 1976). Phenylethylamines, benzylamines and a variety of sulfhydryl reagents such as p-chloromercuribenzoic acid and mercury are known inhibitors of PNMT. Assaying PNMT activity proves to be difficult due to the presence of endogenous inhibitors in the preparations. The effect of these inhibitors is overcome by the administration of MAO inhibitors such as pargyline (Molinoff et al., 1969). Radiochemical and HPLC methods are the two methods of choice to assay

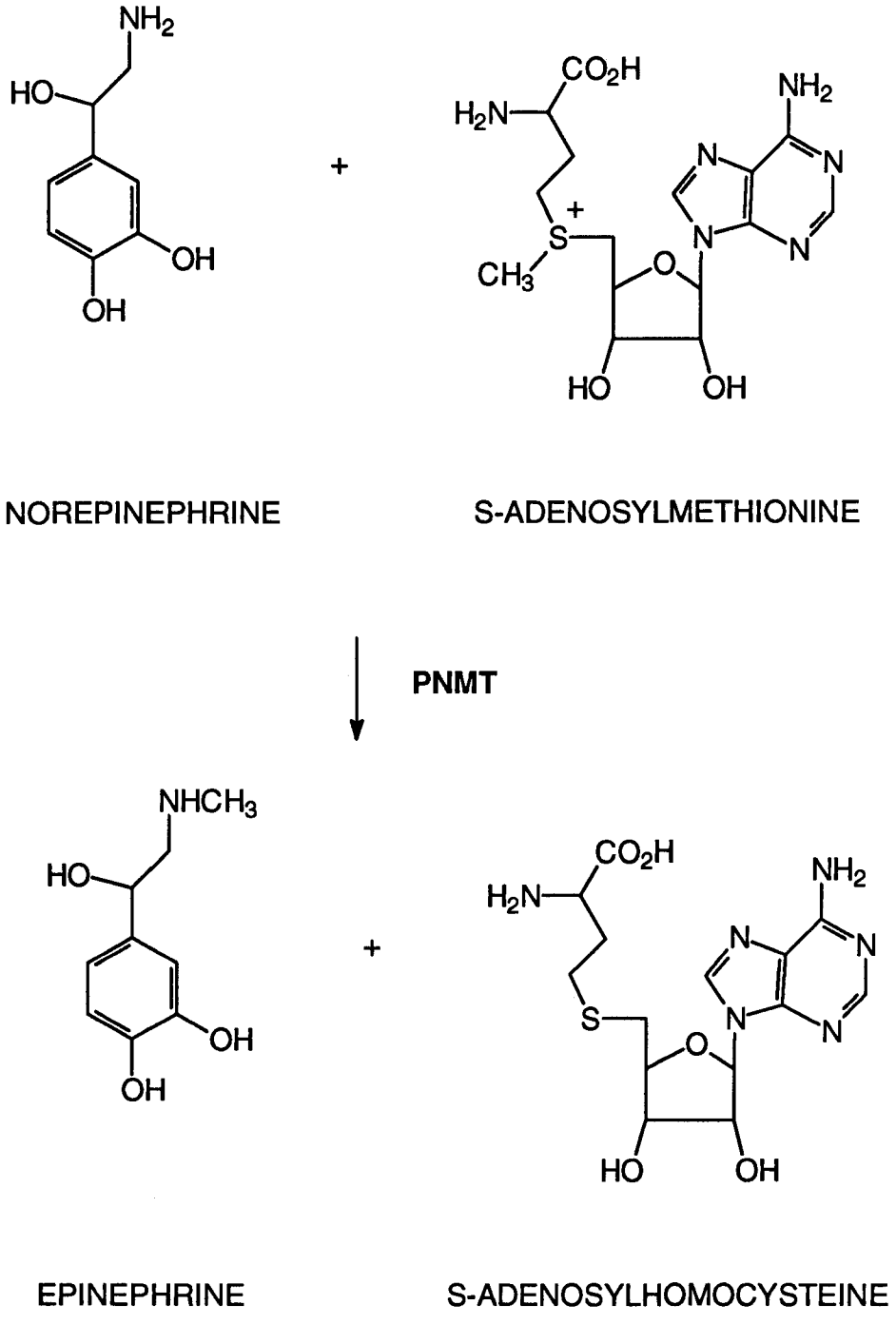

Figure 1.4: The final step in the biosynthesis of epinephrine.

PNMT in various biological matrices (Lee et al., 1985). The physical and chemical characteristics of PNMT have been elucidated utilizing these and many other techniques.

1.4.1. Structure

PNMT has been isolated and purified from various species and molecular weights ranging from 30 to 40 kDa have been reported (Connett and Kirshner, 1970; Park et al., 1982, Kaneda et al., 1998). It is a monomeric protein that has similar features to other N-methyltransferases such as nicotinamide N-methyltransferase (Kaneda et al., 1998). PNMT from different species have been shown to differ in charge and among some species there are multiple forms of the enzmye (Park et al., 1982; Joh and Goldstein, 1973).

The PNMT structure is primarily composed of parallel beta sheets. The PNMT active site consists of a polar amine binding area and two lipophilic pockets (Grunewald et al., 1988). Complete nucleotide sequences of bovine, mouse, rat and human PNMT have been determined (Baetge et al., 1986; Weisberg et al., 1989; Morita et al., 1992). Their amino acid sequences display a shared homology of approximately 80%. Human and mouse PNMT encode 282 and 295 amino acids respectively. In contract to bovine and human PNMT, mouse PNMT has a different C- and N-terminus. The N-terminus of mouse PNMT has direct repeat sequences which are lacking in human and bovine PNMT (Morita et al., 1992). These sequences are believed to be involved in gene variation and may have been removed during evolution.

Reagents that modify cysteine, tyrosine, arginine, lysine and histidine residues have proven useful in elucidating PNMT's structure and function. Modification of lysine residues does not affect PNMT activity however modified cysteine residues have a direct effect (Caine et al., 1996). Cysteine residues have been implicated in a number of other small molecule methyltransferases (Fujioka, 1992). Studies with p-chloromercuribenzoate, a chemical reagent specific for cysteine residues, have demonstrated the importance of cysteine residues for PNMT activity. Primary amino acid sequence analysis of human PNMT has revealed six cysteine residues at positions 48, 69, 91, 131, 139 and 183 (Kaneda et al., 1988). Through the use of site-directed mutagenesis, Kaneda et al. replaced each cysteine residue with a serine and produced mutants in *Escherichia coli*. Their findings located the active site of PNMT in the C-terminal region and concluded that Cys 183 was located in the active site and essential for activity. Comparisons to bovine and mouse PNMT sequences have revealed Cys 183 to be a conserved residue among the different species.

1.4.2. Mechanism of action

PNMT converts NE to EP through the use of SAM as a methyl donor. This reaction is one of several in the body that utilizes SAM. Important methylation reactions *in vivo* include the methylation of DNA, conversion of guanidinoacetate to creatine and conversion of acetylserotonin to melatonin (Hoffman, 1984; Itoh et al., 1997; Jenne et al., 1997). Methionine is a dietary source of methyl groups. The adenosyl group of ATP is transferred to the methionine sulfur group resulting in the formation of SAM. Methyl groups attached to the sulfur group of SAM can be

transferred to a nitrogen, oxygen or carbon atom of an acceptor molecule that yields the methylated product and SAH. SAH is then hydrolyzed to adenosine and homocysteine.

The PNMT reaction is believed to proceed through ordered sequential binding (Grunewald et al., 1996). SAM binds followed by NE (Pendleton et al., 1973). Quantitative structure-activity relationship studies have proven useful in elucidating the required conformation of the aminoethyl side chain of the substrates of PNMT (Grunewald et al., 1988). They have indicated the presence of a compact hydrophilic pocket within the aromatic ring-binding region of the enzyme's active site (Sall and Grunewald, 1987). A coplanar relationship exists between the amine nitrogen, the aromatic ring and the active site. Once epinephrine is formed it returns to the chromaffin granule for storage (Burke et al., 1983). The cells release EP into the bloodstream where it is capable of acting on the liver, skeletal muscle and adipose tissue.

1.4.3. Regulation

The human PNMT gene has been assigned to chromosome 17 and shown to encode a single gene (Kaneda et al., 1988). It is composed of three exons and two introns and spans a length of 2kbp. In the 5' flanking regions regulatory sequences have been identified that represent the Sp1 binding site, TATA box, glucocorticoid response element and a polyadenylation signal (Kaneda et al., 1988; Koike et al., 1995). PNMT is regulated by factors such as cell specific determinants of expression, glucocorticoids, cholinergic stimuli, phorbol esters and forskolin (Nagatsu and

Stjarne, 1998; Wan et al., 1991; Evinger et al., 1994). These factors have been shown to increase steady-state levels of PNMT mRNA. The levels have been shown to increase in epinephrine rich cells with no visible effect in norepinephrine cells (Cahill et al., 1996).

Glucocorticoids have been shown to maintain PNMT activity *in vivo* and in cultured cells (Wurtman and Axelrod, 1966; Betito et al., 1992). They maintain the levels of methionine adenosyltransferase and SAH hydrolase such that more SAM is available for PNMT (Wong et al., 1992). PNMT activity in hypophysectomized rats is drastically decreased. Axelrod and Wurtman displayed the restoration of PNMT activity with the administration of dexamethasone, a synthetic glucocorticoid (Wurtman and Axelrod, 1966). Neuronal regulation of PNMT is viewed through the action of cholinergic stimuli on muscarinic and nicotininc receptors. PNMT expression is enhanced by the independent action of nicotinic and muscarinic agonists. Factors affecting PNMT regulation have been identified and their roles in clinical disorders observed.

1.4.4. Disorders associated with PNMT

Hypertension is a polygenic disease. PNMT plays a role in blood pressure homeostasis (Reis et al., 1988). The ability of PNMT inhibitors to lower blood pressure in spontaneously hypertensive rats has been widely investigated (Saavedra, 1979). PNMT inhibitors reduce central epinephrine levels however they are non-selective and demonstrate α_2-adrenoceptor binding affinity (Toomey et al., 1981). The effects of the PNMT inhibitors investigated have proven to be ambiguous based

upon their display of α_2-adrenoceptor affinity. Pheochromocytomas are common in the adrenal medulla. These benign tumors induce an increase in the secretion of epinephrine. This results in increases in the heart rate, headaches, palpitations and weight loss. Therefore the importance in developing potent and selective PNMT inhibitors will prove vital in the effective intervention of these disorders.

1.5. Immobilized Enzymes

Enzymes are complex proteins that are involved in a variety of chemical transformations. These molecules accelerate chemical reactions within living cells through a process that involves the formation of enzyme-substrate complexes. These complexes lower the kinetic and energetic barriers associated with a chemical transformation and result in product formation. This process is illustrated in Figure 1.5.

In the body, enzymes mediate a variety of processes ranging from digestion to synthesis to degradation. The biological importance of enzymes and their wide utility have made them primary targets for the medical, industrial and analytical fields. Indeed, there have been numerous advances in the isolation, production and purification of enzymes, which have resulted in the development of the field of enzyme technology.

In the past the conventional uses of enzymes within various scientific fields were based upon enzymes in their soluble forms (Katchalski-Katzir, 1993). Even with the enzymes' high efficiency and specificity there are numerous disadvantages and limitations. Enzymes can be costly, unstable, and difficult to recover from reactions

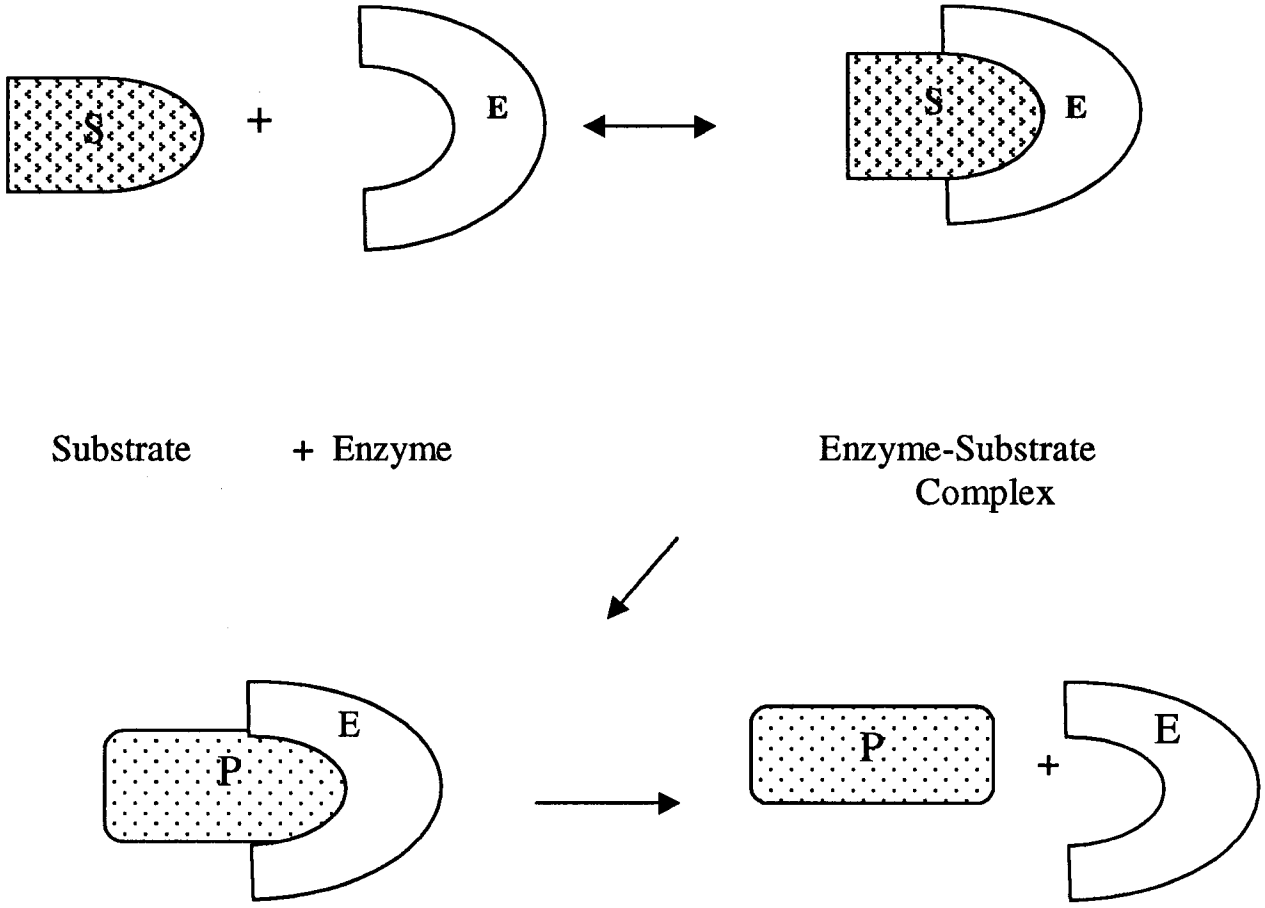

Figure 1.5 : Simplest model accounting for the kinetic properties of enzymes. E combines with S to form an ES complex and then proceeds to form a product, P.

and some are only available in minute amounts. Since enzymes are not altered during the reactions they catalyze, it would be beneficial if they could be reused. With the recent advances in biotechnology there has been an increased interest in the development of immobilized enzymes (Hoffman, 1990; Liang et al., 2000; Turner et al., 1987). Immobilization stabilizes enzymes by restricting their movement and allowing for their reuse.

1.5.1. Immobilized Enzyme Technology

The immobilization of biopolymers was initially reported in the 1960s and initiated their wide use in industrial and scientific fields (Silman et al., 1966). Technological developments in the immobilization of enzymes has allowed for the development of biosensors (Turner et al., 1987), industrial biocatalysts (Bull et al., 1999) and bioreactors in medicine (Hoffman, 1990) (Table 1.1).

1.5.1.1. Biosensors

Biosensors are defined as analytical devices that couple biological elements such as enzymes, antibodies and nucleic acids to transducers in order to monitor changes and/or the presence of substances (Turner et al., 1987). The coupling of the analyte (e.g. substrate) with the biological system (e.g. enzyme) and the transducer elicits a signal. Transducers can be potentiometric, aperometric, optical or physicochemical resulting in charge density, currents, light and enthalpy changes respectively (Wang, 2000).

The concept of a biosensor was first demonstrated in a study performed by Clark and Clark (1973) and involved the measurement of glucose concentration through the use of a glucose oxidase biosensor. Glucose oxidase was entrapped at an oxygen electrode. The amount of glucose formed was proportional to the measured decrease in oxygen concentration, which was detected amperometrically. These findings led to the use of glucose oxidase biosensors for the monitoring of diabetes as well as a wide variety of biosensors including cholesterol biosensors, immunosensors, and biosensors for the detection of gas toxicity (Campanella et al., 1995; Gil et al., 2000; Penalya et al., 1999; Vidal et al., 2000), Table 1.1.

1.5.1.2. Industrial and Pharmaceutical Biocatalysts

Biocatalysts and bioreactors are extensively used for the production of products and their facile isolation from reaction mixtures. In this process, enzymes are coupled to carriers using either physical or chemical methods. The advantages of the resulting solid supports include high specificity, high activity and high turnover number. The most frequently used operations in the industrial and medical fields are continuous fixed-beds and the stirred tank processes.

Numerous immobilized enzymes have been developed for use in industrial processes and in the medical sectors (Liang et al., 2000), and some examples are listed in Table 1.1. They have been found to be useful for the large scale-production of biologically active compounds. For example, immobilized lipases have proven useful for the production of optically pure compounds (Balcao et al., 1996; Rizzi et

Biosensors	**Application**	**Reference**
Glucose oxidase	Detection of glucose	Clark et al., 1973
Amine oxidase	Monitoring of biogenic amines	Niculescu et al., 2000
Tyrosinase	Detection of phenols	Liu et al., 2000
Biocatalysts		
Penicillin acylase	Production of 6-APV	Illanes et al., 1996
Lipases	Hydrolysis, glycerolysis, optically pure compounds	Lehmann et al., 2000 Yamane et al., 1998
Fumarase	Production of malic acid	Takata et al., 1993
Therapeutic Bioreactors		
Heparinase	Heparin neutralization	Freed et al., 1993
Urease	Analysis of blood urea	Kallury et al., 1992
L-asparginase	Cancer treatment	Inada et al., 1975
Bilirubin Oxidase	Treatment of jaundice	Mullon et al., 1989

Table 1.1: Typical applications of various biosensors, biocatalysts and therapeutic bioreactors.

al.,1992), penicillin acylase reactors are used for the production of 6-aminopenicillanic acid (Illanes et al., 1996) and immobilized aspartase has proven to be an ideal source of L-aspartic acid (Cheng et al., 1987).

1.5.1.3. On-line Immobilized Enzyme Reactors

Many of the above mentioned immobilized enzymes were used in straightforward batch-wise reactions, allowing for the easy detection and production of the target products. However, most biological systems are complex in nature and present complicated challenges. For example, many enzymes are cofactor dependent. The delivery and regeneration of cofactors are additional variables that have to be considered when placing enzymes in immobilized formats.

One approach to the use of immobilized cofactor dependent enzymes has been the development of on-line immobilized enzyme reactors (IMERs). IMERs can be used in standard high performance liquid chromatographic (HPLC) systems. In the IMER-HPLC system, cofactors can be delivered and regenerated as part of the circulating mobile phase. IMER-HPLC systems have been used for direct synthesis and purification (Alebic-Kolbah and Wainer, 1993a).

HPLC has been widely employed in enzymatic studies because it reduces analysis time, and the wide selection of detection methods eliminates the need for the use of radioisotopes. In addition, HPLC reduces the need for highly purified enzymes since secondary reactions can be detected and quantified (Lough et al., 1995). One of the main advantages of combining immobilized enzymes in an IMER format and HPLC is that the on-line IMER systems are easy to automate and control.

It has also been demonstrated that on-line chromatography applying IMERs allows for the ideal reflection of biological processes (Alebic-Kolbah and Wainer, 1993a). Studies with the IMER-HPLC systems have shown that the activities of the immobilized enzymes reflect the non-immobilized enzymes. Thus, IMER-HPLC systems can be used to carry out standard Michaelis-Menten enzyme kinetic studies and to quantitatively determine enzyme kinetic constants such as K_m and V_{max} (Thelohan et al., 1989). These systems can also be used to identify specific inhibitors, to provide information regarding the mode of inhibition and to calculate the K_i of the inhibitor (Alebic-Kolbah and Wainer, 1993a; Thelohan et al., 1989).

This is demonstrated by the IMER-HPLC system based upon alpha-chymotrypsin (ACHT) which was used to determine K_i's and other kinetic parameters of ACHT inhibitors including the inhibition mechanism (Alebic-Kolbah and Wainer,1993 b). Other IMER-HPLC systems based upon trypsin (Thelohan et al., 1989), lipase (Zhang and Wainer, 1993), alcohol dehydrogenase (Sotolongo et al., 1999) and beta-glucoronidase (Pasternyk Di Marco et al., 1998) have also demonstrated that the systems could be used for on-line synthesis and purification. Furthermore, the IMER containing beta-glucoronidase was also used for the analysis of urine samples containing chloramphenicol-beta-D-glucoronide (Pasternyk et al., 1998).

IMER-HPLC systems based upon non-solubilized rat liver microsomes have also been developed (Alebic-Kolbah et al.,1993). In addition, solubilized microsomal enzymes on cyanogen bromide-activated Sepharose beads including cytochrome P450 enzymes and UDP-glucuronyltransferase (Lehman et al., 1981) or just UDP-

glucuronyltransferase (Gilissen et al., 1992) have been developed. These systems were active and able to produce glucuronides as well as catalyze the N-demethylation of ethylmorphine and the O-demethylation of p-nitroanisole (Alebic-Kolbah et al., 1993; Lehman et al., 1981).

Interestingly, studies with an IMER containing the NADPH-dependent enzyme horse liver alcohol dehydrogenase (HLADH) indicated that the confluence of cofactor, substrate and supported protein does not pose any difficulties and that on-line co-factor regeneration can be accomplished (Sotolongo et al., 1999). The HLADH-IMER-HPLC system contained an NADPH regenerating system. The development of this system demonstrated the applicability of on-line immobilized enzyme reactors in the study of complex enzyme systems. The reproducibility and applications of IMERs are governed by the method of immobilization chosen and the properties of the support and of the enzyme.

1.5.2. Choice and Method of Immobilization

Numerous experimental methods and supports have evolved over the years for the immobilization of enzymes and other biopolymers. The various methods are classified into two categories, which are based upon the physical or chemical binding of the enzyme to the chosen support (Bickerstaff, 1997). Methods that have been investigated include adsorption, covalent binding, entrapment and cross-linking. (Figure 1.6). There is no ideal method of immobilization and as such the choice made is based upon examination of certain parameters.

METHODS OF IMMOBILIZATION

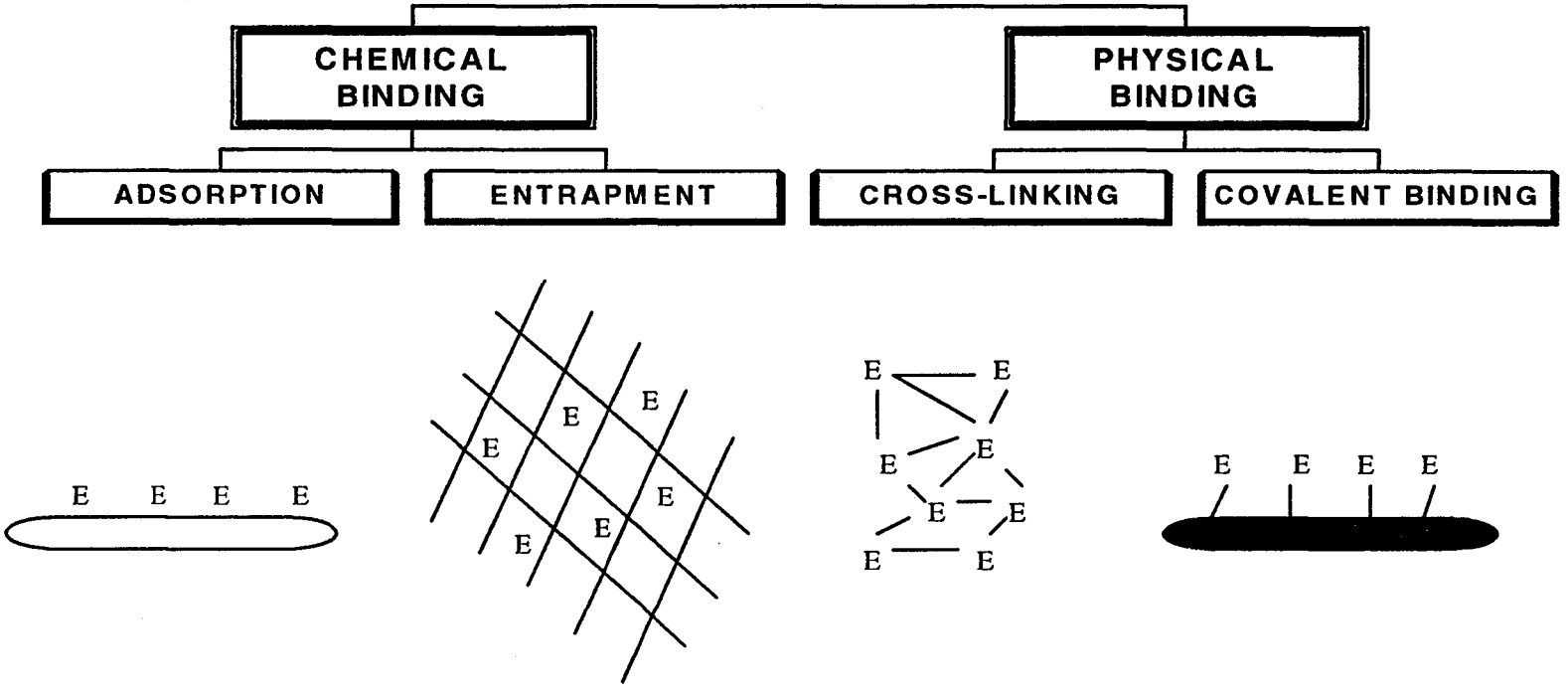

Figure 1.6: Representation of different methods of immobilization.

For instance, information concerning the chemical properties of the enzyme and the support are necessary. Knowledge of the active site is needed to avoid loss of enzymatic activity due to binding of the support to the reactive groups within the active site. The biochemical properties of the enzyme such as molecular mass, purity, and functional groups are among the characteristics concerning the enzyme, which will be important in assessing the method of choice (Bickerstaff et al., 1997). The functional groups, chemical stability and pore size and particle diameter of the support are equally important (Tischer and Kasche, 1999). Finally, the specific application of the immobilized enzyme must also be considered when choosing the correct method of immobilization. Each method of immobilization will have associated advantages and disadvantages.

1.5.2.1. Cross-linking

The first approach to immobilization was cross-linking (Silman and Katchalski, 1966). Cross-linking does not utilize a support. It involves the joining of the enzyme molecules to one another either by physical or chemical methods resulting in the formation of a three-dimensional structure. The chemical methods involve covalent bond formation with reagents such as glutaraldehyde or toluene dissocyanate. Physical cross-linking of enzymes is carried out by flocculations with agents such as polyamines and polystyrene sulfonates (Bickerstaff, 1997).

Cross-linking has been shown to increase enzyme stability. For example, lysozyme found in hen egg white, was cross-linked with glutaraldehyde. This technique resulted in a mechanically stable matrix (Marolia and D'ouza, 1993). In

addition, the cross-linked lysozyme exhibited about 6-times the lytic activity as compared to the non-immobilized form.

However, there are disadvantages associated with the cross-linking methods. For example, when chemical cross-linking is carried out there is the possibility that the reagents used may be toxic for the enzyme and will affect enzymatic activity. Also, some of the cross-linked enzyme may end up acting as a support, which can lead to decreased enzymatic activity. To overcome some of the disadvantages associated with cross-linking, this technique has been used in conjunction with other methods of immobilization with significant increases in the stability of the enzyme (Chernukhin and Klenova, 2000).

1.5.2.2. Adsorption

The simplest method of enzyme immobilization is physical adsorption (Bickerstaff, 1997). This method relies on the knowledge and use of the surface chemistry of the support and enzyme. In this approach, weak bonds are formed between the enzyme and the support. These interactions are governed by electrostatic forces including ionic interactions and van der Waals forces or by hydrogen bonding. The number of bonds formed is influenced by the pH utilized during immobilization and the isoelectric point of the enzyme (Mustranta et al., 1993). In a very simple manner the enzyme is mixed with the support at the appropriate pH and ionic strength for a pre-determined period of incubation. One advantage of this method is that the reaction is reversible and the support can be recycled by simply changing the pH, temperature or ionic strength.

Adsorption is a simple and fast method of immobilization, and it increases the enzyme stability relative to the soluble form of the enzyme. An example of an enzyme immobilized by adsorption is lipase from *Candida cylindracea.* Once the enzyme was adsorbed onto a SiO_2 support, the stability of the enzyme increased five-fold, i.e. the half life of the solubilized enzyme ($t_{1/2}$) was 0.5hr and $t_{1/2}$ = 2.5hr for the immobilized form (Moreno and Sinisterra, 1994).

Disadvantages associated with this form of immobilization include the possibility of non-specific binding onto the support affecting the reaction kinetics of the enzyme. This can result in altered K_m and V_{max} values (Tischer and Kasche, 1999). Other disadvantages include leakage of the enzyme due to the weak bonds formed between the enzyme and the support, substrate-induced changes in enzyme conformation and loss of activity or desorption of the enzyme from the support due to exposure of the enzyme to a toxic substrate or product.

1.5.2.3. Covalent-binding

The most frequently used method of immobilization is the formation of a covalent bond between the enzyme and the support. There are numerous functional groups on the enzyme that can be used to form these bonds, see Table 1.2. A knowledge of the chemistry of the support and the composition of the enzyme's active site allows for the appropriate support to be chosen. Care is taken to avoid a covalent bond being formed between any groups associated with the active site of the enzyme and the support, which would inevitably affect the enzymatic activity of the immobilized enzyme. Once the appropriate support is chosen, covalent binding

compared to other immobilization methods offers increased stability. When lipase was immobilized covalently using trichlorotriazine onto a SiO_2 support the half life of the enzyme in comparison to immobilization by adsorption increased by a factor of 6.4 ($t_{1/2}$ = 16 hr) (Moreno and Sinisterra, 1994).

1.5.2.4. Entrapment

Entrapment is a method of immobilization that involves the trapping of the enzyme in synthetic and natural polymers such as agar and polyacrylamide. The enzyme is not restricted in movement however it is confined in a certain area. There are two methods of entrapment, one involving the embedding of the enzyme in a polymer network (encapsulation) and the other is to confine the enzyme in solution that is separated by a membrane where the membrane is permeable to the substrates and products (Bickerstaff, 1997).

Entrapment is applicable to many enzymes and this method of immobilization has relatively no effect on the enzyme structure. The ratio of enzyme/support is low with this form of immobilization in comparison to the adsorption and covalent binding methods (Chang, 1999). This form of immobilization however limits the diffusion of the substrate. Therefore in contrast to covalent binding the activity of the immobilized enzyme by entrapment is low. Another disadvantage associated with this method of immobilization is the possible loss of enzymatic activity due to leakage of the enzyme from the matrix.

The immobilized artificial membrane HPLC stationary phase (IAM-SP) is a unique example of a phase that has proven ideal for the hydrophobic entrapment of

enzymes. The IAM-SP is derived from the covalent immobilization of 1-myristoyl-2-[(13-carboxyl)tridecanoyl]-sn-3-glycerophosphocholine on aminopropyl silica, and resemble one-half of a cellular membrane (Pidgeon, 1990). In the IAM-SP, the phosphatidylcholine headgroups from the surface of the support and the hydrocarbon side chains produce a hydrophobic interphase that extends from the charged headgroup to the surface of the silica. The IAM-SP can be used in batch-type experiments (i.e. stirred in beakers and flasks) or packed into chromatographic columns for use in liquid chromatography. The phase has been previously used to immobilize hydrolytic enzymes with retention of activity and the resulting IMERs were used in coupled HPLC systems (Alebic-Kolbah et al., 1993; Chui et al., 1992).

1.5.3. Properties of Immobilized Enzymes

There is no ideal method or support that can be used for the immobilization of enzymes. The advantages and disadvantages must be weighed against one another in order to proceed with the ideal method/support for the specified task. It is evident that upon immobilization the possibility of changes in the enzymes physical and chemical properties may be observed. The effects of immobilization on the stability, kinetic properties and productivity of the enzyme all need to be considered.

1.5.3.1. Stability

Immobilization can either increase or decrease the enzymatic stability of an enzyme. The magnitude and direction of this effect is dependent upon the method of immobilization and the resulting environment of the enzyme produced by the support. For example, immobilization of lipase on inorganic supports produces higher enzym-

Functional Group	Amino Acid
α and ε–amino groups	Lysine
	Arginine
α, β, and γ -carboxyl groups	Aspartic acid
	Glutamic acid
Phenol ring	Tyrosine
Thiol group	Cysteine
Hydroxyl group	Serine
	Threonine
Imidazole group	Histidine
Indole group	Tryptophan

Table 1.2: The functional groups of enzymes suitable for covalent binding with various supports.

atic stability than immobilization on organic supports (Moreno et al., 1994). The environment that the enzyme is subjected to may result in protein unfolding and/ or dissociation of the enzymes' subunits. This inevitably decreases the enzyme stability. There are several strategies that can be utilized to improve the stability of immobilized enzymes. They include modifying the enzyme structure, derivatization and the use of stabilizing additives.

A unique way of controlling enzymatic stability is by chemical modification (Bickerstaff, 1997). Cross-linking the subunits of enzymes to form a new quartenary structure can increase enzymatic stability. This is routinely accomplished with the use of polyfunctional macromolecular reagents such as polyaldehydes and polyamines (Balcao et al., 2001). The large molecular size of these reagents and their ability to reach the different residues makes the connection of the subunits feasible. Immobilized glyceraldehyde-3-phosphate dehydrogenase was shown to express increased stability when its subunits were cross-linked with bifunctional reagents (Balcao et al., 2001).

Another way to avoid negative effects arising from the surface environment is to modify the properties of the enzyme surface. Changes in charge and hydrophobicity are among some of the commonly used approaches. For example the introduction of hydrophilic groups on the surface of an enzyme molecule prevents incorrect refolding after reversible denaturation (Mozhaev, 1993). Utilizing this approach reduces contact between any hydrophobic regions on the enzyme surface and water, which can results in protein unfolding.

Further stabilization of enzymes can be achieved by attaching a spacer group also referred to as an "arm" (Penzol et al., 1998). The spacer is composed of reactive end groups and forms a bond between the enzyme and the chosen support. The reactive groups are prominently amine groups, carboxylic acid or hydroxyl groups. Increased steric freedom of the immobilized enzyme is achieved utilizing this approach and distancing the enzyme from the support reduces any possible steric hindrance. Several considerations have to be made when utilizing this approach such as the spacer needs to be flexible, inert, hydrophilic and present in small amounts (Penzol et al., 1998). Dextrans are commonly used as spacers and have been shown to produce stable enzyme-dextran-support bonds (Bickerstaff, 1997). Rennin and protein A have been immobilized with the use of dextrans as spacer arms and were shown to display higher capacities of recognition of their substrates (Penzol et al., 1998).

1.5.3.2 Kinetic behavior of immobilized enzymes

The main advantage of immobilized enzymes is the ability to reuse the enzymes. Similar to solubilized enzymes, the kinetic parameters, temperature and pH effects on immobilized enzymes can be investigated. However, some general considerations must be taken into account since immobilization can introduce new difficulties not associated with the free enzyme. Depending upon the method of immobilization and properties of the support, there may be diffusional restrictions associated with the immobilized enzyme (Goldstein, 1976) and a decrease in enzyme mobility can also affect the mobility of substrates and cofactors.

The mass-transfer of substrates and products influences the reaction system and mass-transfer resistance can arise due to the location of the enzyme in the support or due to the large particle size of the immobilized enzyme. Under these conditions the immobilized enzyme operates under diffusion-limiting conditions as opposed to reaction-limiting conditions whereby diffusion layers that form around immobilized enzymes govern its catalytic rate (Giorno and Drioli, 2000). In this instance, the movement of a substrate from the bulk solution into the unmixed liquid layer surrounding the immobilized enzyme and then through to the active site represents the diffusion layer. A thin diffusion layer in contrast to a thick layer results in limited mass-transfer effects.

For immobilized enzymes the extent of mass-transfer effects are expressed by the efficiency coefficient, η.

$$\eta = \frac{\text{rate of immobilized enzyme}}{\text{rate of non-immobilized enzyme}}$$

The rates of the immobilized and non-immobilized enzyme are determined under identical assaying conditions such as enzyme concentration, substrate concentration, pH, temperature, etc (Tischer and Kasche, 1999). Mass-transfer resistance has been shown to decrease with increased flow-rates and increased stirring (Castner et al., 1984). As such, in the kinetic analysis of immobilized enzymes in flow reactors (IMERs), the investigation of the effect of flow-rate is important in the determination of K_m and V_{max} of the immobilized enzyme. Other methods exist that can increase the reaction efficiency of an immobilized enzyme. They include decreasing the particle

size of the support, reduction of the enzyme load, and manipulation of the binding of the enzyme to the support (Tischer and Kasche, 1999).

In general, enzymes are susceptible to thermal degradation (Dixon, 1979). Generally, as the temperature increases, so does the enzymatic activity due to increased movement of the enzyme and substrate molecules. As a result there are more collisions between the substrate and the enzyme yielding product formation. Exceeding certain temperatures however, results in the inactivation of the enzyme. At the higher temperatures hydrogen bonds are disrupted and the shape of the enzyme is altered. This diminishes the enzymes' affinity for its substrate producing a rapid decline in activity.

Immobilized enzymes have been shown to display increases in thermal stability (Markoglou and Wainer, 2001). Immobilization results in limiting the thermal movement of the enzyme at the higher temperatures. As a result, thermal denaturation may not be visible at the higher temperatures for the immobilized enzyme. Thermostable enzymes allow for higher reaction rate, lower diffusional restrictions, increased stability and greater yields.

Strategies to further improve the thermostability of immobilized enzymes in industrial sectors include the use of popular techniques such as gene cloning and protein engineering (Schulein, 2000; Taylor et al., 1999). For example, with protein engineering techniques thermostable proteases have been produced allowing for their use at higher temperatures offering the added advantage of higher reaction rates, and higher product yields (Van den Burg et al., 1998). Another example, includes the use of immobilized beta-galactosidase in the dairy industry for the production of lactose-

hydrolysed milk (Park and Hoffman, 1990). Thermostable biocatalysts in beer brewing (malting of barley) and in the production of cheese flavor (proteases and peptidases) have proven to be vital in these industries (Hagedorn and Kaphammer, 1994).

Enzymes are complex proteins composed of charged hydrophilic and neutral hydrophobic constituents. The tertiary structure of an enzyme is produced by intra-molecular interactions that include hydrogen bonding, pi-pi stacking, disulfide bridging and electrostatic interactions. The electrostatic interactions are sensitive to the pH of the surrounding environment and pH changes in this environment can result in changes in the pattern of charges on the enzyme, and, consequently, a change in the teritary structure of the enzyme. Finally a change in the tertiary structure of the enzyme can alter the active site producing either an increase or decrease in enzymatic activity. Therefore, the effect of pH on the structure of an enzyme is most often reflected in bell-shaped pH-activity profiles in which the enzyme displays maximal activity at an optimal pH. The shape of the curve and the optimal pH is dependent upon the enzyme.

The immobilization of an enzyme on a solid support can radically change the enzyme's microenvironment. This effect has been studied and it has been demonstrated that the use of charged supports can cause shifts in an enzyme's optimum pH (Chapman et al., 1975). For example, the immobilization of trypsin on a cation-exhange carrier shifted the pH optimum (Goldstein, 1976). In addition, the immobilization process itself can alter the pH of the enzyme's microenvironment. There are a number of approaches to the solution of reaction-produced or solid

support-produced pH changes. These include changing the type and ionic strength of the immobilization buffer, altering the particle size of the support, and co-immobilization of enzymes that consume any protons generated from the immobilization or enzymatic reaction (Tischer and Kasche, 1999).

This thesis will describe the development and use of immobilized DBH and PNMT. The immobilized enzymes are compared to the soluble enzymes to examine the effects of immobilization on the enzymes.

1.6. Research Objectives

The objective of this research project was to develop immobilized enzyme reactors of the enzymes involved in the biosynthesis of catecholamines. This was accomplished by: 1) developing enzyme assays utilizing HPLC 2) selecting the appropriate immobilization support for optimal conditions and 3) applying the IMER onto a chromatographic system via switching valve technology.

Each immobilized enzyme reactor was examined with numerous experimental tests including the effect of substrate/enzyme contact time at different flow rates and substrate concentrations. The IMERs were used individually and collectively for the screening of substrates and the examination of kinetic parameters. The aim of these studies was a better understanding of the enzymatic processes involved in the biosynthesis of catecholamines.

1.6.1. Project Outline

In order to accomplish the goals of this project, the following experimental objectives were pursued:

Objective I: The development of a validated standard HPLC method for the catecholamines involved in the pathway of interest. Assessing the chromatographic conditions for the separation of the substrates and products on chiral and achiral stationary phases.

Objective II: The setup of previously reported or newly developed chromatographic enzyme assays for each enzyme involved in the pathway. *In vitro* assays were carried out for each enzyme with different substrates and inhibitors. Kinetic parameters were determined (K_m, V_{max}, pH, effect of cofactors, etc).

Objective III: The appropriate immobilization supports and optimal immobilization procedures were selected. Immobilized enzymes on loose packing material were examined for activity. Once active, similar experiments that were carried out for the non-immobilized enzyme were performed and compared.

Objective IV: Immobilized enzymes were packed into chromatographic columns. Utilizing switching valve technology appropriately, on-line extraction and synthesis could then be carried out. Enzyme/substrate contact time, flow rate, temperature and pH effects were examined. The IMERs were studied in a similar fashion. Experiments were carried out on the individual reactors and coupled IMERs.

1.6.2. Contributions of Authors

CHAPTER 2

This chapter has been published in the *Journal of Biochemical and Biophysical Methods* 48:61-75, 2001. I performed all the experimental work and prepared the first draft of the manuscript. Dr. I.W. Wainer provided supervision in the planning of the experiments and is the corresponding author. Dr. I.W. Wainer and Dr. Yanxiao Zhang assisted with the revisions of the manuscript. Journal reviewers requested several clarifications and revisions, which I completed.

CHAPTER 3

This chapter was submitted to the *Journal of Chromatography B.* Dr. I.W. Wainer and I discussed the setup of the on-line system. In addition, I had a few discussions with André Pageau from ThermoQuest concerning specifics on the instrumental setup. I performed all the experiments and prepared the first draft of the manuscript. Following review of the manuscript by Dr. I.W. Wainer, I made the necessary changes and submitted the manuscript to the Journal.

CHAPTER 4

This chapter has been published in the Journal *Analytical Biochemistry* 288: 83-88, 2001. I performed all the experimental work and planned the experiments with Dr. I.W. Wainer. I prepared the first draft of the manuscript and worked with Dr. I.W. Wainer on revising the manuscript. Journal reviewers requested minor revisions and

some clarifications. I responded to the reviewers' questions and made the necessary changes.

CHAPTER 5

This chapter has been submitted to the *Journal of Chromatography A*. Dr. I.W. Wainer and I discussed the development of the PNMT-IMER and the coupled IMER system, and we outlined all the necessary experiments to be performed. I prepared the first draft of the manuscript, and made the necessary revisions that Dr. I.W. Wainer recommended before sending it to the Journal.

Chapter 2

SYNTHESIS AND CHARACTERIZATION OF IMMMOBILIZED DOPAMINE β-HYDROXYLASE IN MEMBRANE-BOUND AND SOLUBILIZED FORMATS

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2.1. Abstract

Dopamine β-hydroxylase (DBH) catalyzes the β-hydroxylation of dopamine to norepinephrine. The enzyme in chromaffin granules occurs in a soluble form and a form confined to the surrounding membrane. DBH was noncovalently immobilized in the hydrophobic interface of an immobilized artificial membrane (IAM) liquid chromatographic stationary phase and the resulting DBH-IAM stationary phase was enzymatically active and was shown to mimic the membrane-bound form of the enzyme. DBH was also covalently immobilized onto a silica-based support containing, glutaraldehyde-P (Glut-P). The resulting DBH-Glut-P interphase was also enzymatically active, reproducible and shown to display characteristics of the solubilized enzyme. The results demonstrate that the different immobilization methods utilized for the enzyme can be used to quantitatively and qualitatively determine the enzyme kinetic constants associated with enzyme/substrate and enzyme/inhibitor interactions for the two distinct forms of the enzyme. These new entities can be used in basic biochemical studies as well as in high throughput screening of substances for DBH substrate/inhibitor properties.

Key Words: Dopamine beta-hydroxylase, Glutaraldehyde-P, Immobilization, Immobilized Artificial Membrane, Screening.

2.2. Introduction

Dopamine is hydroxylated to norepinephrine by the catalytic effect of dopamine beta-hydroxylase (DBH), one of the enzymes involved in catecholamine biosynthesis (Wong et al., 1990). The enzymatic process involves the oxidation of ascorbic acid to dehydroascorbate and the reduction of Cu^{2+} to Cu^{+} (Friedman et al., 1965). The enzyme is involved in the regulation of blood pressure by the nervous system and a target for antihypertensive drugs (Lewis et al., 1992). DBH is situated within catecholamine-containing chromaffin granules and exists in two forms: a membrane-bound form that is reinternalized upon exocytosis and a soluble form that is stored in the granule and secreted (Helle et al., 1984). The two states of the enzyme in the chromaffin granules are immunochemically identical, however they display differences in pH stability and substrate affinity (Nagatsu et al., 1972).

With the key role that DBH plays in the biosynthesis of catecholamines, this process has been subject of a number of studies. The majority of the assays utilize crude extracts giving rise to the possibility of endogenous inhibitors in the extracts that can interfere with DBH activity (Molinoff et al., 1971). When highly purified enzyme is used it involves long and complicated purification procedures, which are costly. The enzymatic assays can also be cumbersome, for example, one assay utilizes a two-step enzyme method, using phenylethanolamine N-methyltransferase and S-adenosyl-L-methionine (Molinoff et al., 1971). However, with these techniques saturating substrate concentrations for DBH cannot be examined due to the inhibition of the second enzyme, phenylethanolamine N-methyltransferase.

The main problem faced by researchers when characterizing DBH is the repeated need for large amounts of highly purified enzyme. However there are recent applications that utilize enzymes in one or more immobilized form (Bickerstaff, 1984, Bickerstaff, 1997). The enzymes were immobilized onto solid supports and used in batch incubations (Bickerstaff, 1997; Lowe et al., 1990) or as biosensors (Bickerstaff, 1984; Johansson et al., 1993; Lowe et al., 1990; Marko-Varga et al., 1994; Nordling et al., 1993; Tischer et al., 1999). These applications do not require highly purified enzymes and decrease the amounts of enzyme required. The immobilized enzymes retain their activity and can be reused following a simple washing procedure.

In this study, the hydrophobic character of the IAM stationary phase was used to immobilize commercially available partially purified dopamine beta-hydroxylase. The IAM interphase is derived from the covalent immobilization of 1-myristoyl-2-[(13-carboxyl) tridecanoyl]-sn-3-glycerophospholine on aminopropyl silica, and resembles one-half of a cellular membrane (Pidgeon et al., 1992). The hydrocarbon chains create interstitial spaces that allow for the insertion of DBH.

DBH was also immobilized onto glutaraldehyde-P (Glut-P), a wide-pore silica that has been covalently clad with polyethyleneimine, a hydrophilic polymer (Narayanan et al., 1990). The reactive amine groups of the polymer form a covalent bond with glutaraldehyde. This particular support is ideal for immobilization of proteins with primary amino groups that form an amine-aldehyde Schiff linkage with Glut-P (Narayanan et al., 1990).

The utility of the DBH-IAM phase and the DBH-Glut-P phase was investigated by determining both qualitative and quantitative aspects of enzyme

kinetics. In particular, studies were carried out comparing both the free and immobilized enzyme. The results of these studies confirm that the DBH-IAM and DBH-Glut-P interphases retained their enzymatic activities.

A chiral assay was developed to demonstrate that the immobilized enzyme retained its stereospecificity. Investigations into the effect of the enzyme activity by changes in pH, buffer type, substrate concentration, cofactor concentration and sensitivity to inhibitors were carried out. The results demonstrate that the IAM-immobilized and Glut-P immobilized dopamine beta-hydroxylase can be utilized for the screening of possible substrates and inhibitors for membrane-bound DBH and soluble DBH, and to provide information concerning their pharmacological properties.

2.3. Materials and Methods

2.3.1. Materials

Dopamine-beta-hyrdoxylase (from bovine adrenals), catalase (from bovine liver), DL-octopamine hydrochloride, tyramine hydrochloride, (±)- norepinephrine bitartrate salt, (-)-norepinephrine bitartrate salt, dopamine hydrochloride, ascorbic acid, and other chemicals unless otherwise stated were obtained from Sigma Chemical Company (St. Louis, MO, USA). Hexane (95% n-hexane), methanol and glacial acetic acid, all HPLC grade were manufactured by J.T. Baker (Phillipsburg, NJ, USA) and purchased through Moquin Scientific (Montreal, QC, Canada). The IAM.PC (12 μm, 300 Å) non-endcapped chromatographic support was obtained from Regis Chemical Co. (Morton Grove, IL, USA). The IAM.PC bonded phase, according to the manufacturer, contains a near monolayer of C14 saturated phosphatidylcholine,

covalently linked to silica through an amide link. Glutaraldehyde-P affinity packing (40 μm, 300 Å) was obtained from J.T. Baker Inc.

2.3.2. Apparatus

The chromatographic experiments were carried out using a Thermo Separation Products P1000 pump (ThermoQuest, San Jose, CA, USA) and a Thermo Separation Products AS3000 autosampler equipped with a 100μl loop. The solutes were detected using an ABI fluorescence detector (ABI Analytical, Ramsy, NJ). Data was collected using a Thermo Separation Products Chromjet integrator interfaced with a Spectra 486 computer equipped with OS2 software for data collection. A 5μm Prodigy C18 stationary phase packed in 250 x 4.6 mm i.d. column (Phenomenex, Torrance, CA, USA) and a 5μm nitrile guard cartridge (Regis Chemical Co.,Morton Grove, IL, USA) were used for the chromatography.

2.3.3. Chromatographic Procedures

The chromatographic separation of the different substrates and products was achieved with a mobile phase consisting of potassium phosphate buffer (25 mM) adjusted to pH 2.0 with trifluoroacetic acid. The solutes were quantitated using fluorescence detection with excitation at λ = 265 nm and no cut-off filter for emission. A flow rate of 1ml/min and ambient temperature were used throughout the study. Under these chromatographic conditions the relative chromatographic retentions (k' vlaues) of norepinephrine, octopamine, dopamine and tyramine are 0.68, 1.27, 4.79, and 8.86, respectively.

Examination of the enzyme's stereospecificity was performed on the same chromatographic system described above using a column containing a chiral crown ether based chiral stationary phase, Crownpak CR(+) packed in 250 x 4.6 mm i.d. column. (Regis Chemical Co.) The mobile phase consisted of aqueous perchloric acid (0.01M, pH 2) at a flow rate of 1ml/min and at ambient temperature. The relative chromatographic retentions (k' values) of R-(+)-norepinephrine and S-(-)-norepinephrine were 3.98 and 4.38, respectively and the observed enantioselectivity (α) was 1.10. The solutes under chiral conditions were monitored using UV detection at λ =278nm.

2.3.4. Extraction of Catecholamines

Enzymatic conversions utilizing DBH require cupric sulphate, catalase, ascorbic acid and sodium fumarate. An off-line extraction method utilizing solid phase extraction cartridges containing a phenylboronic acid stationary phase was developed. The procedure consisted of the following: 1-ml cartridges were conditioned with 2 ml of methanol followed by 2 ml of sodium phosphate buffer (0.1M, pH 8.4). One ml of reaction mixture was added, the cartridge was then washed with 1ml sodium phosphate buffer (0.1M, pH 8.4) and the substrate and product were eluted using 1ml of 0.1N HCl. The eluate was directly injected onto the HPLC system.

2.3.5. Assay for Dopamine beta-hydroxylase activity

The activity of dopamine beta-hydroxylase activity was assayed using a modified procedure derived from the work of Nagatsu (Nagatsu, 1991). The enzyme was assayed as follows: four different solutions were prepared A, B, C and D (final concentration). (A) 500 µL of enzyme solution (10µg/ml); B: 275 µL sodium acetate buffer (10mM, pH 5.5), 50 µL cupric sulphate (5µM); C: 25µL catalase (5µg/ml), 50 µL ascorbic acid (10mM), 50 µl sodium fumarate (10mM); D: 50 µl substrate (5mM). Solution B was added to solution A, the resulting solution was mixed for 1 min. Solution C was added and the resulting solution was mixed for an additional minute. The reaction was started with the addition of the substrate (D), after which the reaction solution was incubated for 5 min at 37°C in a shaking bath. The reaction was stopped by the addition of 100 µl of cold hexane, the resulting mixture was centrifuged at 3000xg for 10 min and the supernatant transferred to the pre-conditioned phenylboronic acid cartridges in order to extract the product formed and the remaining substrate. Extracted samples were directly injected onto the HPLC under either chiral or achiral conditions.

2.3.6. Immobilization of DBH onto IAM

The packing material was washed three times with sodium acetate buffer (0.1M, pH 5.5). The washing was carried out by adding 1ml of buffer to the packing material, the suspension was vortexed for 1min, centrifuged at 3000xg for 10min and the supernatant decanted. The enzyme solution (8-10 µg in 1ml sodium acetate buffer) was added to the packing material, the mixture was vortexed for 15 min and

then placed in a shaking bath for 2 h at ambient temperature. At the end of 2 h, the suspension was centrifuged, the supernatant decanted and the packing material washed three additional times with buffer. The amount of enzyme immobilized on the packing material was determined by measuring the amount of residual enzyme in the supernatant using the BioRad Protein Assay. The difference in the absorbance reading before immobilization and the total of absorbances after immobilization determined the amount of enzyme bound on the packing material.

2.3.7. Immobilization of DBH onto the Glut-P interphase

The packing material was washed three times with sodium acetate buffer (0.1M, pH 6). The washing was carried out by adding 1ml of buffer to the packing material, the suspension was mixed for 2min, centrifuged at 3000xg for 10min and the supernatant decanted. The enzyme solution (8-10 μg in 1ml sodium acetate buffer] was added to the packing material, the mixture was vortexed for 2 min and then placed on a shaker/rotator for 6 h at ambient temperature. At the end of 6 h, the suspension was centrifuged, the supernatant decanted and the packing material washed three additional times with the buffer. The amount of residual enzyme was determined utilizing the same procedure as that carried out for the enzyme immobilized onto IAM.

2.3.8. Regeneration of Enzyme Activity

After each reaction, the activity of the immobilized enzyme was regenerated using a simple washing procedure. One ml of sodium acetate buffer at the appropriate pH was added to the DBH-IAM or DBH-Glut-P material, the mixture was mixed for 2

min, centrifuged for 15 min, and the supernatant was discarded. After this process was repeated an additional 3 times, the enzyme was active. The DBH-IAM phase remained active for a six-month period when stored in sodium acetate buffer (0.1M, pH7) at 4°C. The DBH-Glut-P material was stored in sodium acetate buffer (0.1M, pH6) at 4°C and remained active for over 3 months.

2.4. Results

2.4.1. Chromatographic results

Octopamine, norepinephrine, tyramine and dopamine were resolved, under the chromatographic conditions used in this study. Standard curves for the substrates and products were linear over the range investigated with regression equations and correlation coefficients: y =9E+06 x + 3E+06, r = 0.9777; y =1.3E+07x-9.6E+03, r = 1.000; y = 3E+07x-21963, r = 1.000 and y=1.12E+07x-6.7E+04, r = 0.9978 for dopamine, tyramine, octopamine and norepinephrine respectively. Recoveries for both the substrates and products exceeded 75%; dopamine, 94±2.1 % , norepinephrine, 76 ± 1.6 %, octopamine, 84 ± 1.1 % and tyramine, 89 ± 2.4 %.

The reaction mixture and a blank were analyzed. Typical chromatograms resulting from the analysis of blank reaction mixture containing boiled enzyme and reaction mixture are shown in Figures 2.1A and 2.1B. The results show that the product enzymatically formed by DBH can be isolated by HPLC and determined by fluorescence detection. A chiral HPLC assay was developed utilizing a Crownpak CR(+) chiral stationary phase in order to determine if the immobilized enzyme retains its native stereospecificity. Figure 2.1C displays the formation of R–(+)-norepinephrine by immobilized dopamine beta-hydroxylase when dopamine is used as

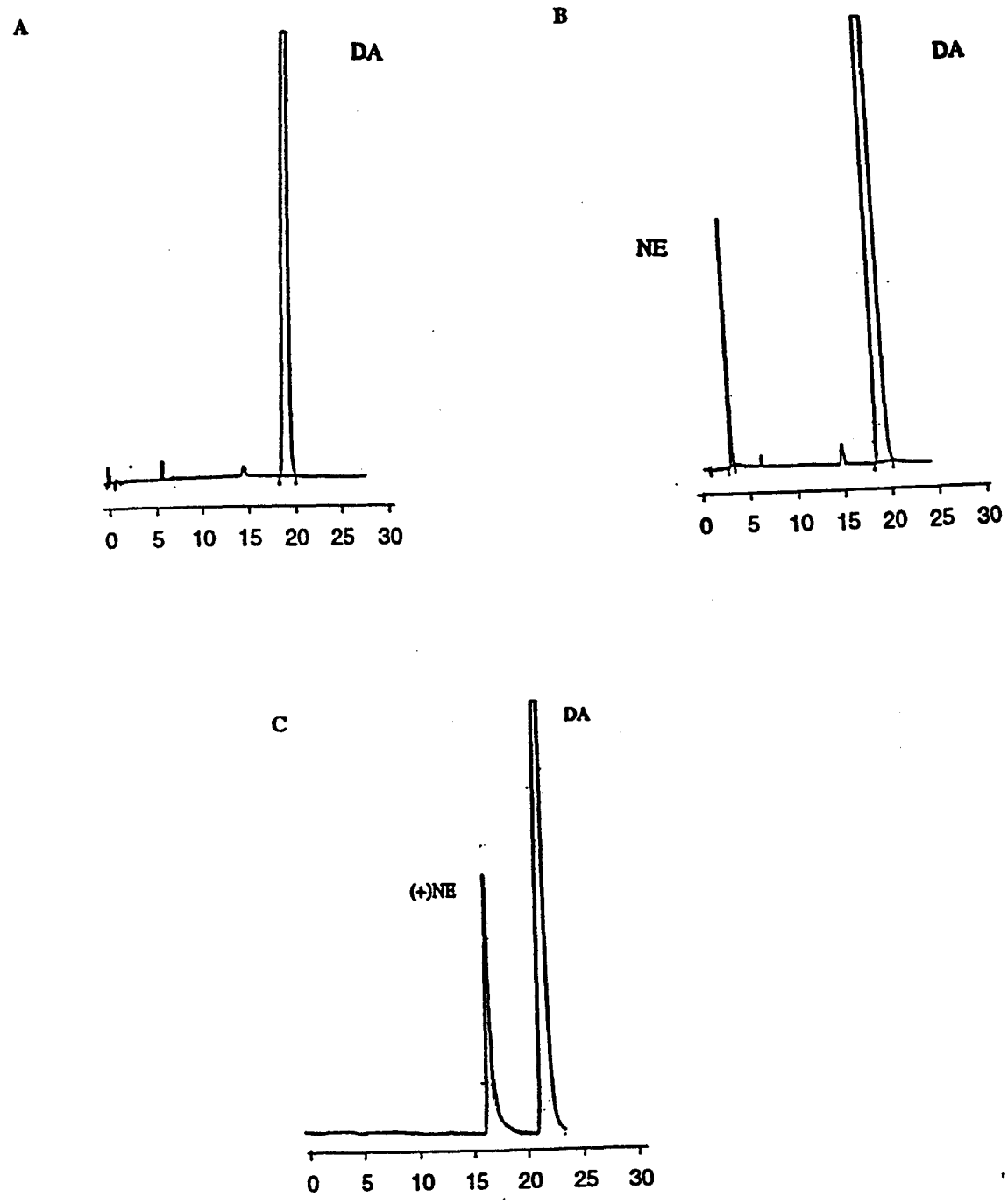


Figure 2.1: Representative chromatograms from DBH assays analyzed: (A) blank reaction mixture containing a boiled enzyme; (B) reaction mixture; (C) reaction mixture under chiral conditions (Crownpak CR(+) column).

a substrate. Therefore, immobilization of DBH on the IAM stationary phase does not alter the enzymes activity or stereospecificity. DBH was also shown to retain its stereospecificity when immobilized on the Glut-P interphase (Appendix A).

2.4.2. Optimization of DBH Immobilization

The quantity of DBH, which could be immobilized on the IAM, was investigated. DBH (10 μg in 1ml of sodium acetate buffer (0.1M, pH 7)) was stirred with 1-10mg of IAM. The amount of immobilized enzyme and the rate of the reaction were investigated for the different amounts of IAM packing material. The amount of immobilized enzyme was determined utilizing the BioRad assay. DBH (8.67 ± 0.55μg) was immobilized on 2 mg of the IAM material. When greater than 2 mg of IAM were used to immobilize 10 μg of enzyme, over 85% of the enzyme was immobilized. However, it was found that with increasing amounts of IAM there was a decrease in the rate. Under the experimental conditions, the enzyme and substrate concentrations were held constant with increasing amounts of IAM.

The amount of DBH immobilized on the Glut-P interphase was also investigated. DBH (10 μg in 1ml of sodium acetate buffer (0.1M, pH 6)) was mixed with different amounts of Glut-P utilizing a rotator/stirrer. When greater than 10mg of Glut-P were used to immobilize 10 μg of DBH, over 70% of the enzyme was immobilized. Increases in the rate were visible with decreasing amounts of Glut-P. The optimal conditions were therefore set at 10 μg of DBH being immobilized onto 50mg of Glut-P for the remainder of the experiments.

2.4.3. Comparison of immobilized and non-immobilized DBH

2.4.3.1. Effect of buffer composition on DBH activity

The hydroxylation activity of free and immobilized DBH was determined by examining a series of compounds known to be substrates and products of the enzyme. The free and immobilized enzyme activities were determined by following the formation of the norepinephrine or octopamine from the β -hydroxylation of dopamine or tyramine, respectively. The effect of concentration and composition of buffer on enzymatic activity was examined. Phosphate buffer was found to be inhibitory in comparison to sodium acetate when dopamine and tyramine were utilized as substrates. Similarly at sodium acetate buffer concentrations exceeding 0.2M an inhibitory effect was visible for both the free and immobilized enzyme. Therefore, 0.1M sodium acetate buffer was used for the assays allowing for maximal enzyme activity.

2.4.3.2. Effect of incubation and enzyme concentration on DBH activity

The length of incubation and the amount of enzyme used per assay were varied independently. Optimal conditions consisted of a 5-min incubation with 10 μg of dopamine β-hydroxylase (Appendix B). The amount of dopamine β-hydroxylase that was prepared for each experiment was determined utilizing the BioRad assay each time. The activity of free and immobilized DBH as measured with substrates, dopamine and tyramine, is linear upto 20 μg of enzyme under the assay conditions.

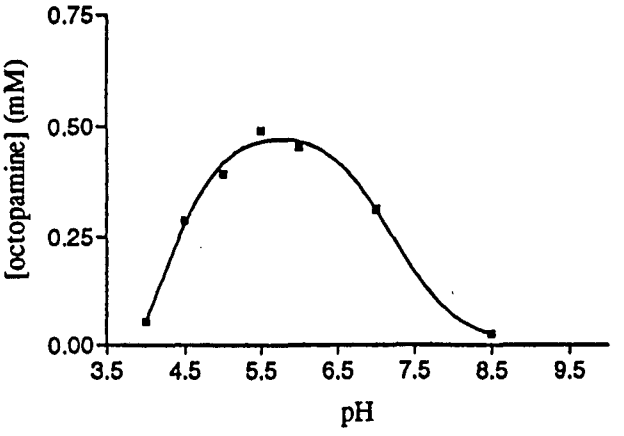
2.4.3.3. Effect of pH on DBH Activity

The activity of the free and the two forms of the immobilized dopamine β-hydroxylase was measured at a series of pHs (with constant ionic strength buffers) to determine the optimum pH for the two forms of the enzyme (DBH-IAM and DBH-Glut-P). Figures 2.2 A-C demonstrate the general profiles that have been obtained under non-immobilized and immobilized conditions. A pH optimum of 5.5 was found for non-immobilized dopamine β-hydroxylase, which is consistent with previously reported values (Nagatsu, 1991; Cooper et al., 1996). There was a shift of 1.5 pH units for the immobilized enzyme onto IAM, pH 7. A pH optimum of 6.0 was found for the DBH-Glut-P interphase. Similar pH profiles were obtained when substrates, dopamine and tyramine were utilized. The optimum pH was utilized at optimal conditions for the non-immobilized and the two forms of immobilized DBH.

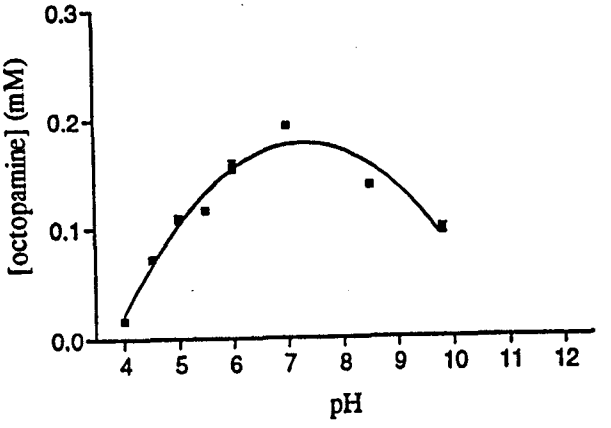
2.4.3.4. Effect of catalase on DBH Activity

Catalase has been reported to stimulate *in vitro* the activity of DBH (Nagatsu, 1991). For the free enzyme it was found that with increasing concentrations of catalase there was an increase in the enzymes' activity. In contrast, the enzyme immobilized onto IAM did not display increased activity with increased amounts of catalase (Figure 2.3A, 2.3B). Similar results were obtained when the effect of sodium fumarate was examined. Unlike the free enzyme, the immobilized enzyme does not require the presence of catalase and sodium fumarate to stimulate its activity. Similar findings were obtained with the enzyme immobilized onto Glut-P.

A



B



C

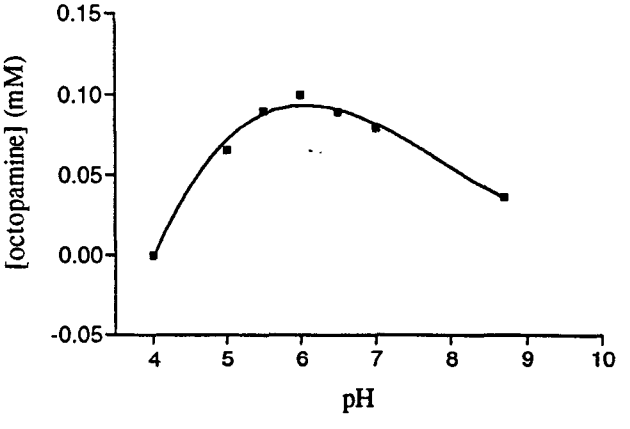


Figure 2.2 : Effect of pH on DBH activity. (A) Non-immobilized DBH; (B) DBH-IAM; (C) DBH-Glut-P.

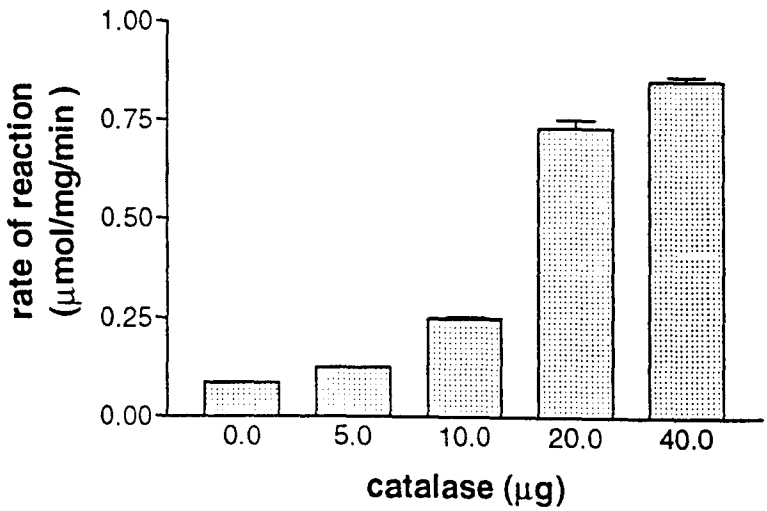


Figure 2.3 A: Effect of catalase on non-immobilized DBH activity.

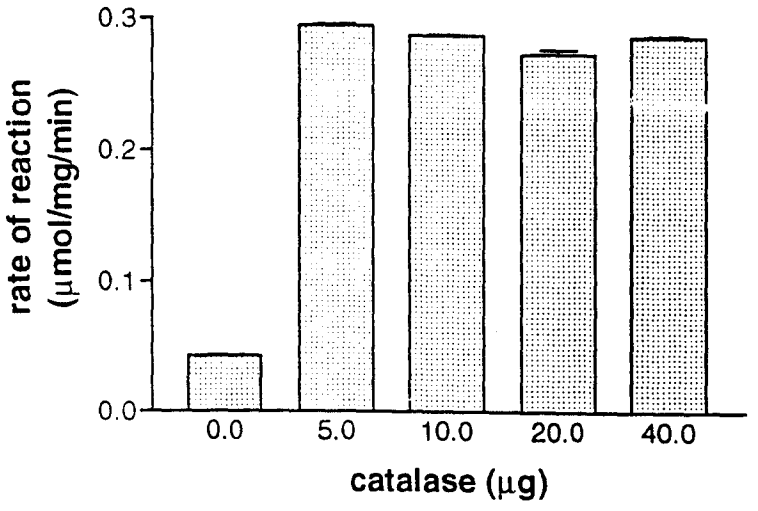


Figure 2.3 B: Effect of catalase on DBH-IAM activtiy (10mg/ml DBH immobilized onto 2mg IAM). Utilizing 5mM tyramine and 5mM ascorbic acid.

2.4.3.5. Effect of cupric ions on DBH activity

For all forms of the enzyme optimal enzyme activity was reached with a Cu^{2+}concentration of 5μM. However, with increasing concentrations of cupric sulphate inhibition is visible for all forms of the enzyme (Nagatsu, 1991). This was consistent with previously reported findings.

2.4.3.6. Effect of Substrate and Cofactor Concentration on DBH Activity

The effect of tyramine and dopamine concentrations on the enzymatic activities of non-immobilized and immobilized DBH was examined. The kinetic parameters were calculated for all forms of the enzyme using the standard Michaelis-Menten approach (Appendix C). Lineweaver-Burke plots were used to calculate the Michaelis constant (K_m) (Dixon and Webb, 1979). The rates of reaction (V_{max}) were calculated using μmol/mg/min for non-immobilized DBH (specific activity) and immobilized DBH (apparent-specific activity). The data was calculated using mg of immobilized or non-immobilized DBH in order to accurately compare the enzymatic activities in the two formats. The results are listed in Table 2.1 and expressed as mean ±.standard error of the mean (SEM). Parallel line patterns were obtained in double reciprocal plots with dopamine or tyramine as a varied substrate at fixed ascorbic acid concentrations. The kinetic properties of the DBH-Glut-P interphase are similar to the non-immobilized enzyme, giving rise to a ping-pong mechanism. The DBH-IAM interphase does not represent a ping-pong mechanism consistent with previous reports on membrane-bound DBH (Miras-Portugal et al., 1975).

Non-immobilized DBH	K_m (mM)	V_{max} (μmol /mg /min)
Tyramine	2.85	0.208
Dopamine	5.03	0.320
Ascorbic Acid	0.62	0.185

DBH-IAM	K_m (mM)	V_{max} (μmol /mg /min)
Tyramine	1.64 ± 0.23	0.101 ± 0.023
Dopamine	2.53 ± 0.45	0.160 ± 0.014
Ascorbic Acid	0.52 ± 0.14	0.112 ± 0.004

DBH-Glut-P	K_m (mM)	V_{max} (μmol /mg /min)
Tyramine	1.04 ± 0.09	0.112 ± 0.032
Ascorbic acid	1.10 ± 0.12	0.047 ± 0.006

Table 2.1: Kinetic parameters (K_m and V_{max}) of non-immobilized and immobilized forms of dopamine β-hydroxylase (DBH, DBH-IAM, DBH-Glut-P).

2.4.3.7. Effect of temperature on DBH activity

The non-immobilized enzyme was shown to have an optimal activity at 40°C after which the activity decreased with increasing temperature (Figure 2.4A). Similar results were observed for the DBH-Glut-P interphase (Figure 2.4B). The enzyme immobilized onto IAM however displayed an increase in its thermal stability (Figure 2.4C). Maximal activity was maintained between 35°C and 55°C followed by a decrease in activity at temperatures greater than 60°C.

The Arrhenius plots for the enzyme forms were drawn. The logarithm of the enzyme activity is plotted against the reciprocal of the absolute temperature (Dixon and Webb, 1979). Data yielded a straight line for the non-immobilized enzyme with the activation energy calculated to be 20.54 kJ/mol. A straight line was also obtained for the enzyme immobilized onto the Glut-P interphase, yielding an activation energy of 38.37 kJ/mol. The Arrhenius plot for the enzyme immobilized onto IAM gave rise to a break in the curve at 37°C. An activation energy of 11.36 kJ/mol was observed above 37°C and the activation energy below this temperature was 15.78 kJ/mol.

2.4.3.8. Effect of Inhibitor Concentration on DBH Activity

Fusaric acid is a known inhibitor of dopamine β-hydroxylase (Nagatsu et al., 1970). The effects of this compound on the enzymatic activities of non-immobilized and DBH-IAM was examined and the results are presented in Table 2.2. Fusaric acid was found to inhibit the DBH mediated formation of norepinephrine from dopamine at concentrations as low as 10^{-6} M, the inhibition for both the immobilized and non-immobilized enzyme was approximately 50%.

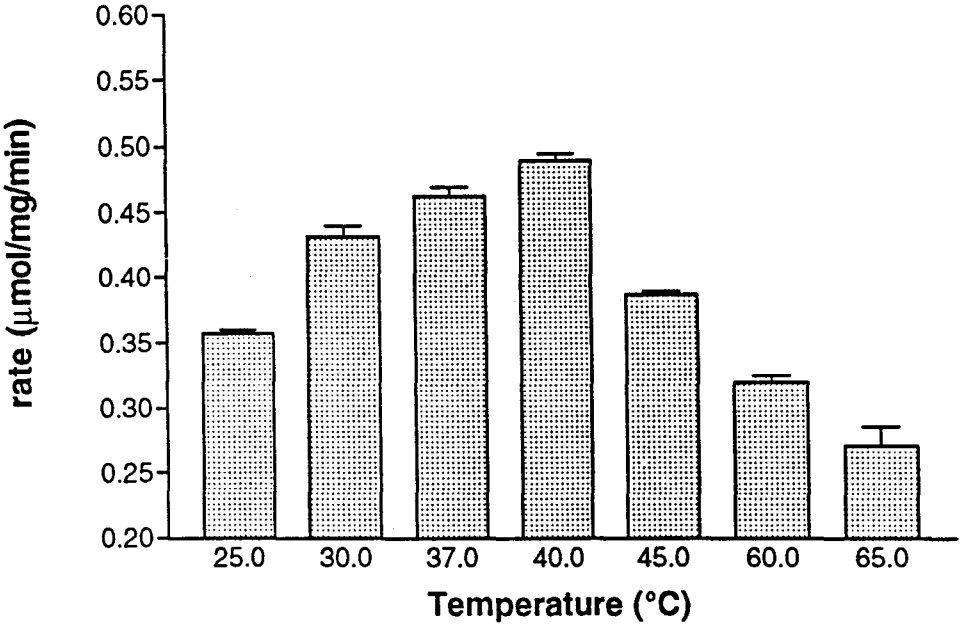


Figure 2.4 A: Effect of temperature on non-immobilized DBH activity.

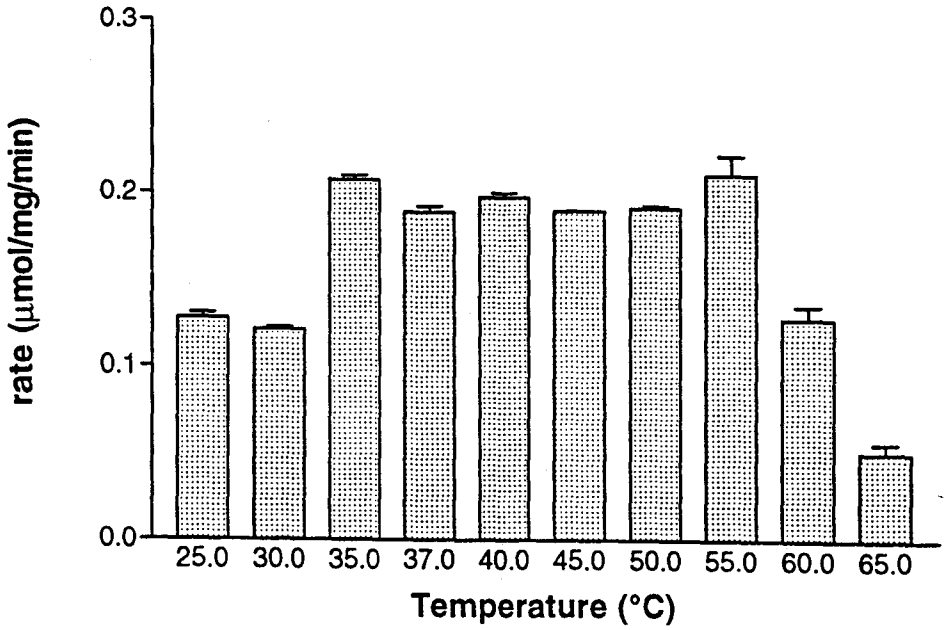


Figure 2.4 B: Effect of temperature on DBH-Glut-P activity.

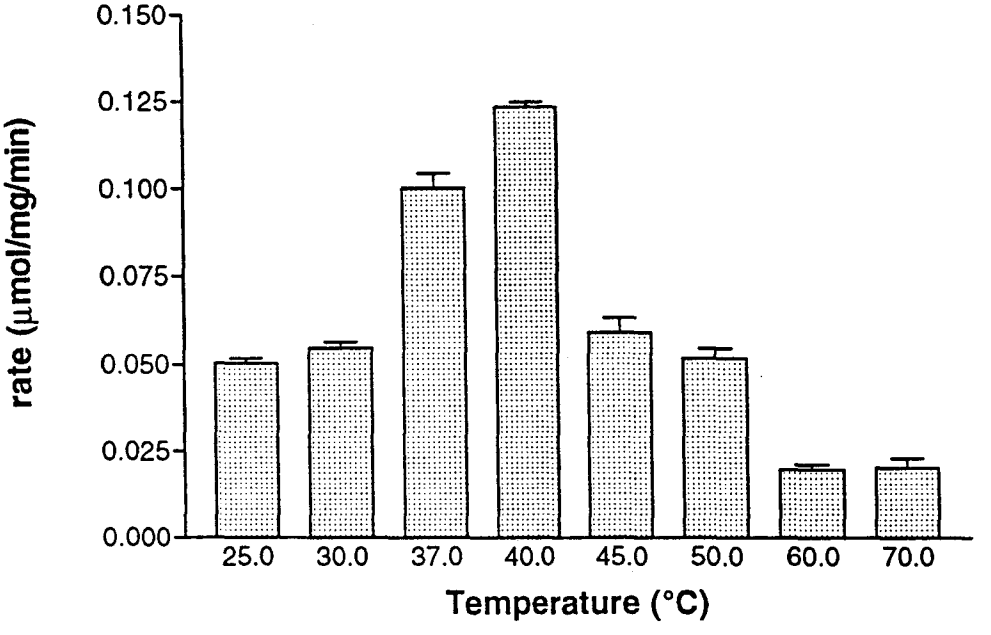


Figure 2.4 C: Effect of temperature on DBH –IAM activity.

[Fusaric acid] (nM)	% Control Activity DBH	% Control Activity DBH-IAM
0	100	100
1.6	99	91
3.2	84	73
6.3	40	56
12.5	21	51
25.0	19	46
50.0	17	20
100	0	0
200	0	0

Table 2.2: The effect of fusaric acid on the enzymatic activities of non-immobilized dopamine (DBH) and immobilized dopamine beta-hydroxylase onto IAM (DBH-IAM).

Captopril is another known inhibitor of dopamine β-hydroxylase (Mueller et al., 1999). The effect of captopril on immobilized DBH kinetics is represented in the form of a reciprocal velocity plot versus the reciprocal of the concentration of tyramine. The plot indicates noncompetitive inhibition with respect to tyramine. The slope and intercept replots were linear ($r > 0.995$). Captopril was found to inhibit the DBH mediated formation of octopamine from tyramine at concentrations as low as 150 μM, the inhibition for both the immobilized and non-immobilized enzyme was approximately 50%. The results are consistent with previously reported results demonstrate that captopril is capable of inhibiting DBH (Mueller et al., 1999).

2.5. Discussion

The results of this study demonstrate that the immobilized enzymesare indeed active and retain their stereospecificity. Maximal adsorption and maximal activity was achieved at 2mg of IAM and at 50 mg Glut-P. Decreasing amounts of IAM and Glut-P at a fixed enzyme concentration gave rise to an increased rate. At a fixed enzyme concentration and increasing amounts of IAM and Glut-P, accessibility of the substrate and cofactors is reduced therefore diluting the rate. The immobilized enzymes were shown to be reusable and capable of testing different substrates after regeneration was carried out. A variety of substrates can therefore be examined once the enzyme is immobilized limiting the amount of enzyme to be used.

Many assays in the literature for DBH require the use of catalase to protect the active site from hydrogen peroxide, which is a by-product in the initial step of the reaction. For the non-immobilized enzyme there was a visible increase in the rate with

increasing amounts of catalase. However, for both types of immobilized enzyme minimal amounts of catalase are required for maximal activity. The rates for both forms of immobilized enzyme (see Table 2.1) are lower relative to the non-immobilized DBH. This suggests the enzyme is working slower and therefore the amount of hydrogen peroxide produced as a by-product is minimal and does not affect the enzyme activity. The immobilized enzymes have conformations that do not require large amounts of catalase for protection.

Under ideal assay conditions the K_m and V_{max} for different substrates were determined for all the enzyme forms. In both cases, immobilization onto Glut-P and IAM, the K_m and V_{max} showed a decrease. This is understandable when one considers the effects of diffusion. The diffusion of the substrate from the bulk solution to the microenvironment of the immobilized enzyme can limit the rate. This in turn affects the concentration of the substrate/cofactor in the vicinity of the enzyme. However, differences in the kinetic properties of the DBH-Glut-P and DBH-IAM interphases were observed. The DBH-Glut-P interphase similar to the non-immobilized enzyme gave rise to a ping-pong mechanism. The DBH-IAM interphase did not display a ping-pong mechanism, which is consistent with previous findings (Miras-Portugal et al., 1975). The different forms of immobilization explain these findings. When DBH is immobilized on the Glut-P interphase the enzyme is outside the stationary phase whereas with the IAM interphase the enzyme is embedded within the interphase surroundings. As such the DBH-Glut-P interphase can be utilized to characterize the enzyme found in the cytosol and information concerning the membrane-bound enzyme can be obtained with the DBH-IAM interphase.

Fusaric acid, an antibiotic produced by fungus, has been found to be a potent inhibitor of DBH. Inhibition of DBH has been shown to result in decreased sympathetic activity and marked hypotensive effects (Nagatsu et al., 1970). The results are consistent with previously reported results demonstrating that fusaric acid is capable of reducing endogenous levels of norepinephrine (Nagatsu et al., 1970). Fusaric acid was found to inhibit the DBH mediated formation of NE for DA at concentrations as low as 10^{-6} M. Fifty percent inhibition was achieved at similar concentrations for both enzyme forms.

Captopril contains a sulfhydryl moiety, which has been shown to be responsible for the attenuation of the vasoconstriction induced by sympathetic nerve stimulation (Mueller et al., 1999). Sulfhydryl compounds are known to inhibit DBH *in vivo* and *in vitro*. Noncompetitive inhibition with respect to tyramine was viewed with inhibitions achieved at concentrations as low as 150 μM. The inhibitory effect of captopril was shown to be reversed in a dose-dependent manner by cupric ions. The reversal of the inhibition was achieved with 2.5 μM cupric sulphate. Palatini et al showed reversal of the inhibition by 140 μM captopril at 1.5 μM Cu^{2+}, as $CuSO_4$ (Palatini et al., 1989).

DBH in chromaffin granules of the adrenal medulla occurs in a soluble form and a membrane bound form. The amino acid compositions of these two states of DBH are essentially identical (Aunis et al., 1977). However the two states have shown differences in pH stabilities. The non-immobilized enzyme was shown to have a pH optimum at pH 6.0. Consistent with our results the DBH-IAM interphase displayed a shift of 1.5 pH units, pH 7. The observed optimum pH for immobilized

DBH mimics physiological conditions for the membrane bound DBH. The support utilized for immobilization, IAM, mimics the membrane environment that membrane bound DBH is accustomed too. The IAM support contains covalently bound phospholipids which is an ideal support due to the fact that the membrane bound DBH has been shown to be linked with lipids of the membrane (Pidgeon et al., 1992).

Arrhenius plots for the immobilized and non-immobilized enzyme displayed different profiles. A continuous profile was visible for the non-immobilized DBH with an activation energy of 20.54 kJ/mol. At increased temperatures the non-immobilized enzymes displays a decrease in activity due to the thermal denaturation of the enzyme. The DBH-Glut-P interphase displayed a continuous profile as well with an activation energy of 38.37 kJ/mol. However, DBH-IAM interphase showed an Arrhenius plot with a break in the slope, and thermal denaturation was observed at temperatures exceeding 55°C. Immobilization onto the IAM interphase stabilizes the enzyme therefore limiting the thermal movement of molecules at the higher temperatures.

In a study carried out by Aunis et al. the properties of soluble DBH and membrane bound were examined (Aunis et al., 1977). The membrane bound enzyme was shown in contrast to the soluble form to have thermal denaturation at higher temperatures of 43.5-44°C. Similar discontinuities in the Arrhenius plots were obtained for the membrane bound DBH by the authors. Our findings demonstrate that the DBH-IAM and DBH-Glut-P interphases are representative of the membrane-bound and soluble enzyme.

Based on these results, the immobilized DBH interphases have been formatted for a flow system. In-line immobilized reactors based upon the two interphases have been developed and attached to an HPLC analytical column. The results of these studies will be presented elsewhere. The HPLC system will be utilized for the generation, separation and identification of substrates as well as for the identification of inhibitors of enzymatic activity. This will prove beneficial for the screening of substances for their pharmacological properties for membrane bound and soluble forms of DBH.

2.6. Acknowledgements

We would like to extend our gratitude to Gilles Gariepy and André Pageau from ThermoQuest for all their technical assistance and support throughout the study. We would also like to thank Dr. Yanxiao Zhang, Peter Michalatos and Vasilios Markoglou for their assistance with the preparation of this manuscript.

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Connecting Text

In the preceding chapter, DBH was immobilized by covalent immobilization and hydrophobic entrapment on silica-based liquid chromatographic supports. The synthesized phases were used in batch-wise (i.e. non-flow formats) formats for both qualitative and quantitative determinations of enzymatic activity. Both IMERs were enzymatically active and reproducible. The results obtained demonstrate that the two supports developed can be utilized for the screening of possible substrates and inhibitors for membrane-bound DBH and soluble DBH. The next chapter describes the development of on-line immobilized enzyme reactors based upon DBH that can be utilized through the use of switching valve technology to characterize mDBH and sDBH.

Chapter 3

ON-LINE SYNTHESIS UTILIZING IMMOBILIZED ENZYME REACTORS BASED UPON IMMOBILIZED DOPAMINE BETA-HYDROXYLASE IN MEMBRANE BOUND AND SOLUBILIZED FORMATS

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3.1. Abstract

Immobilized enzyme reactors (IMERs) based upon dopamine beta-hydroxylase (DBH) have been developed. Immobilized artificial membrane (IAM) and glutaraldehyde-P (Glut-P) stationary phases have been used to immobilize DBH. When DBH is immobilized on the Glut-P interphase the enzyme is outside the stationary phase whereas with the IAM interphase the enzyme is embedded within the interphase surroundings. The activity of each IMER and their ability for on-line hydroxylation has been investigated. The resulting IMERs are enzymatically active and reproducible. The IMERs can be utilized through the use of coupled chromatography to characterize the cytosolic (DBH-Glut-P-IMER) and membrane-bound (DBH-IAM-IMER) forms of the enzyme. The substrate is injected onto the individual IMERs and the reactants and products are eluted onto a phenylboronic acid column for on-line extraction. The substrates and products are then transported via a switching valve to coupled analytical columns. The results demonstrate that enzyme/substrate and enzyme/inhibitor interactions can be investigated with the on-line system. These IMERs can be utilized for the discovery and characterization of new drug candidates specific for the soluble form and membrane-bound form of DBH. The effects of flow rate, contact time, pH and temperature have also been investigated.

Running Title: dopamine beta-hydroxylase IMERs

Keywords: dopamine-beta hydroxylase, enzymes, high-throughput screening, reactors, switching valve technology.

3.2. Introduction

Dopamine is converted to norepinephrine by dopamine beta-hydroxylase (DBH). DBH is a key enzyme involved in the regulation of blood pressure by the nervous system and a target for antihypertensive drugs (Lewis et al., 1992). Compounds such as benzylhydrazines, benzyloxyamines as well as substances acting by copper chelation such as tropolone are known inhibitors *in vivo* and *in vitro* of DBH (Lewis et al., 1992). DBH is a copper containing protein and does not show a high degree of substrate specificity oxidizing a variety of substrates. The resultant structurally analogous metabolites are capable of replacing norepinephrine at noradrenergic nerve endings therefore functioning as "false neurotransmitters" (Helle et al., 1984).

The enzyme is situated within catecholamine-containing chromaffin granules in contrast to the other catecholamine-synthesizing enzymes (tyrosine-hydroxylase, dopa decarboxylase and phenylethanolamine N-methyltransferase) that are present in the cytoplasm (Nagatsu et al., 1995). DBH exists in two forms, the membrane bound (mDBH) which is reinternalized upon exocytosis and the soluble form (sDBH) that is stored in the granule and secreted (Helle et al., 1984). sDBH and mDBH are composed of four subunits (Richard et al.,1988) and display differences in pH stability and substrate affinity (Nagatsu et al., 1972).

The majority of the information obtained concerning DBH has been through enzymatic assays that have utilized the soluble form of the enzyme. Since the two forms have distinct differences it is important to carryout assays that investigate the

effects of substrates and inhibitors on both enzyme forms. We have previously reported the immobilization of DBH onto solid supports that could be used in batch incubations to investigate the qualitative and quantitative aspects of sDBH and mDBH kinetics (Markoglou and Wainer, 2001). The immobilized artificial membrane (IAM) and glutaraldehyde-P (Glut-P) stationary phases were used. The findings of the study demonstrated that through the use of different immobilization procedures and different supports the two enzyme forms could be characterized.

The IAM-SP is derived from the covalent immobilization of 1-myristoyl-2-[(13-carboxyl)tridecanoyl)]-sn-3-glycerophosphocholine on aminopropyl silica, and resembles one-half of a cellular membrane (Pidgeon, 1990). In the IAM-SP, the phosphatidylcholine headgroups form the surface of the support and the hydrocarbon side chains produce a hydrophobic interface that extends from the charged headgroup to the surface of the silica. With the IAM interphase, DBH is embedded within the interphase surroundings (Figure 3.1A). The results of the study confirmed that information concerning mDBH could be obtained with the DBH-IAM interphase (Markoglou and Wainer, 2001)

Glutaraldehyde-P is a wide pore silica that has been covalently clad with a hydrophilic polymer, polyethleneimine (Narayanan et al., 1990). Immobilization of DBH onto the interphase results in formation of an amine-aldehyde Schiff linkage with Glut-P (Markoglou and Wainer, 2001; Narayanan et al, 1990). The DBH-Glut-P interphase proved to be useful in the characterization of the soluble form of the enzyme (Figure 3.1B).

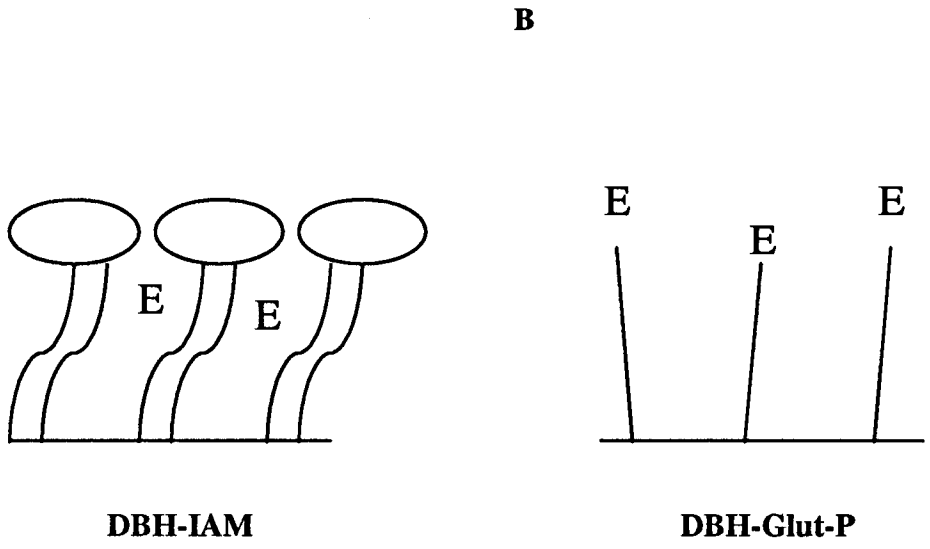

Figure 3.1: Representation of DBH immobilized onto two different supports in order to mimic mDBH and sDBH. A: DBH- IAM; B: DBH-Glut-P.

The aim of the present study was to develop immobilized sDBH and mDBH-based liquid chromatographic phases that could be attached on-line to HPLC analytical columns for screening of DBH inhibitors. The DBH-IAM and DBH-Glut-P stationary phases were prepared and packed into columns. Using switching valves, the individual IMERs were linked to a phenylboronic acid column and coupled analytical columns. The resulting IMERs retained their catalytic activities displaying distinct sensitivity to pH, temperature and inhibitors.

3.3. Experimental

3.3.1. Materials

Dopamine-beta-hyrdoxylase (from bovine adrenals), catalase (from bovine liver), DL-octopamine hydrochloride, tyramine hydrochloride, (±)- norepinephrine bitartrate salt, (-)-norepinephrine bitartrate salt, dopamine hydrochloride, fumaric acid, fusaric acid, captopril, ascorbic acid, and other chemicals unless otherwise stated were obtained from Sigma Chemical Company (St. Louis, MO, USA). Glacial acetic acid, HPLC grade, was manufactured by J.T. Baker (Phillipsburg, NJ, USA) and purchased through Moquin Scientific (Montreal, QC, Canada). The IAM.PC (12 μm, 300 Å) non-endcapped chromatographic support was obtained from Regis Chemical Co. (Morton Grove, IL, USA). The IAM.PC bonded phase, according to the manufacturer, contains a near monolayer of C14 saturated phosphatidylcholine, covalently linked to silica through an amide link. Glutaraldehyde-P affinity packing (40 μm, 300 Å) was obtained from J.T. Baker Inc.

3.3.2. Instrumentation and Operating Conditions

Three modular HPLC systems were setup in order to carry out on-line hydroxylation of tyramine by the DBH-IMERs. System 1 consisted of a Thermo Separation Products P1000 pump (ThermoQuest, San Jose, CA, USA), a Rheodyne 7125 injector with a 100 µl sample loop (Rheodyne, Cotati, CA, USA), and the DBH-IMER of interest. System 2 consisted of a Thermo Separation Products P2000 binary pump and a phenylboronic acid column (PBA). System 3 consisted of a Thermo Separation Products P1000 pump, a 5µm cyano (CN) stationary phase packed in 150 x 4.6 mm i.d. column (Regis Chemical Co. Morton Grove, IL), a 5µm octadecyl (ODS) stationary phase packed in a 250 x 4.6 i.d. mm column (Regis Chemical Co. Morton Grove, IL) connected in series, a SpectraSystem FL2000 fluorescence detector, and a Thermo Separation Products Chromjet integrator interfaced with a computer equipped with WOW software for data collection. The eluent from system 1 was directed onto system 2 then onto system 3 through Rheodyne 7000 switching valves (SV).

System 3 was used independently of systems 1 and 2 by replacing the latter systems with a Rheodyne 7125 injector (i) in order to analyze the results obtained from incubations involving non-immobilized DBH and DBH immobilized onto the loose Glut-P stationary phase. For the temperature studies, the DBH-IMER temperature was controlled with a Fiatron System CH-50 Column Heater (Fiatron, Wisconsin, USA).

3.3.3. Chromatographic Conditions

The mobile phase on system 1 consisted of sodium acetate buffer [10mM at the appropriate pH for each DBH-IMER] with a flow rate of 0.2 ml/min. System 3

contained two mobile phases A and B. Mobile phase A consisted of sodium phosphate buffer [25 mM, pH 8.4] and mobile phase B consisted of sodium phosphate buffer [25 mM, pH 4]. A mobile phase consisting of potassium phosphate buffer [25 mM adjusted to pH 2.0 with trifluoroacetic acid] was utilized for system 3 to achieve the desired chromatographic separation of the products from the substrates. The solutes were quantitated using fluorescence detection with excitation at $\lambda = 266$nm and emission at $\lambda = 380$nm. A flow rate of 0.7ml/min and ambient temperature were used for system 2 throughout the study.

3.3.4. Enzyme Immobilization on Loose Packing Material

3.3.4.1. Immobilization of DBH onto IAM

DBH was immobilized onto IAM stationary phase utilizing a previously described method (Markoglou and Wainer. 2001). IAM stationary phase (200-250mg) was washed five times with sodium acetate buffer [0.1M, pH 5.5]. The washing was carried out by adding 2ml of buffer to the packing material, the suspension was centrifuged at 3000 xg for 5 min and the supernatant decanted. The enzyme solution (1.65 mg in 2 ml sodium acetate buffer [0.1M, pH 5.5]) was added to the stationary phase, the mixture was placed in a rotator/stirrer for 12h at ambient temperature. At the end of 12 h, the suspension was centrifuged for 5min, the supernatant was collected and the stationary phase was washed an additional five times with buffer. The amount of enzyme immobilized was determined by measuring the amount of residual enzyme present in the supernatant using the Bio-Rad Protein Assay (Bio-Rad Laboratories Ltd, Mississauga, Ontario, Canada). The difference in the absorbance

reading before immobilization and the combined absorbances of the washings after immobilization determined the amount of enzyme bound on the IAM stationary phase.

3.3.4.2. Immobilization of DBH onto the Glut-P Interphase

The immobilization of DBH onto the Glut-P interphase involved a similar approach to that utilized for immobilization onto IAM. The washing of the stationary phase involved the addition of 2ml of sodium acetate buffer to the material [0.1M, pH 6.0]. The enzyme solution (1.65 mg in 2 ml sodium acetate buffer [0.1M, pH 6.0]) was added to the stationary phase, the mixture was placed in a rotator/stirrer for 12h at ambient temperature. The amount of enzyme immobilized was measured utilizing a similar approach described for immobilization onto IAM material.

3.3.5. Preparation of DBH Immobilized Enzyme Reactors

DBH immobilized on the IAM or Glut-P stationary phase was packed into a 1 cm x 10 mm i.d. column (Regis Technologies) to create the DBH-IMERs. The IMERS were separately connected to a chromatographic system. The DBH-IAM-IMER and the DBH-Glut-P-IMER was washed with sodium acetate buffer [0.1M, pH 5.5] and sodium acetate buffer [0.1M, pH 6.0], respectively. The eluent from both IMERs was collected in order to determine if any of the DBH had been washed off the columns. The Biorad assay was utilized to measure the amount of non-immobilized enzyme. When the columns were not in use they were washed with sodium acetate buffer at the respective pHs and stored at 4°C.

3.3.6. Procedure for on-line injection

3.3.6.1. DBH-IAM-IMER and DBH-Glut-P-IMER

A schematic diagram of the coupled HPLC system is presented in figure 3.2. Pumps 2 and 3 on systems 2 and 3 were stopped. 100 µl of a substrate/cofactor mixture is loaded into the injector (i) and the valve is switched to the inject position. At the same time, the switching valve (SV) is switched such that substrate/ product are eluted from the DBH-IMERs at a flow rate of 0.2 ml/min for 20 min, and concentrated onto the PBA column of system 2. The second pump was started and mobile phase A was pumped through the PBA column at a flow rate of 0.1 ml/min for 30 sec in order to elute, ascorbic acid and any other by-products produced from the reaction. The pump was then switched to mobile phase B with a flow rate of 0.1 ml/min for 2 min and simultaneously SV1 was switched such that unreacted substrate and product formed were concentrated on the analytical columns of System 3. When the specific contact time elapses the SV2 is switched back to the original position and the pump 3 is started.

3.3.7. Effect of flow rate and contact time on on-line system.

The effect of flow rate through the DBH-Glut-P-IMER and DBH-IAM-IMER was investigated at flow rates ranging from 0.1 to 1.0 ml/min at 0.1ml increments yielding a fixed elution volume of 3 ml at the respective flow rates.

The effect of contact time through the DBH-IMERs was also investigated at a fixed flow rate of 0.2 ml/min. Contact times from 5 to 30 min were investigated at 5 min increments.

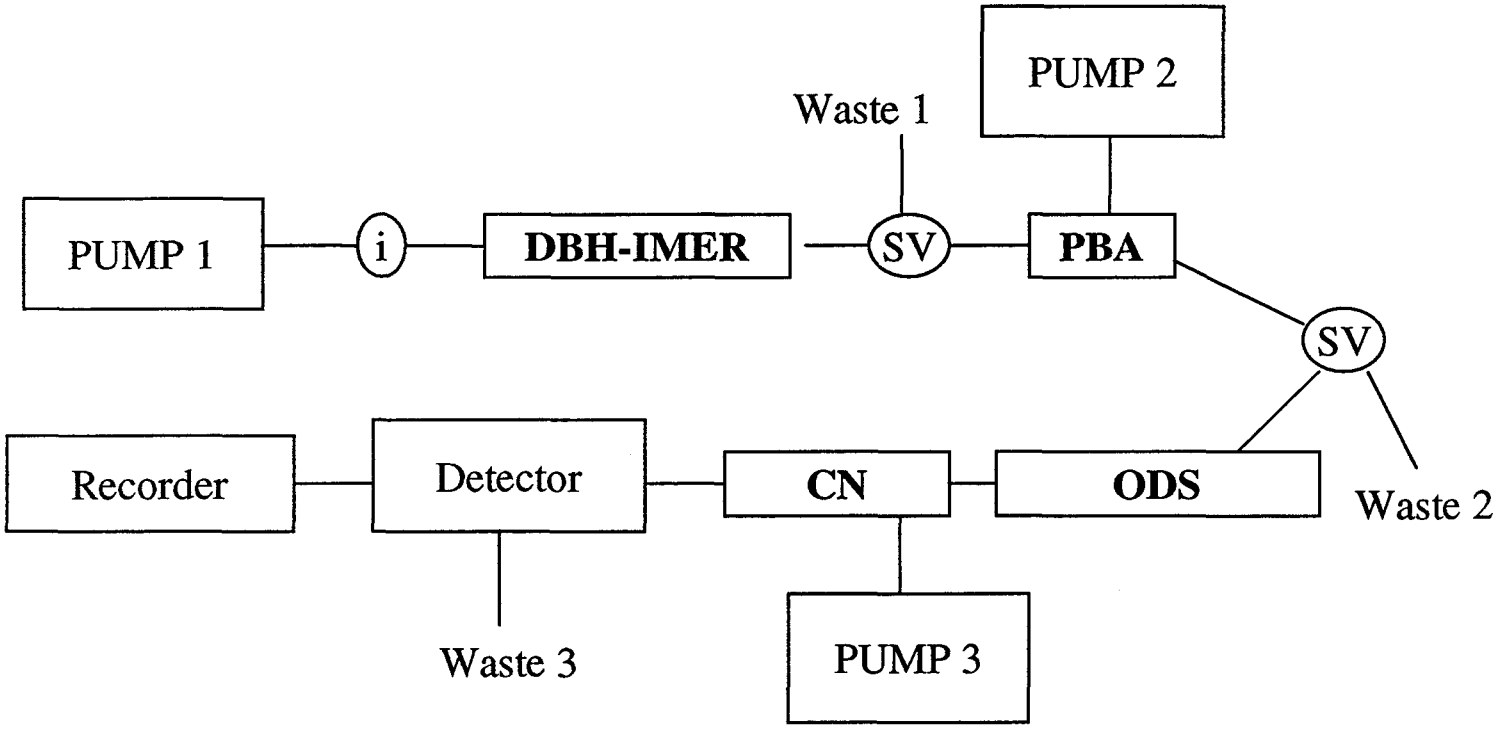

Figure 3.2.: Schematic representation of on-line DBH-IMER HPLC system.

The effect of flow rate and contact time was also examined on the PBA column to determine conditions that would yield maximal recovery of unreacted substrate and product formed. Flow rates ranging from 0.1 to 0.4 ml/min and contact times of 30 sec to 3 min were examined.

3.3.8. Effect of pH and temperature on the DBH-IMERs

The activities of the DBH-IMERs were measured at a series of pHs (with 0.1M buffers) to determine the optimum pH. The temperature of the IMERs was kept at 37°C.

The effect of temperature was also examined for the individual DBH-IMERs using temperatures ranging from 25°C (RT) to 60°C.

3.3.9. Enzyme activity and Inhibition Studies on DBH-Glut-P-IMER

The enzymatic activity on the DBH-Glut-P-IMER was determined by quantification of the amount of product formed with a given substrate. The temperature of the IMER unless otherwise stated was kept at 37°C with a column heater. Stock solutions of tyramine were prepared in water. The substrate concentrations examined ranged from 0.1-10 mM and that of the cofactor, ascorbic acid, ranged from 0.1-10 mM. Enzymatic activity was examined carrying out injections of a series of substrate/cofactor mixtures. The mixtures were injected onto the DBH-IMER at a flow rate of 0.3ml/min for a contact time of 10min. The kinetic parameters were determined using standard Michaelis-Menten approach (Dixon and Webb, 1979). Lineweaver-Burke plots were used to calculate the Michaelis constant

(K_m). The rates of reaction (V_{max}) were calculated using μmol/mg/min. Results are expressed as mean ± standard error of the mean (SEM).

The effect of known inhibitors, fusaric acid and captopril on the enzymatic activity of the DBH-Glut-P IMER was also examined. The inhibition of the IMER was carried out using injections of a series of substrate/cofactor/inhibitor mixtures.

3.4. Results and Discussion

DBH was previously reported to be immobilized covalently onto Glut-P silica based chromatographic phase and immobilized by hydrophobic entrapment onto IAM stationary phase (Markoglou and Wainer, 2001). In this study, 0.76 ± 0.21 mg of DBH was immobilized onto 320 ± 3.3 mg (n=3) of Glut-P and packed into a column to form the DBH-Glut-P-IMER. The DBH-IAM-IMER was formed in a similar manner with 0.89 ± 0.40 mg of DBH immobilized onto 305 ± 4.7 mg of IAM (n=3). These DBH interphases have been formatted into flow systems (see Figure 3.2).

Immobilized DBH in the flow systems was shown to be active. Chromatographic studies with the two IMERs are depicted in figure 3.3. A mixture of tyramine and ascorbic acid was injected onto the DBH-IMERs and the eluent from the IMERs were concentrated onto system 2 containing a PBA column for on-line extraction of ascorbic acid and any by-products produced during catalysis. Unreacted substrate and product are then concentrated onto coupled analytical columns for separation and analysis. Figures 3.3 B and C display typical chromatographic profiles achieved on the DBH-Glut-P-IMER and the DBH-IAM-IMER respectively.

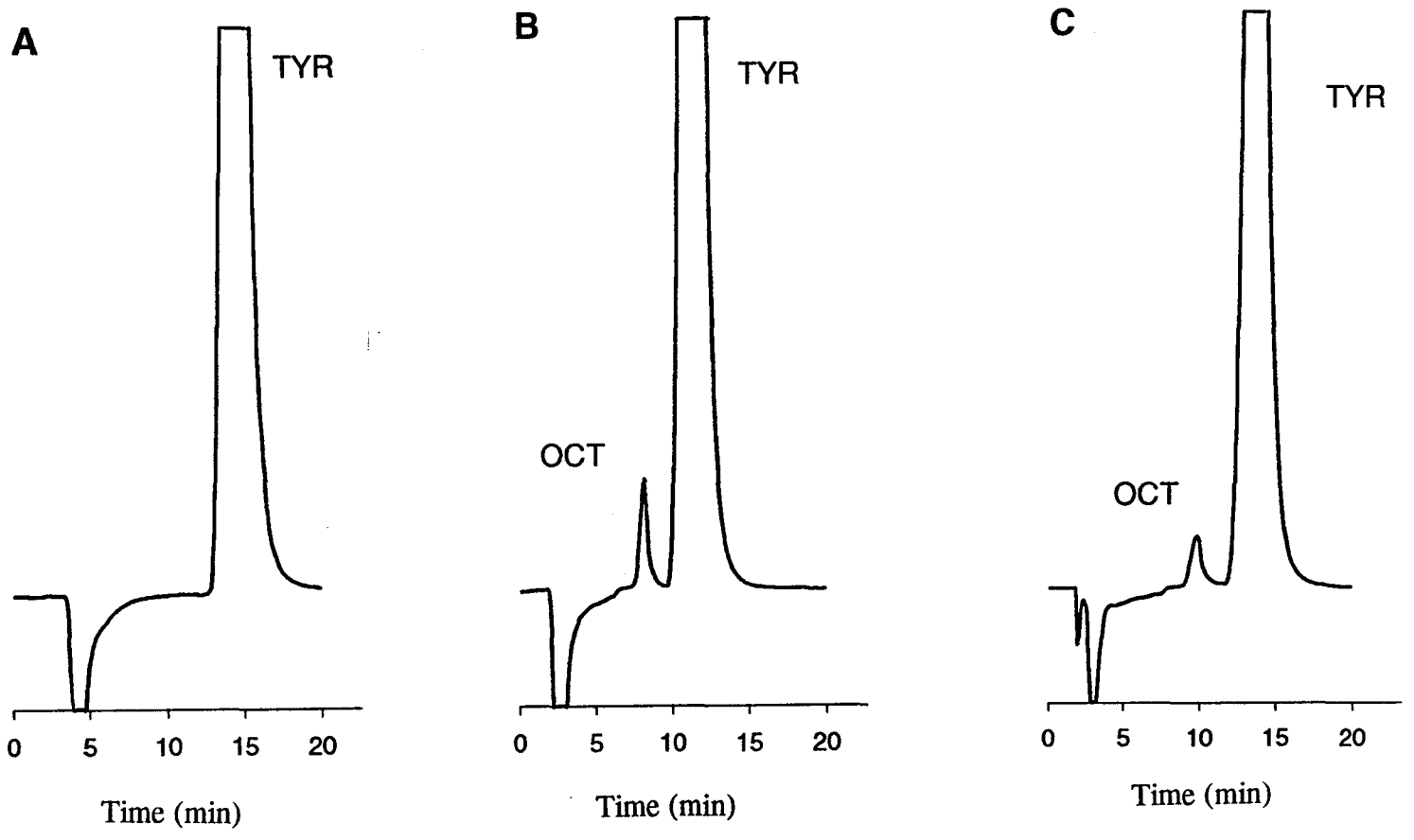

Figure 3.3: Representative chromatograms of on-line hydroxylation of tyramine. **A**: Control (Injection of 5mM tyramine no ascorbic acid present); **B**: Reaction on DBH-Glut-P-IMER (injection of 5mM tyramine / 1mM ascorbic acid); **C**: Reaction on DBH-IAM-IMER.

A positive control was carried out by injecting tyramine onto the system without the presence of the cofactor, ascorbic acid. No product formation was observed under these conditions as seen in figure 3.3 A.

Optimal conditions of flow rate and contact time had to be determined for each subset (i.e. systems 1 through 3) of the on-line system in order to achieve the maximal productivity. A flow rate of 0.2 ml/min through both IMERs with a contact time of 20 min resulted in the maximal recovery of the product formed. Extraction of the cofactor and other by-products was achieved on-line through the use of the PBA column. At a flow rate of 0.1 ml/min and a contact time of 30 sec over 95% of the unreacted substrate and product were extracted on-line.

The kinetic parameters, K_m and V_{max} were determined for the DBH-Glut-P-IMER. For the substrate, tyramine, the observed V_{max} was reduced by approximately half and the K_m remained similar to the non-immobilized enzyme, Table 3.1. Immobilization places the enzyme in a new microenvironment, which can impede the rate at which the substrate reaches the active site of the enzyme. Comparisons of the K_m and V_{max} values obtained with the DBH-Glut-P-IMER and the DBH-Glut-P-SP shows a reduction of the affinity and activity of the DBH-Glut-P-IMER.

The changes seen with the DBH-Glut-P-IMER must be due to the experimental format i.e. the change from a non-flowing system (non-immobilized DBH and DBH-Glut-P-SP) to a flowing system (DBH-Glut-P-IMER). They can be attributed to the kinetics of the distribution of the substrate from the mobile phase to the stationary phase and the shearing forces produced by the moving phase.

Non-immobilized DBH *	**K_m (mM)**	**V_{max} (µmol/mg/min)**
Tyramine	2.85	0.208
Ascorbic acid	0.62	0.185
DBH-Glut-P-SP *	**K_m (mM)**	**V_{max} (µmol/mg/min)**
Tyramine	1.04 ± 0.09	0.112 ± 0.032
Ascorbic acid	1.10 ± 0.12	0.047 ± 0.006
DBH-Glut-P-IMER	**K_m (mM)**	**V_{max} (µmol/mg/min)**
Tyramine	2.76 ± 0.46	0.079 ± 0.004
Ascorbic acid	0.54 ± 0.09	0.084 ± 0.002

* (Data obtained from Markoglou and Wainer, 2001, see page 69)

Table 3.1: Kinetic parameters for non-immobilized, immobilized DBH onto the Glut-P interphase (DBH-Glut-P-SP) and DBH Immobilized Enzyme Reactor (DBH-Glut-P-IMER). In the experiments with the DBH-Glut-P-IMER n = 3.

The effect of temperature and pH on the DBH-Glut-P-IMER was also examined. The DBH-Glut-P IMER displayed an optimal activity at 40°C after which the activity decreased with increasing temperature. Similar results were previously reported for the DBH-Glut-P-SP (Markoglou and Wainer, 2001). The activity of the DBH-Glut-P IMER was measured at a series of pHs to determine the optimum pH. A pH optimum of 6.0 was found for the IMER, which is consistent with previous finding for the DBH-Glut-P-SP (Markoglou and Wainer, 2001).

The ability to examine DBH activity on an on-line chromatographic system allows for the examination of possible inhibitors of the enzyme. The effect of fusaric acid and captopril, known inhibitors of DBH, was examined on the DBH-Glut-P IMER (Table 3.2). Fusaric acid and captopril were shown to inhibit the IMER at concentrations as low as 3nM and 50 μM, respectively. The IMER can therefore allow for the screening and characterization of potent inhibitors.

Previous findings have demonstrated the DBH-IAM and DBH-Glut-P interphases are representative of the membrane-bound and soluble enzyme (Markoglou and Wainer, 2001). In this study, two individuals IMERs, DBH-Glut-P-IMER and DBH-IAM-IMER were prepared and formatted onto an on-line system for the synthesis of octopamine from tyramine. Both IMERs can be used on the system for the generations, separation and identification of inhibitors and substrates. The individual IMERs will prove useful for the screening of substances for their pharmacological properties for membrane bound and soluble forms of DBH.

Inhibitor	IC_{50} (Non-immobilized DBH)	IC_{50} (DBH-Glut-P-IMER)
Captopril	150 μM	120 ± 1.2 μM
Fusaric Acid	7.5 nM	5.6 ± 0.3 nM

Table 3.2: The effect of known inhibitors on the activity of non-immobilized DBH and the DBH Immobilized Enzyme Reactor (DBH-Glut-P-IMER). In the inhibition studies with the DBH-Glut-P-IMER n = 3.

3.5. References

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Connecting Text

Chapters 2 and 3 concentrated on DBH, the enzyme responsible for the synthesis of epinephrine. The next chapter will focus on the second enzyme of interest, PNMT. The synthesis and characterization of immobilizing PNMT onto a silica-based support will be described. The immobilized enzyme will be utilized in a batch-wise format to investigate the effect of immobilization on the enzymatic activity and the potential applications.

Chapter 4

SYNTHESIS AND CHARACTERIZATION OF AN IMMOBILIZED PHENYLETHANOLAMINE N-METHYLTRANSFERASE LIQUID CHROMATOGRAPHIC STATIONARY PHASE

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4.1. Abstract

Norepinephrine is N-methylated to epinephrine by the catalytic effect of the terminal enzyme in catecholamine biosynthesis, phenylethanolamine N-methyltransferase (PNMT). PNMT has been covalently immobilized onto a silica-based liquid chromatographic support, glutaraldehyde-P (Glut-P). The resulting PNMT-Glut-P stationary phase (PNMT-SP) was enzymatically active, stable and reusable. Standard Michaelis-Menten kinetic studies were performed with both free and immobilized PNMT and known substrates and inhibitors were examined. The results demonstrate that the PNMT-SP can be utilized for the rapid screening of potential PNMT substrates as well as the screening of compounds for PNMT inhibitory activity.

Running Title: Immobilized Phenylethanolamine N-methyltransferase

Key Words: chromatography ; glutaraldehyde-P; immobilization; phenylethanolamine N-methyltransferase.

4.2. Introduction

Phenylethanolamine N-methyltransferase (PNMT) is the enzyme responsible for the N-methylation of norepinephrine to epinephrine. Enzymatic activity requires the presence of a hydroxyl group beta to the amino moiety and S-adenosyl-L-methionine as a methyl donor (Boulton et al., 1996). PNMT displays poor substrate specificity, transferring the methyl group to the nitrogen atom of a variety of β-hydroxylated amines (Grunewald et al., 1992).

PNMT plays a role in neuroendocrine and blood pressure regulation in the CNS. Increases in epinephrine and PNMT content have been demonstrated in numerous hypertensive animal models (Cahill et al., 1992). Inhibitors of PNMT have been shown to lower blood pressure, suggesting PNMT involvement in the maintenance of hypertension (Cahill et al., 1992).

Numerous methods have been developed to assay PNMT activity. Radiochemical methods and chromatographic methods coupled to electrochemical detection are among the most commonly used analytical methods (Molinoff et al., 1969; Ray et al., 1979; Saavedra et al., 1974; Trocewisz et al., 1982; Vogel et al., 1976). However, these methods either require the use of radioactive substrates and/or large amounts of purified enzyme. In addition, these methods use solubilized enzymes and tend to be complicated, costly and time-consuming.

One way to avoid problems associated with solubilized enzymes is their use in an immobilized form. In this approach, highly purified enzymes are not necessary and the immobilized enzymes are active and reusable following a simple washing procedure. An example of this approach is the work by Wainer et al. who have developed

immobilized enzyme reactors based upon α–chymotrypsin (Chui et al., 1992; Jadaud et al., 1989; Wainer et al., 1998), trypsin (Thelohan et al., 1989), lipase (Johnson et al., 1997; Sotolongo et al., 1999; Zhang et al., 1993), alcohol dehydrogenase (Alebic-Kolbah et al., 1993a ; Alebic-Kolbah et al., 1993b) β-glucoronidase (Alebic-Kolbah et al., 1993c; Pasternyk Di Marco et al., 1998; Pasternyk et al., 1998) and cytochrome P450s (Alebic-Kolbah et al., 1993d; Alebic-Kolbah et al., 1993e). These immobilized enzyme reactors have proven useful for the on-line or batch-wise generation, separation and identification of metabolites as well as for the identification of inhibitors.

In these studies, α–chymotrypsin was covalently immobilized onto a silica based liquid chromatographic stationary phase, glutaraldehyde-P (Glut-P) The immobilization was accomplished through the formation of a Schiff-base between an amine group on the α–chymotrypsin molecule and a glutaraldehyde moiety covalently linked to the stationary phase. The resulting liquid chromatographic stationary phase was shown to be enzymatically active and capable of the on-line liquid chromatographic stereochemical resolution of substrate analog amino acids and amino acid deriavatives.

In the present work, we report the use of the glutaraldehyde-P liquid chromatographic stationary phase for the covalent immobilization of PNMT. The resulting PNMT-Glut-P stationary phase (PNMT-SP) was stable and capable of the transmethylation of normetanephrine. Standard Michaelis-Menten kinetic studies were carried out for both free and immobilized PNMT. Known substrates and inhibitors for PNMT were examined, and the results demonstrate that the PNMT-SP can be utilized for both qualitative and quantitative determinations of enzymatic activity in batchwise

(i.e. non-flow) and flow formats. The PNMT-SP can be utilized for the rapid screening of potential PNMT substrates and inhibitors.

4.3. Materials and Methods

4.3.1. Materials

Phenylethanolamine-N-methyltransferase, S-adenosyl-L-methionine(SAM), DL-normetanephrine hydrochloride, DL-metanephrine, benzylamine hydrochloride, N-ethylmaleimide, p-chloromercuriphenylsulfonic acid monosodium salt and other chemicals unless otherwise stated were obtained from Sigma Chemical Co. (St.Louis, MO, USA). Glutaraldehyde-P 40μM affinity packing, 300Å was obtained from J.T. Baker Inc. (Phillipsburg, NJ, USA).

4.3.2. Apparatus

The chromatographic experiments were carried out using a Thermo Separation Products P1000 pump, a Thermo Separation Products AS3000 autosampler equipped with a 100 μl loop, a SpectraSystem FL2000 fluorescence detector and data collection was carried out using a Thermo Separation Products Chromjet integrator interfaced with a computer equipped with WOW software for data collection (ThermoQuest, San Jose, CA, USA). The chromatographic separations were performed using a 5μm phenyl stationary phase packed in 150 x 4.6 mm i.d. column (Regis Chemical Co. Morton Grove, IL) and a 5μm C18 stationary phase packed in 250 x 4.6 mm i.d. column (Regis Chemical Co.) connected in series.

4.3.3. Chromatographic Procedures

A mobile phase consisting of potassium phosphate buffer (50 mM) adjusted to pH 2.0 with trifluoroacetic acid was utilized to achieve the desired chromatographic separation of the products from the substrates. The solutes were quantitated using fluorescence detection with excitation at λ= 266 nm and emission at λ=380 nm. A flow rate of 0.7ml/min and ambient temperature were used throughout the study.

4.3.4. Assay for Phenylethanolamine N-methyltransferase activity

The activity of phenylethanolamine N-methyltransferase was assayed as follows: [final concentration] To 500 µL of enzyme solution [163 µg] was added 50 µL S-adenosyl-L-methionine [20µM] and the solution was vortexed for 1 min. The reaction was started with the addition of the 50 µL substrate [1mM]. The reaction solution was incubated for 10 min at 37°C in a shaking bath. The resulting solution was centrifuged at 3000xg and the supernatant were directly injected onto the HPLC under the above mentioned conditions.

4.3.5. Covalent Immobilization of PNMT

Immobilization onto the Glut-P liquid chromatographic stationary phase was accomplished in the following manner: (1) the stationary phase (10-100mg) was washed three times with 0.1 M sodium phosphate buffer adjusted to pH 8.30 with 5M NaOH. The washing was carried out by adding 1ml of buffer to the stationary phase, the suspension was mixed for 1min, centrifuged at 3000xg for 10 min, and the supernatant decanted. (2) The enzyme solution [98 µg in 0.6ml sodium phosphate

buffer (0.1M, pH 8.30)] was added to the packing material, the mixture was mixed gently for 5 min and then placed in a rotator/stirrer bath for 18 h at ambient temperature. (3) At the end of 18 h, the suspension was centrifuged, the supernatant decanted and the packing material was washed three additional times with buffer. (4) The amount of enzyme immobilized on the packing material was determined by measuring the amount of residual enzyme in the supernatant using the BioRad Protein Assay. The difference in the absorbance reading before immobilization and the combined absorbances of the washings after immobilization determined the amount of enzyme bound on the packing material.

4.3.6. Regeneration of Enzyme Activity

A simple washing procedure was utilized to regenerate the activity of the immobilized enzyme. To the PNMT-Glut-P material was added 1ml of sodium phosphate buffer [0.1M, pH 8.30]. The mixture was mixed for 1 min, centrifuged at 3000xg for 10 min, and the supernatant was discarded. The PNMT-Glut-P material was stored in sodium phosphate buffer [0.1M, pH8.30] at 4°C and remained active. When the enzyme was stored at room temperature for an 18-day period almost 75% of the enzyme activity was lost. However, storage of the material at 4°C retained over 85% enzymatic activity for over a three-month period.

4.4. Results

4.4.1. Chromatographic results

Under the chromatographic conditions used in this study, normetanephrine and metanephrine were resolved from each other with relative retentions, k', of 7.63 and 13.04, respectively. Standard curves for metanephrine ranged from 1.25 x 10^{-5}mM - 1mM and for normetanephrine ranging from 6.25 x 10^{-3}mM-10mM. Standard curves for the substrate and product were linear over the range investigated. The results demonstrate that the product enzymatically formed by immobilized PNMT can be isolated by HPLC and determined by fluorescence detection. The boiled enzyme control and the reaction mixture contained no interfering peaks.

4.4.2. Optimization of PNMT Immobilization

The amount of PNMT immobilized onto the Glut-P stationary phase was examined using PNMT (98 μg in 0.6ml of sodium phosphate buffer [0.1M, pH 8.30] and 10-100mg of Glut-P. The amount of immobilized enzyme and the rate of the reaction were investigated for the different amounts of Glut-P packing material. When greater than 50mg of Glut-P were used to immobilize 163 μg of PNMT, over 80% of the enzyme was immobilized i.e. 135± 0.16 μg of PNMT was immobilized on 50 mg of the Glut-P material. However, there was a decrease in the enzymatic activity when greater than 50mg of the Glut-P material was used. The optimal conditions were therefore set at 163 μg of DBH being immobilized onto 50mg of Glut-P for the remainder of the experiments.

4.4.3. Effect of incubation and enzyme concentration on PNMT activity

The optimal assay conditions for both forms of the enzyme were determined by varying independently the length of incubation and amount of enzyme. The optimal conditions were determined to be a 10-min incubation utilizing 120-250μg of phenylethanolamine N-methyltransferase (Figures 4.1A and 4.1B). The amount of phenylethanolamine N-methyltransferase that was prepared for each experiment was determined utilizing the BioRad assay each time. The activity of free and immobilized PNMT as measured with the substrate normetanephrine, is linear upto 250 μg of enzyme under the assay conditions.

4.4.4. Effect of pH on PNMT Activity

The activity of the free and of the immobilized phenylethanolamine N-methyltransferase was measured at a series of pHs (with 0.1M ionic strength buffers) to determine the optimum pH. A pH optimum of 8.30 was found for both the immobilized and non-immobilized enzyme (Figure 4.2).

4.4.5. Effect of Substrate and Cofactor Concentration on PNMT Activity

The effect of normetanephrine concentrations on the enzymatic activities of non-immobilized and immobilized PNMT was examined. The standard Michaelis-Menten approach was utilized to determine kinetic parameters for both forms of the enzyme. The results are listed in Table 4.1.

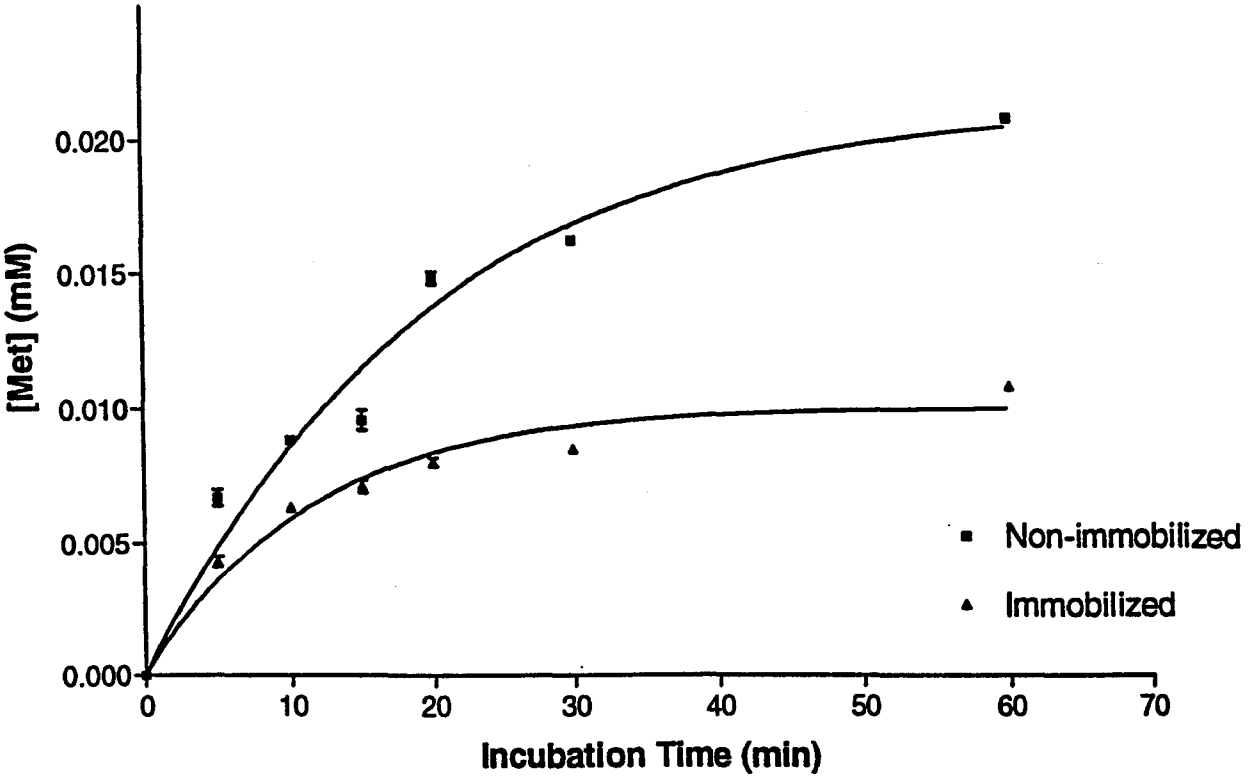

Figure 4.1. A: Effect of incubation time on PNMT activity for the immobilized and non-immobilized enzyme.

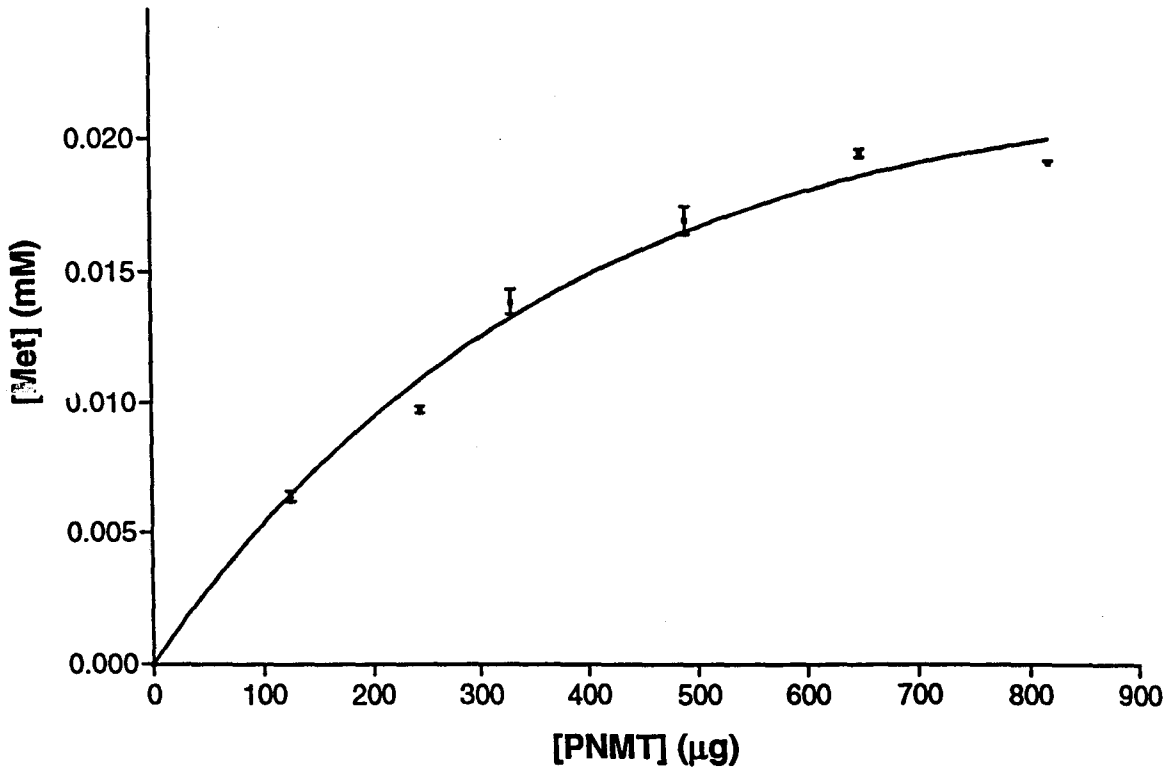

Figure 4.1. B: Effect of enzyme concentration on reaction rate for non-immobilized PNMT.

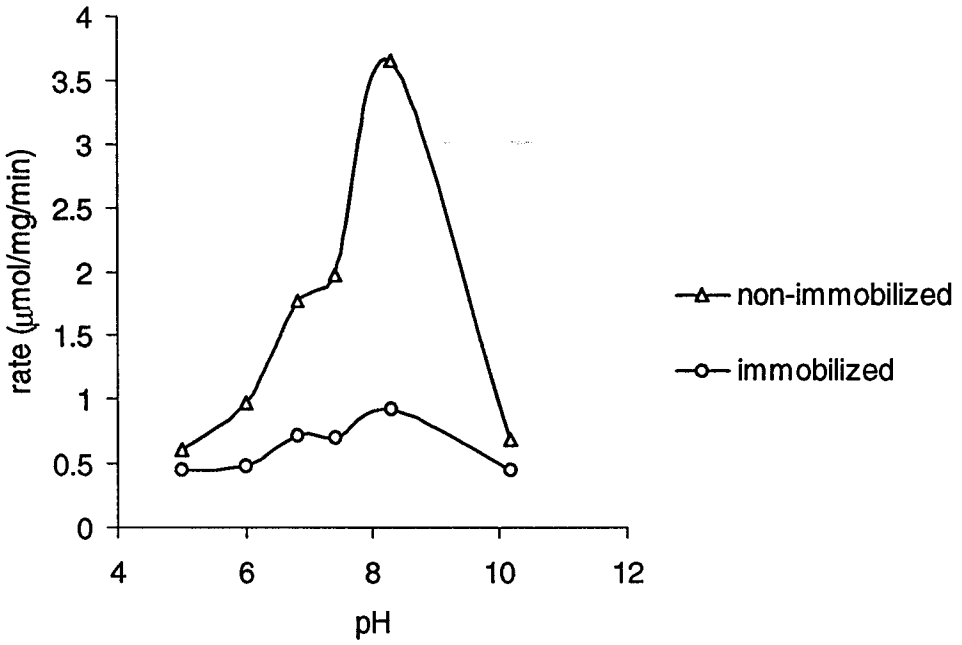

Figure 4.2: Effect of pH on PNMT activity for immobilized and non-immobilized PNMT (1mM normetanephrine, 20µM SAM, 163 µg PNMT, 10 min incubation).

4.4.6. Effect of temperature on PNMT activity

The effect of temperature was also examined for both the free and immobilized enzyme. The non-immobilized enzyme was shown to have optimal activity at 60°C after which the activity decreased with increasing temperature (Figure 4.3). The PNMT-Glut-P interphase however displayed optimal activity at 37°C with little change at increased temperatures. The Arrhenius plots for both forms of PNMT were drawn. Data yielded straight lines yielding activation energies of 11.04 kJ/mol and 7.61 kJ/mol for the non-immobilized and immobilized PNMT, respectively.

4.4.7. Effect of Inhibitor Concentration on PNMT activity

Benzylamine, p-chloromercuriphenylsulfonic acid, and N-ethylmaleimide are known inhibitors of phenylethanolamine N-methyltransferase (Grunewald et al., 1999). The effect of these compounds on the enzymatic activities of non-immobilized and PNMT-Glut-P was examined. The inhibition of PNMT activity by benzylamine was found to be three times higher with the immobilized PNMT in comparison to the non-immobilized PNMT (Figure 4.4). Similarly, the inhibitory effects of N-ethylmaleimide and p-chloromercuriphenylsulfonic acid were found to be higher for the immobilized form of the enzyme (Table 4.2).

5.0. Discussion

In this study PNMT has been immobilized onto a glutaraldehyde-P stationary phase and the enzyme remained active and retained its enzymatic characteristics. The kinetic parameters for the substrate and cofactor were determined for the immobilized

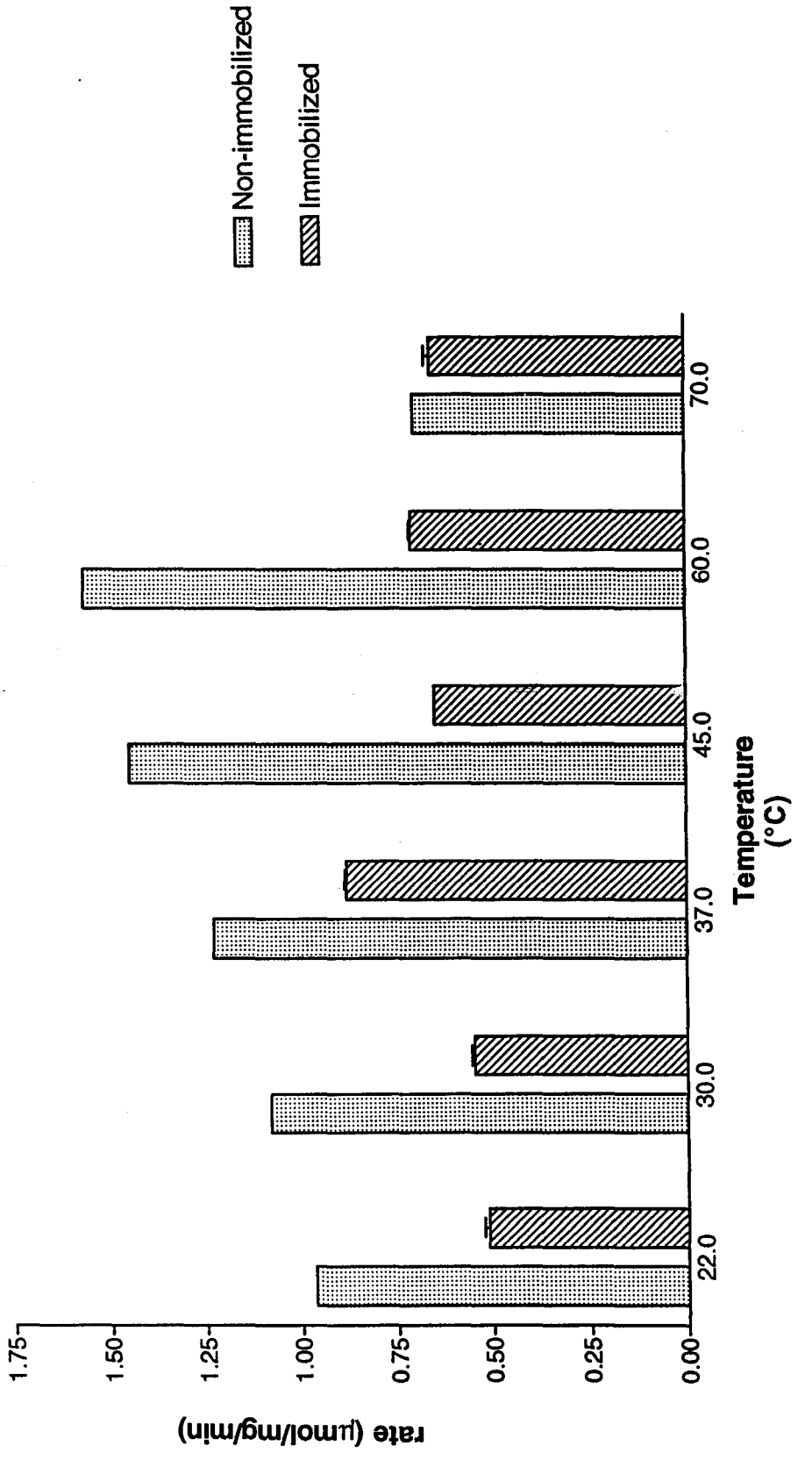

Figure 4.3 : Effect of temperature on immobilized and non-immobilized PNMT.

	PNMT	PNMT-SP
Normetanephrine		
K_m (mM)	0.109	0.152 ± 0.034
V_{max} (μmol/mg/min)	1.136	0.823 ± 0.012
SAM		
K_m (mM)	14.17	10.56 ± 0.14
V_{max} (μmol/mg/min)	1.249	0.654 ± 0.044

Table 4.1: Kinetic parameters for non-immobilized phenylethanolamine N-methyltransferase (PNMT) and immobilized phenylethanolamine N-methyltransferase (PNMT-SP).

and non-immobilized enzyme. The results obtained on the PNMT-SP are comparable to those obtained with the non-immobilized enzyme although the observed V_{max} and K_m values for the immobilized enzyme (Table 4.1) were lower relative to the non-immobilized enzyme. The relative differences in these enzymes may be due to the restrictions imposed by the immobilization. For example, the microenvironment of the immobilized enzyme can impede the rate at which the substrate and cofactor reach the active site. The conformational mobility of the enzyme may also be hindered by the covalent attachment to the chromatographic support.

The PNMT-Glut-P interphase displayed a similar pH optimum to that of the non-immobilized enzyme. Under the conditions utilized the optimal pH for activity was found to be 8.30 for both forms of the enzyme. As the non-immobilized enzyme is heated past 60°C the enzyme activity is markedly decreased. A decrease in the activity is due to the thermal denaturation of the enzyme. However, for the immobilized enzyme optimal activity is visible at 37°C with very little change in the rate at the higher temperatures (Figure 4.3). Arrhenius plots for both enzyme forms displayed continuous profiles. Activation energies of the free and immobilized PNMT were 11.04 kJ/mol and 7.61 kJ/mol, respectively.

Thermal movement of molecules at higher temperatures is limited due to the immobilization of the enzyme. As a result, thermal denaturation is not visible at the higher temperatures for the immobilized enzyme. Similarly, previous reports have demonstrated the protective effect of S-adenosyl-L-methionine on the heat inactivation of the enzyme. The microennvironment of the immobilized enzyme may amplify the protective effect of S-adenosyl-L-methionine.

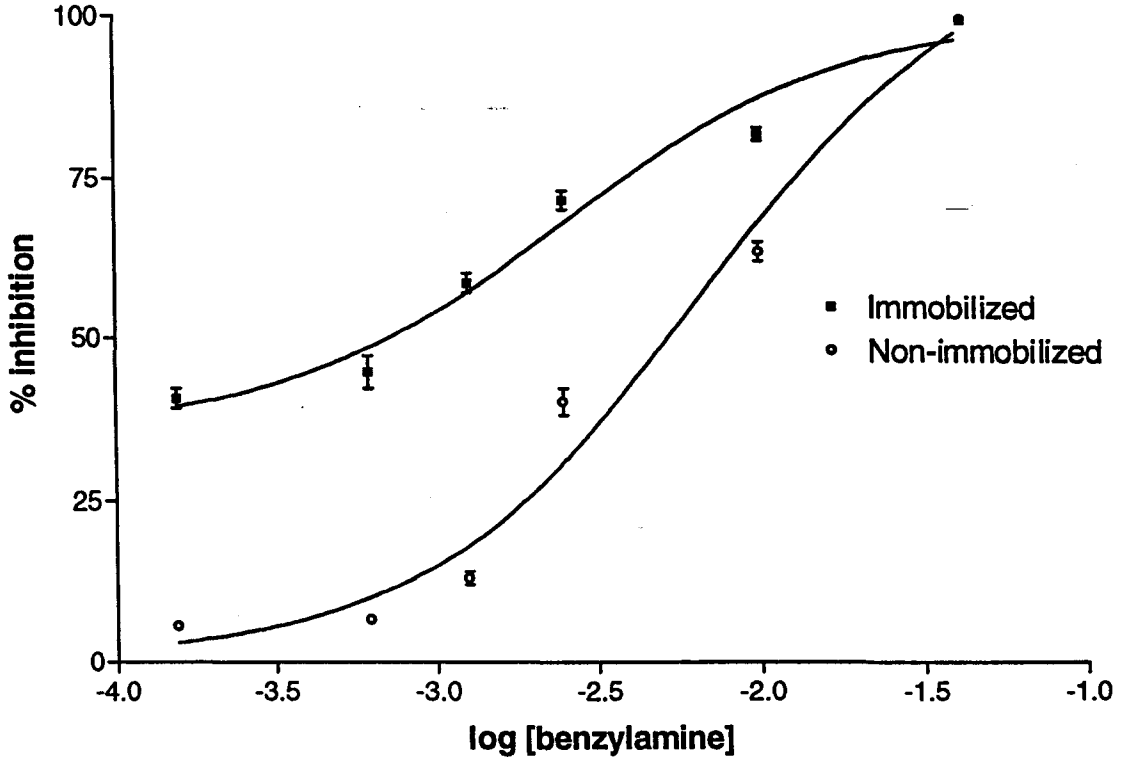

Figure 4.4: Inhibition of PNMT activity of both PNMT and PNMT-SP as a function of benzylamine.

The correct method of immobilization and choice of support is important when a comparison of the free enzyme to the immobilized enzyme is carried out. PNMT is localized in the soluble fraction of the adrenal medulla. When PNMT is immobilized on the Glut-P interphase the enzyme is outside the stationary phase and not embedded within the interphase surroundings. As such this form of immobilization is comparable to the non-immobilized cytosolic enzyme.

Several phenylethylamines, benzylamines and numerous conformational analogs of these compounds are used as *in vitro* and *in vivo* inhibitors of PNMT (Grunewald et al., 1999). The inhibitory effect of three PNMT inhibitors was investigated for both the immobilized and non-immobilized enzyme. Benzylamine was capable of inhibiting both types of enzymes at concentrations as low as $1x\ 10^{-4}$ M. Sulfhydryl reagents such as N-ethylmaleimide and p-chloromercuriphenylsulfonic acid were shown to inhibit both types of enzyme. These compounds are well-known in vitro inhibitors of PNMT because the enzyme in known to possess an essential sulfhydryl group (Boulton et al., 1996).

Numerous studies with hypertensive animals have been carried out to investigate the ability of PNMT inhibitors to lower blood pressure. Initial findings demonstrated a reduction in central epinephrine levels by PNMT inhibitors. Toomey et al. however discovered that the existing PNMT inhibitors were in fact non-selective and demonstrated α_2 - adrenoceptor binding affinity (Toomey et al., 1981). As such, the effects of the PNMT inhibitors could prove to be ambiguous if they display α_2-adrenoceptor affinity. Our studies demonstrate that the PNMT-SP can be utilized to screen for potent and selective inhibitors of the enzyme.

Inhibitor	PNMT	PNMT-SP
	Ki	Ki
Benzylamine	0.22 ± 0.11 mM	0.69 ± 0.10 mM
N-ethylmaleimide	1.04 ± 0.49 μM	4.92 ± 0.16 μM
p-chloro-mercuri-phenylsulfonic acid	0.12 ± 0.14 mM	0.52± 0.19 mM

Table 4.2: Effect of known inhibitors on immobilized (PNMT-SP) and non-immobilized phenylethanolamine N-methyltransferase (PNMT).

In this study, PNMT-SP was used in a batchwise (non-flow) format. Based upon our results, the immobilized PNMT-SP has been formatted for a flow system. Thus, an in-line immobilized enzyme reactor based upon the Glut-P stationary phase has been developed and attached to an HPLC analytical column (these studies will be reported elsewhere). This on-line system will prove to be a vital pharmacological tool for the development of potent PNMT inhibitors with minimal α_2 - adrenoceptor binding affinity.

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Connecting Text

In the preceding chapter, PNMT was immobilized by covalent immobilization on a silica-based liquid chromatographic support. The synthesized phase was used in batch-wise (i.e. non-flow formats) format for both qualitative and quantitative determinations of enzymatic activity. The phase was enzymatically active and reproducible. The following chapter describes the development of an on-line system based upon a PNMT-IMER that can be utilized through the use of switching valve technology for screening of possible substrates and inhibitors. With the individual IMERs developed and characterized the on-line coupling of the DBH-IMER and PNMT-IMER systems will be examined. A modular liquid chromatographic system for the two-step synthesis will be described.

Chapter 5

BIOSYNTHESIS USING AN ON-LINE IMMOBILIZED ENZYME REACTOR CONTAINING PHENYLETHANOLAMINE N-METHYLTRANSFERASE IN SINGLE ENZYME AND COUPLED ENZYME FORMATS

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5.1. Abstract

An immobilized enzyme reactor (IMER) based upon phenylethanolamnie N-methyltransferase (PNMT) has been developed. The activity of the PNMT-IMER and its applicability for on-line N-methylation of normetanephrine was investigated. The reactor was connected through a switching valve to a cyano (CN) and ODS stationary phase connected in series. The substrate was injected onto the PNMT-IMER and the unreacted substrate and product were eluted and transported via a switching valve onto the analytical columns. The results from the PNMT-IMER/ CN-ODS chromatographic system demonstrate that the enzyme retained its catalytic activity. Known substrates and inhibitors for PNMT were examined and the chromatographic system was utilized to carry out both quantitative and qualitative determinations. The PNMT-IMER/ CN-ODS system proves to be useful in basic biochemical studies, an ideal for the high throughput screening of substances for PNMT substrate/ inhibitor properties. The PNMT-IMER was then coupled in series using switching valve technology with a previously developed dopamine beta-hydroxylase immobilized enzyme reactor (DBH-IMER) and used to carry out the on-line two-step synthesis of epinephrine from dopamine.

Keywords: immobilization, enzymes, phenylethanolamine N-methyltransferase, screening, dopamine beta-hydroxylase, coupled system.

5.2. Introduction

Enzymes are primary targets of exploitation by the industrial and analytical fields due to their involvement in a wide variety of chemical transformations. However, the use of enzymes in these fields is limited by several factors such as the high cost of isolation and purification stability and difficulty to recover the enzymes for reuse. Enzyme immobilization is one approach that can be used to overcome these problems. In this technology the enzyme is associated with a matrix in order that the enzyme can be stabilized recovered from a reaction mixture and reused. Immobilized enzymes can be used in batch-wise experiments or packed into columns and used in flow systems as immobilized enzyme reactors (IMERs).

IMERs have been developed by numerous groups and have proven to be useful and economic alternatives to conventional enzymatic synthesis or screening for inhibitors and substrates. For example, IMERs have been developed based upon α–chymotrypsin (Chui et al., 1992; Jadaud et al., 1989; Wainer et al., 1988), trypsin (Thelohan et al., 1989), lipase (Johnson et al., 1997; Sotolongo et al., 1999; Zhang and Wainer, 1993), alcohol dehydrogenase (Alebic-Kolbah et al., 1993; Alebic-Kolbah and Wainer, 1993) and dopamine beta-hydroxylase (Markoglou and Wainer, 2001a). The IMERs were employed in HPLC systems coupled to analytical columns via switching valve technology. The systems proved ideal in obtaining both quantitative and qualitative information concerning biosynthetic and metabolic processes.

The enzymes, tyrosine hydroxylase, dopa-decarboxylase, dopamine beta-hydroxylase and phenylethanolamine N-methyltransferase are involved in the synthesis of catecholamines, dopamine, norepinephrine and epinephrine. These

enzymes are located in the adrenal medulla and in dopaminergic, noradrenergic and adrenergic nerves (Goldstein et al., 1972). Numerous studies have revealed their involvement in a broad range of physiological processes as well as being implicated in diseases such as Schizophrenia, Parkinson's disease and hypertension (Elenkov et al., 2000; Serova et al., 1998). As such, the development of drugs that can alter the function of these catecholamine-synthesizing enzymes are key targets for new drug development.

PNMT catalyzes the methylation of norepinephrine and its conversion to epinephrine. The conversion occurs in the adrenal medulla and in certain nuclei in the central nervous system. The addition of a methyl group significantly alters the pharmacology of the catecholamines. PNMT action has proven important in neuroendocrine and blood pressure regulation, and the importance of developing selective and potent inhibitors has become increasingly evident (Grunewald et al., 1999).

We have previously reported the use of the glutaraldehyde-P liquid chromatographic stationary phase for the covalent immobilization of PNMT (Markoglou and Wainer, 2001b). The resulting PNMT-Glut-P stationary phase (PNMT-SP) was stable and capable of the transmethylation of normetanephrine. Stnadard Michaelis-Menten kinetic studies were carried out for both free and immobilized PNMT. Known substrates and inhibitors for PNMT were examined, and the results demonstrated that the PNMT-SP can be utilized for both qualitative and quantitative determinations of enzymatic activity in batch-wise (i.e. non-flow) format.

The aim of the present study was to develop an IMER containing the phenylethanolamine N-methyltransferase (PNMT) based liquid chromatographic phase. The experimental goals were the demonstration that the PNMT-IMER could be used as a probe of biochemical and pharmacological properties and as an on-line high throughput screening for PNMT inhibitors. The PNMT-Glut-P stationary phase (PNMT-SP) was prepared and packed into a column. The resulting immobilized enzyme reactor (PNMT-IMER) was linked to coupled analytical HPLC columns through a switching valve and used for on-line N-methylation of known substrates of PNMT. The PNMT-IMER retained its catalytic activity and displayed sensitivity to pH, temperature and inhibitors. The results demonstrate that the PNMT-IMER can be utilized as a chromatographic probe of enzyme/substrate and enzyme/ inhibitor interactions. The HPLC system allows for the generation, separation and identification of substances as well as the identification of inhibitors.

The experimental utilization of the PNMT-IMER was expanded by coupling it to another previously reported IMER based upon DBH (DBH-IMER) (Markoglou and Wainer, 2001a). The coupled system was shown to be capable of carrying on-line synthesis of epinephrine from dopamine in a continuous flow system. The immobilized enzyme reactors used independently or as a combination will provide a unique opportunity to explore the interrelationships between these enzymes.

5.3. Experimental

5.3.1. Chemicals

Phenylethanolamine N-methyltransferase (from bovine adrenal medulla), s-adenosyl-L-methionine p-toluenesulfonate salt (SAM), DL-normetanephrine hydrochloride, DL-metanephrine hydrochloride, S-adenosyl-L-homocysteine (SAH), methyl-dopa, dopamine, norepinephrine, epinephrine and other chemicals unless otherwise stated were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Glutaraldehyde-P 40 μM affinity packing 300Å was obtained from J.T. Baker Inc. (Phillipsburg, NJ, USA). A 1cm phenylboronic acid cartridge from Varian Inc.(Palo Alto, California, USA) was used for on-line extraction for the coupled IMER system.

5.3.2. Instrumentation and Operating Conditions

5.3.2.1. PNMT-IMER system

Two modular HPLC systems were setup in order to carry out the chromatographic experiments (Figure 5.1A). System 1 consisted of a Thermo Spearation Products P1000 pump (ThermoQuest, San Jose, CA, USA), a Rheodyne 7125 injcetor with a 100 μl sample loop (Rheodyne, Cotati, CA, USA), and the PNMT-IMER. System 2 consisted of a Thermo Separation Products P1000 pump, a 5μm cyano (CN) stationary phase packed in 150 x 4.6 i.d. mm column (Regis Technologies, Morton Grove, IL), a 5 μm octadecyl (ODS) stationary phase packed in a 250 x 4.6 i.d. mm column (Regis Technologies, Morton Grove, IL) connected in series, a SpectraSystem FL2000 fluorescence detector, and a Thermo Separation

A

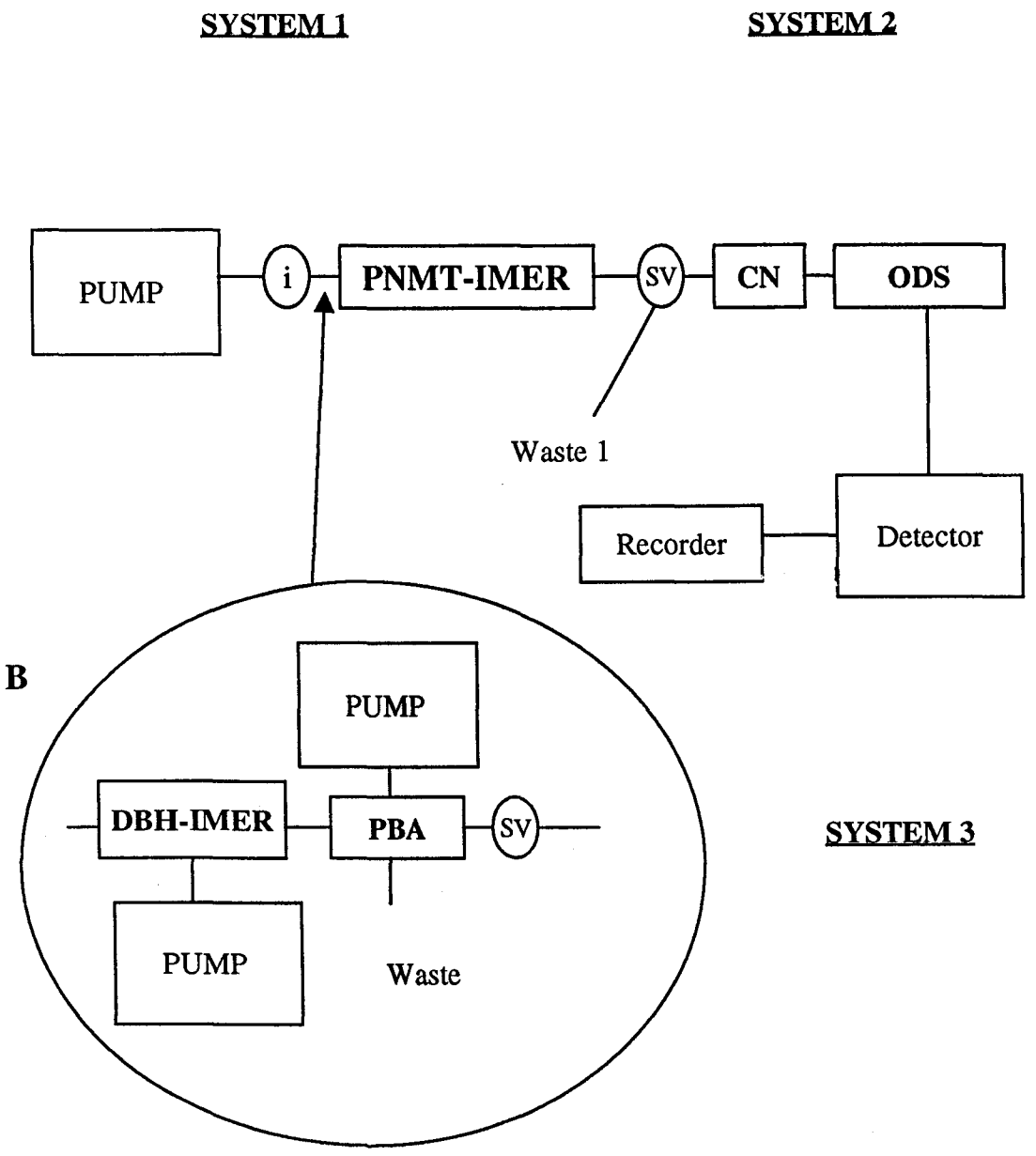

Figure 5.1: A: Schematic representation of on-line phenylethanolamine N-methyltransferase immobilized enzyme reactor (PNMT-IMER) HPLC system. B: Schematic representation of a dopamine beta-hydroxylase immobilized enzyme reactor (DBH-IMER) and phenylboronic acid (PBA) system that can be incorporated to the existing PNMT-IMER system for the on-line synthesis of epinephrine from dopamine.

Products Chromjet integrator interfaced with a computer equipped with WOW software for data collection. The eluent from system 1 was directed onto system 2 through a Rheodyne 7000 switching valve (SV).

System 2 was used independently of system 1 by replacing the latter system with a Rheodyne 7125 injector (i) in order to analyze the results obtained from incubations involving non-immobilized PNMT and PNMT immobilized onto the loose Glut-P stationary phase. For the temperature studies, the PNMT-IMER temperature was controlled with a Fiatron System CH-50 Column Heater (Fiatron, Wisconsin, USA).

5.3.2.2. Coupled IMER system

To the existing PNMT-IMER system was added the dopamine beta-hydroxylase immobilized enzyme reactor (DBH-IMER) coupled to a phenylboronic acid column (Figure 5.1B). Previously reported instrumentation and operating conditions were used for the DBH-IMER (Markoglou and Wainer, 2001a).

5.3.3. Chromatographic Conditions for PNMT-IMER system

The mobile phase on system 1 consisted of potassium phosphate buffer (0.1 M, pH 8.30) with a flow rate of 0.2 ml/min. A mobile phase consisting of potassium phosphate buffer (25 mM) adjusted to pH 2.0 with trifluoroacetic acid was utilized for system 2 to achieve the desired chromatographic separation of the products from the substrates. The solutes were quantitated using fluorescence detection with excitation at

$\lambda = 266$ nm and emission at $\lambda = 380$ nm. A flow rate of 0.7ml/min and ambient temperature were used for system 2 throughout the study.

5.3.4. Immobilization of PNMT on Loose Packing Material

PNMT was immobilized onto Glut-P stationary phase utilizing a previously reported method (Markoglou and Wainer, 2001b). Briefly, the following procedure was used: (1) the Glut-P stationary phase (300-350mg) was washed five times with sodium phosphate buffer [0.1M, pH 8.3]. In this step, 2 ml of buffer was added to the stationary phase, the suspension was vortex-mixed for 15 min, centrifuged and the supernatant decanted. (2) The enzyme solution (1.96 mg in 2ml sodium phosphate buffer [0.1M, pH 8.3]) was added to the packing material, the mixture was mixed gently for 15 min and then placed in a rotator /stirrer for 24h at ambient temperature. (3) At the end of 24h, the suspension was centrifuged, the supernatant decanted and the packing material washed three additional times with buffer. (4) The amount of enzyme immobilized on the stationary phase was determined by measuring the amount of residual enzyme present in the supernatant using the Bio-Rad Protein Assay (Bio-Rad Laboratories Ltd, Mississauga, ON, Canada). The difference in the absorbance reading before immobilization and the combined absorbances of the washings after immobilization determined the amount of enzyme bound on the Glut-P stationary phase.

5.3.5. Preparation of PNMT Immobilized Enzyme Reactor

PNMT immobilized on the Glut-P stationary phase was packed into a 1cm x 10mm guard (Regis Technologies). The guard was put into a holder and the column

was placed onto the chromatographic system. The PNMT-IMER was washed with phosphate buffer [0.1M, pH 8.3]. The eluent was collected in order to determine if any of the enzyme was being washed off the column. The Biorad assay was utilized to measure the amount of non-immobilized enzyme. When the column was not in use it was washed with phosphate buffer [0.1M, pH 8.3] and stored at 4°C.

5.3.6. Procedure for on-line injection

5.3.6.1. PNMT-IMER

A schematic diagram of the coupled HPLC system is presented in Figure 5.1. The pump on system 2 is stopped. 100 µl of a substrate/cofactor mixture is loaded into the injector (i) and the value is switched to the inject position at the same time the switching valve (SV) is switched such that the substrate/product are eluted from the PNMT-IMER and concentrated onto the analytical columns of System 2 for the specified contact time. When the specific contact time elapses the SV is switched back to the original position and the pump on system 2 is started. The unreacted and product formed are separated on the coupled analytical columns.

5.3.6.2. Coupled IMERs

A representation of the coupled IMER system is illustrated by the incorporation of DBH-IMER (Figure 5.1B) into the existing PNMT-IMER system (Figure 5.1A). In order to carry out the on-line synthesis of epinephrine from dopamine the following procedure was used: The first pump connected to the DBH-IMER had a mobile phase of sodium acetate buffer [10mM, pH 5.5] with a flow rate

of 0.3 ml/min. All the other pumps in the system were stopped. 100 µl of a mixture of dopamine and ascorbic acid was loaded into the injector (i) and the valve position switched such that the substrate/product were eluted from the DBH-IMER onto the PBA column where they were trapped.

Following a contact time of 10 min, the second pump was started and mobile phase A [sodium phosphate buffer 25 mM, pH 8.4] was pumped through the PBA column at a flow rate of 0.1ml/min for 30 sec in order to elute the cofactor, ascorbic acid, and any other by-products from the DBH catalyzed reaction. The pump was then switched to mobile phase B [sodium phosphate buffer 25 mM, pH 4] with a flow rate of 0.1 ml/min for 2 min and simultaneously SV1 was switched such that any unreacted substrate and product were eluted onto the PNMT-IMER. The PNMT-IMER system is treated as described above. The only consideration is the addition of SAM into the mobile phase of the pump connected to the PNMT-IMER. Unreacted dopamine and norepinephrine and epinephrine formed are concentrated and separated on the coupled analytical columns.

5.3.7. Effect of flow rate and contact time on the PNMT-IMER activity

The effect of the flow rate through the PNMT-IMER was investigated at flow rates ranging from 0.1 to 0.4 ml/min at 0.1ml/min increments. The contact time was 20min yielding elution volumes of 2, 4, 6 and 8 ml at the respective flow rates.

The effect of contact time through the PNMT-IMER was also investigated at a fixed flow rate of 0.2 ml/min. Contact times from 5 to 30 min were investigated at

5min increments. The recoveries of the substrate and product were determined (Appendix D).

5.3.8. Effect of pH and temperature on the PNMT-IMER activity

The activity of the PNMT-IMER was measured at a series of pHs (with 0.1M buffers) to determine the optimum pH. The temperature of the PNMT-IMER was kept at 37°C.

The effect of temperature was also examined for the PNMT-IMER using temperatures ranging from 25°C (RT) to 60°C (Appendix E). The effect of temperature on PNMT-SP and the PNMT-IMER was compared using Student's t-test for unpaired data. All p values < 0.05 were considered statistically significant.

5.3.9. Enzyme activity and Inhibition Studies on PNMT-IMER

The enzymatic activity on the PNMT-IMER was determined by quantification of the amount of product formed with a given substrate. The temperature of the PNMT-IMER unless otherwise stated was kept at 37°C with a column heater. Stock solutions of normetanephrine were prepared in water. The substrate concentrations examined ranged from 0.15-10mM and that of the cofactor, S-adenosyl-L-methionine, ranged from 5 - 100μM.

Enzymatic activity was examined carrying out injections of a series of substrate/cofactor mixtures. The mixtures were injected onto the IMER at a flow rate of 0.2ml/min for a contact time of 20min. The kinetic parameters were determined by drawing Lineweaver-Burke plots utilizing Microsoft Excel. Results are expressed as mean ± standard error of the mean (SEM).

The effect of known inhibitors, S-adenosylhomocysteine and methyldopa on the enzymatic activity of the PNMT-IMER was also examined. The inhibition of the PNMT-IMER was carried out using injections of a series of substrate / cofactor / inhibitor mixtures.

5.4. Results and Discussion

In the previously reported studies on the development of the PNMT-SP, 135 ± 0.16 μg of PNMT was immobilized onto 50mg of Glut-P (Markoglou and Wainer, 2001b). In this study, the same enzyme:support ratio was utilized such that 0.86 ± 0.13 mg of PNMT was immobilized onto 350 ± 5.4 mg of Glut-P and packed into a column to form the PNMT-IMER.

Chromatographic studies with the PNMT-IMER and the coupled-system depicted in Figure 5.1, demonstrated that the immobilized PNMT was active in the flow system. When a mixture containing normetanephrine (NM) and S-adenosyl-L-methionine (SAM) was injected onto the PNMT-IMER and the eluent from the PNMT-IMER analyzed on system 2, M appeared in the chromatogram, Figure 5.2 A. As a positive control, NM was injected onto the coupled system without the cofactor, SAM. Under these conditions, no product formation was observed in the resulting chromatogram, Figure 5.2B. The negative control consisted of a column packed with immobilized PNMT that had been heat inactivated before immobilization. Injections of NM/SAM mixtures onto the system containing the inactive PNMT-IMER did not result in the production of metanephrine (M). Thus, the production of M in this system was due to the activity of the PNMT-IMER. The productivity of the PNMT-IMER

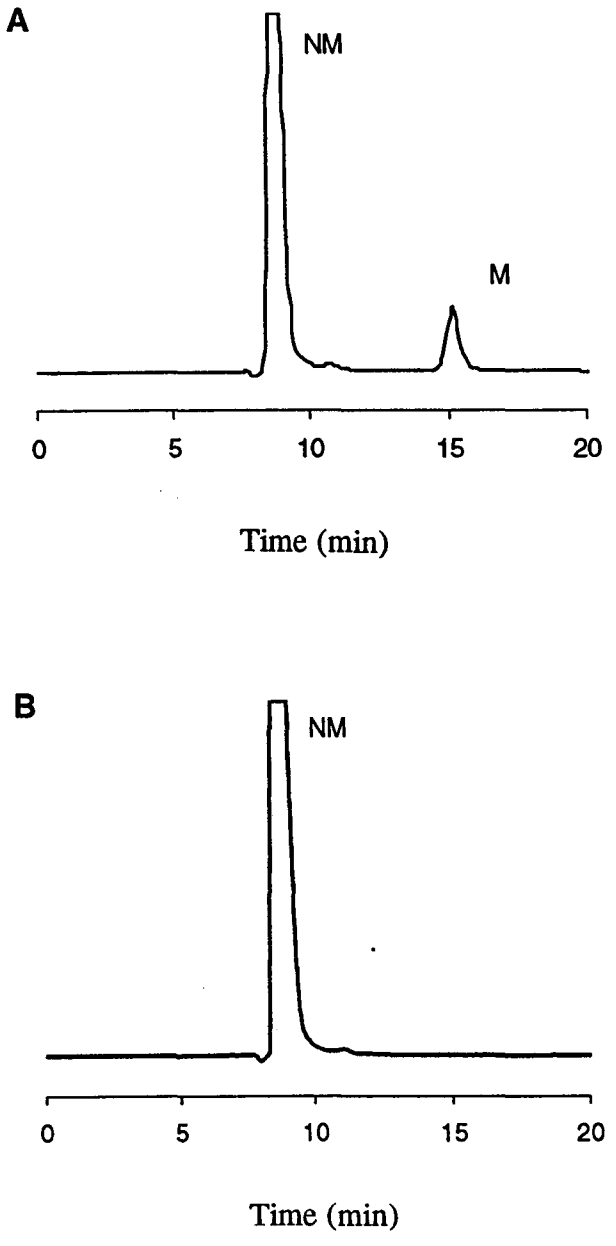

Figure 5.2: Representative chromatograms of the on-line N-methylation of normetanephrine (NM): (A) reaction mixture (injection of a 1mM NM/ 20μM S-adenosyl-L-methionine (SAM) mixture); (B) Control (injection of 1mM NM and no SAM present).

depends upon the time that the substrate/ cofactor mixture is in contact with the immobilized enzyme. Therefore, the flow rate through the PNMT-IMER is a key experimental variable. In order to optimize this factor, flow rates ranging from 0.1 to 0.4 ml/min at 0.05 ml/min increments were investigated using a fixed contact time of 20 min. A flow rate of 0.2 ml/min allowed for maximal recovery of product formed as well as any unreacted substrate (Figure 5.3).

The affinity (expressed as the Michaelis-Menten constant, K_m) and the enzymatic activity (expressed as maximum velocity, V_{max}) of the immobilized PNMT in the IMER format was determined. For the substrate, NM, the observed Km value was increased and the V_{max} reduced, both by a factor of approximately 4, relative to the non-immobilized enzyme, Table 5.1. An increase in K_m indicates a reduced affinity while a decrease in V_{max} indicates a reduced activity. Thus, the immobilization of PNMT negatively affected the enzyme's activity. However, the magnitudes of the observed effects were not solely due to the immobilization of the enzyme. Comparisons of the K_m and V_{max} values obtained with the PNMT-IMER and the PNMT-SP also show a reduction of the affinity and activity of the PNMT-IMER relative to the PNMT-SP. In this case, the values differed by a factor of approximately three, Table 5.1.

The immobilization of an enzyme places the protein in a new microenvironment that can impede the rate at which the substrate reaches the active site of the enzyme. This is demonstrated by changes in the K_m and V_{max} values between the non-immobilized PNMT and the PNMT-SP, Table 5.1. However, these

	PNMT*	PNMT-SP*	PNMT-IMER
Normetanephrine			
K_m (mM)	0.109	0.152	0.384
V_{max} (μmol/mg/min)	1.136	0.823	0.292
SAM			
K_m (μM)	14.17	10.56	7.31
V_{max} (μmol/mg/min)	1.249	0.254	0.424

(* Data obtained from reference Markoglou and Wainer, 2001b)

Table 5.1. Kinetic parameters for non-immobilized (PNMT), immobilized (PNMT-SP) and the immobilized phenylethanolamine N-methyltransferase reactor (PNMT-IMER)

values differ by less than 50%. Therefore, the magnitude of changes seen with the PNMT-IMER must be due to the experimental format i.e. the change from a non-flowing system (non-immobilized PNMT and PNMT-SP) to a flowing system (PNMT-IMER). In this case, the key factors may be the kinetics of the distribution of the substrate from the mobile phase to the stationary phase and the shearing forces produced by the moving phase.

The effect of temperature on the PNMT-IMER was also examined. The PNMT-IMER was shown to display maximum product formation at 37°C with limited changes in production at higher temperatures. Both the PNMT-IMER and PNMT-SP displayed no significant difference in the amount of product formed at temperatures exceeding 37°C (Figure 5.4). The non-immobilized enzyme however shows a considerable decrease in production of M at temperatures exceeding 60°C (Markoglou and Wainer, 2001b). The increase in stability of the immobilized enzymes is due to the environment that the enzymes are subjected to upon immobilization. Upon immobilization the enzyme is restricted in movement, which accounts for the lack of thermal denaturation at the higher temperatures.

PNMT is known to be inhibited by its own substrates and products at certain concentrations. The inhibitory effect of two PNMT inhibitors, S-adenosyl-L-homocysteine (SAH) and methyldopa was investigated for both PNMT-IMER and non-immobilized enzyme. Fifty percent inhibition was achieved at similar concentrations for both enzyme forms see Table 5.2. The PNMT-IMER was shown to be inhibited by SAH at concentrations as low as 5 μM and methyldopa concentrations

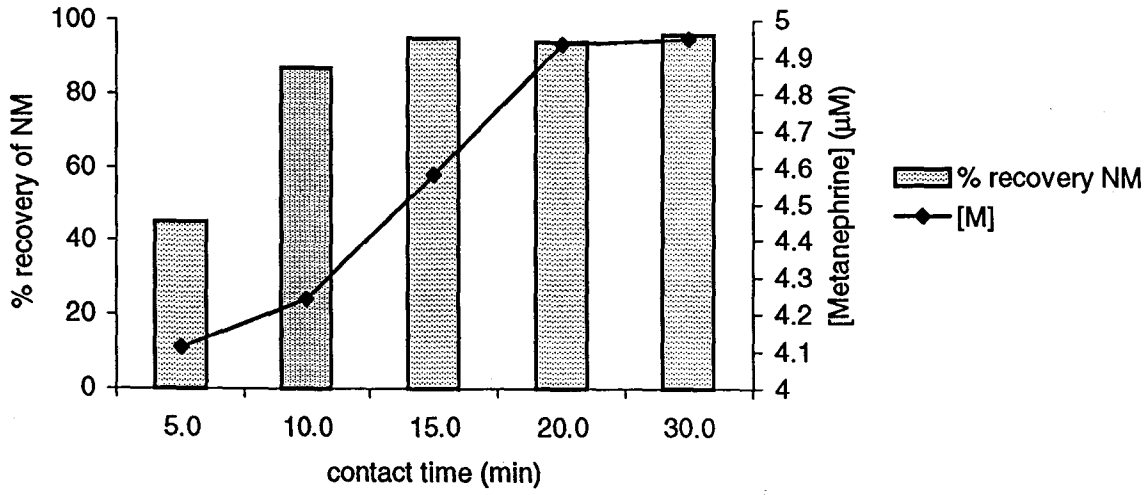

Figure 5.3: Effect of contact time on phenylethanolamine N-methyltransferase immobilized enzyme reactor (PNMT-IMER) activity (At a fixed flow rate of 0.2ml/min).

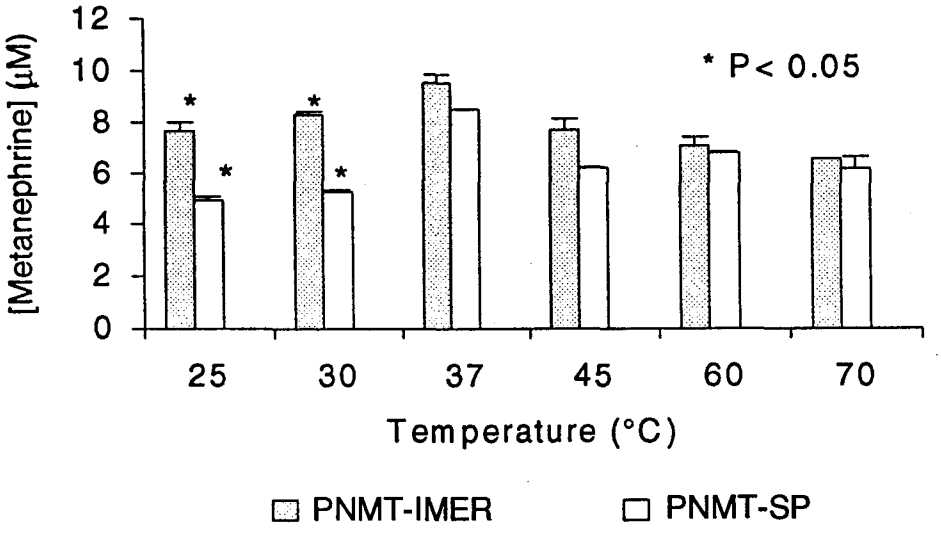

Figure 5.4: Effect of temperature on immobilized phenylethanolamine N-methyltransferase (PNMT-SP) in non-flow format (i.e.batch-wise) and immobilized PNMT in a column on the on-line system (PNMT-IMER) (n=3).

	IC_{50}	
Inhibitor	**PNMT**	**PNMT-IMER**
Methyl-dopa	10.4 μM	7.6 ± 0.2 μM
SAH	40.1 μM	50.5 ± 1.5 μM

Table 5.2: The effect of known inhibitors on the activity of non-immobilized phenylethanolamine N-methyltransferase (PNMT) and on the phenylethanolamine N-methyltransferase immobilized enzyme reactor (PNMT-IMER) (n=3).

of 1 μM. The PNMT-IMER can therefore be used to designate the relative affinities of potential PNMT inhibitors.

In this study, two individual IMERs based upon DBH and PNMT were coupled using switching valve technology and shown to carry out the on-line synthesis of epinephrine from dopamine (Figure 5.1A). Dopamine was injected onto the DBH-IMER and the reactants and products were eluted onto a phenylboronic acid column for on-line extraction. The substrates and products were transported via a switching valve to the PNMT-IMER. Norepinephrine was then converted into epinephrine by the PNMT-IMER and directed onto the analytical columns for analysis (Figures 5.5A and 5.5 B). The system allows for the analysis of the IMERs individually or as a combination. The construction of a coupled system of this nature provides a number of approaches to basic research into synthetic and metabolic pathways as well as a rapid method for the discovery of new pharmaceutical substances.

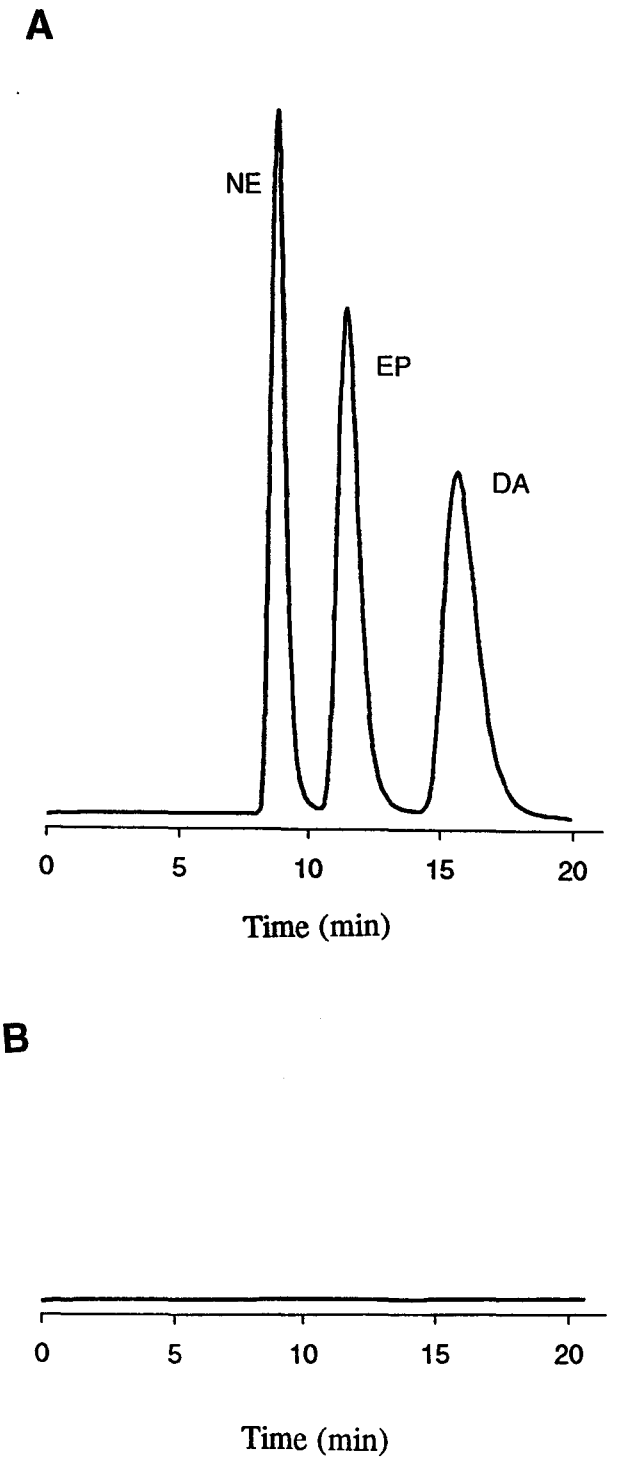

Figure 5.5.: Representative chomatograms of the coupled IMER system. A: On-line synthesis of epinephrine from dopamine. B: Control reaction (no dopamine present).

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Chapter 6

Discussion

6.1. Discussion

A drug molecule's pathway *in vivo*, from absorption to elimination, leads to interactions with biopolymers eliciting a wide variety of effects (Wainer et al., 1993). The characterization of these drug-biopolymer interactions is important in understanding their physiological and clinical significance. The experimental methods developed to evaluate the drug-biopolymer interactions are well established. However, the main disadvantages associated with the existing methods are that they are often complicated and costly.

The basic mechanisms and interactions that govern chromatographic systems resemble mechanisms viewed in biological processes. For instance, intermolecular interactions such as electrostatic and hydrogen bonding, are common in the biological and chromatographic environments. These similarities have prompted the inclusion of biomolecules in chromatographic systems (Wainer et al., 1993). The combination of biopolymers and the experimental techniques of HPLC allow for the rapid, reproducible and accurate reflection of drug-biopolymer interactions.

Biopolymers were originally exploited for the utilization of their high enantioselectivity and their potential for analytical and preparative separation and isolation of enantiomers (Seller et al., 1975). Numerous chiral stationary phases have been developed using enzymes, proteins, and macrocyclic antibiotics (Hermansson et al., 1984; Domenici et al., 1991; Ekborg-Ott et al., 1998). A unique example, is the immobilization of human serum albumin (HSA) and the development of a HSA-SP. Studies with the HSA-SP revealed its use in measuring protein binding of a

compound, enantioselectivity of the binding process and prediction of drug-drug protein binding interactions (Domenici et al., 1991; Aubry et al., 1995; Hage, 2001).

There have been an increased number of compounds generated as a result of combinatorial chemistry, genomics, informatics and high throughput screening techniques. Modern drug discovery has been transformed by the automation and industrialization of research techniques, and the pharmaceutical industries are interested in managing the abundance of possible drug candidates. Innovative technologies that accelerate the development of high-priority compounds through the drug discovery cycle are crucial. Immobilized biopolymer-based liquid chromatographic phases have proven to be ideal probes of biochemical and pharmacological properties governing drug-biopolymer interactions. Thus, their importance in modern drug discovery is evident.

Development of a drug from the discovery stage to reaching the market can average approximately 10-15 years (Figure 6.1). Acceleration of the drug development process is important for two main reasons. It is primarily important for the rapid discovery of new therapeutic agents to meet the unmet needs of patients with various diseases, and secondly for the obvious economic benefits for the pharmaceutical industry (Cancilla et al., 2000). High-throughput models that can assess and eradicate unfavorable properties from vast quantities of potential drug candidates are vital. However, analytical processes for the analysis and screening drug-biopolymer interactions are lacking (Cancilla et al., 2000). Techniques that can characterize these interactions without the need to isolate and dissociate drug/ biopolymer interactions are ideal innovations in reducing the drug discovery timeline.

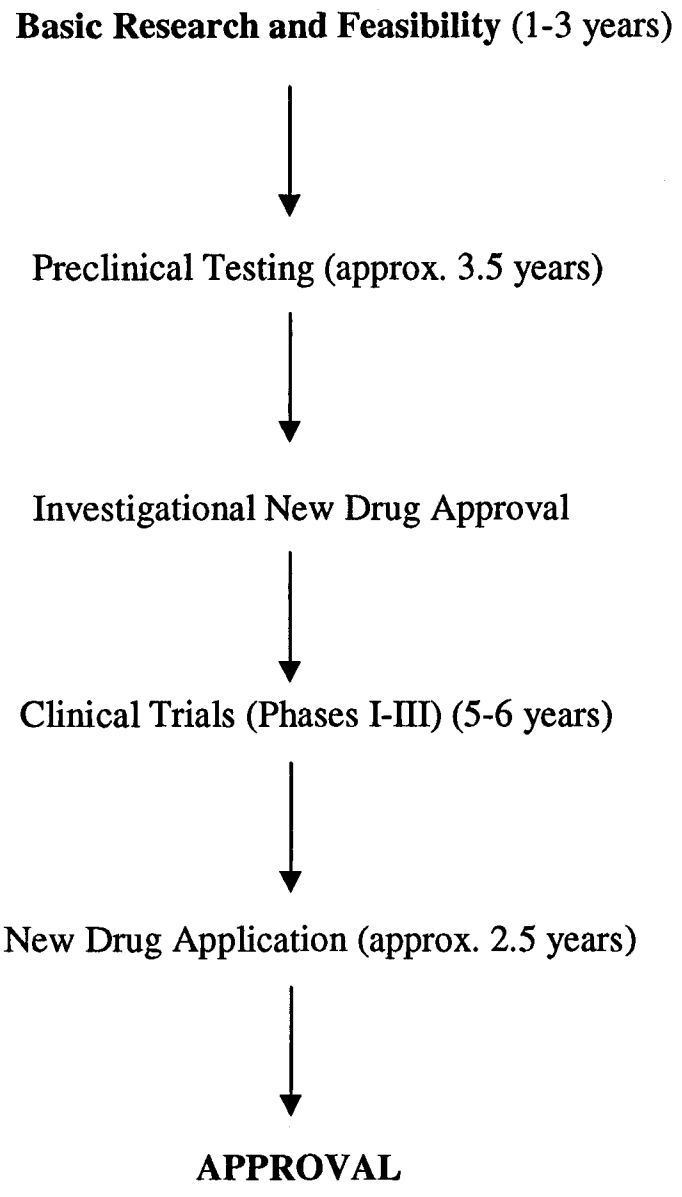

Figure 6.1: Drug Discovery Timeline: From the discovery phase to marketing of the drug.

The overall objective of this thesis work was the development of a prototype comprised of immobilized enzyme reactors that allows for the comprehensive study of a biosynthetic process. This prototype also demonstrates it usefulness in the drug discovery timeline. The objectives included the construction of several IMERs channelled via switching valve technology. The IMERs combined or individually gave rise to data that can be utilized with quantitative structural activity relationship and molecular modeling techniques. The data obtained from the IMERs along with the existing knowledge will aid in the understanding of the enzyme reactions as well as provide information into their mechanisms of action. The experimental procedures and analyses developed can then be utilized to investigate the pharmacokinetics and pharmacodynamics of potential therapeutic agents. This prototype will prove useful in the drug discovery process for the accurate and rapid determination of potential drug candidates.

In recent years a great deal of information has become available concerning the biochemistry and functional organization of catecholamine systems. This has lead to the increased interest in investigating the effects drugs have on the biosynthetic enzymes and their therapeutic roles in various catecholamine disorders. The multiple enzyme system chosen for the development of the prototypical multiple-IMER-HPLC system was the biosynthetic pathway for the formation of epinephrine from dopamine. This pathway was chosen because it has been extensively studied and substrates and products of each enzyme have been characterized. Thus, the effect of immobilization on the selectivity and activity of each enzyme could be readily assessed.

The final two enzymes in the pathway, DBH and PNMT, display distinct activities and co-factor requirements. Thus, the multiple-IMER-HPLC system demonstrated the ease to connect vastly different enzymes in a single on-line system and the ability to deal with each enzyme individually if needed. Any one of the steps involved in the biosynthesis of catecholamines could assume a rate-limiting role depending upon pathological or pharmacologically induced situations. For instance, reserpine can transform DBH into a rate-limiting step by blocking the access of DA to its site for the conversion into NE. The IMERs can be used to investigate the potential of newly synthesized compounds that are believed to have reserpine-like effects. This would allow for the discovery of new therapeutic agents.

Complete characterization of immobilized dopamine β-hydroxylase was carried out and comparisons to the non-immobilized enzyme were made. Using immobilized artificial membrane (IAM) and glutaraldehyde-P (Glut-P) stationary phases to immobilize DBH allowed for the development of stationary phases that can be utilized to discover and characterize new drug candidates specific for membrane-bound and soluble forms of the enzyme. *In vivo*, most of DBH is largely recovered as a component of the vesicle membrane i.e. in its membrane-bound form, mDBH (Dixon et al.,1975; Edwards et al., 1980). Most studies that have investigated DBH activity and the development of potential inhibitors for various clinical conditions have been based upon the use of the soluble forms of the enzyme, sDBH. This is due to the ease of purification from granule lysates by adsorption onto concanavalin A-sepharose (Rush et al., 1980; Wallace and Lovenberg, 1974).

However, mDBH and sDBH have been shown to display differences in pH stability and substrate affinities. The development of inhibitors for the soluble form of the enzyme should therefore not be definitively considered as potent and effective inhibitors for the membrane-bound form. The development of the two IMER systems (DBH-Glut-P-IMER and DBH-IAM-IMER) allows for the selective screening of potential inhibitor candidates for the soluble and membrane-bound form of the enzyme respectively. It would seem reasonable that since the majority of the enzyme is in its membrane-bound form and the research has concentrated on the soluble form that future investigations concentrate on their effects on the mDBH. The IMERs are unique tools for the rapid re-investigation of drugs that have already been investigated for sDBH and to obtain information concerning their effect on the membrane-bound form of the enzyme.

Similar to the DBH-IMERs, a PNMT-IMER was developed by covalently immobilizing the enzyme onto a silica-based support, Glut-P. The development of the IMER was shown to be advantageous relative to the soluble enzyme. The immobilized enzyme allowed for its reuse, isolation of the products from the reaction mixture, and the process could be run continuously. The IMER was used to quantitatively determine enzyme kinetic constants associated with enzyme/substrate and enzyme/inhibitor interactions. The PNMT-IMER can be used for the screening of potent and selective inhibitors of PNMT. The existing PNMT inhibitors are in fact non-selective and demonstrate α_2 –adrenoceptor binding affinity (Toomey et al., 1981). The on-line system is a vital pharmacological tool for the development of PNMT inhibitors that display minimal α_2 –adrenoceptor binding affinity.

Immobilizing multiple-enzyme systems have been previously reported. However, these studies dealt with the co-immobilization of a variety of enzymes (i.e. the enzymes were immobilized together on one support)(Chang, 1987). Although this may prove useful in basic research the method would be difficult in the investigation of different enzymes consisting of different concentrations and reaction conditions. An ideal example is the co-immobilization of hexokinase and pyruvate kinase within microcapsules (Campbell et al., 1975). Differences in K_m and V_{max} were visible when the co-immobilized enzymes were compared to the individual immobilized enzymes. This method of co-immobilization requires the balance of too many variables and makes quantitative determinations difficult. The IMERs developed and described in this thesis can be used independently or as a combination to provide unique opportunities to explore interrelationships between enzymes, which would not be possible, if they are co-immobilized. This system demonstrates the advantages of coupling the biological and chemical sciences.

Future Directions

A technological revolution has occurred in recent years resulting in greater productivity, reduction in time and cost and the development of novel drugs. One key advance in high throughput screening has been miniaturization and parallel processing. In relation to the supports described in this thesis, they can also be scaled down to create microscale analyzers. Microfabrication and micromachinery allows for the acceleration of large-scale screening of potential inhibitors and substrates of

the enzymes. The potential of microsystems is of great interest in the field of separation sciences.

Open-tubular columns, columns of small internal diameter, can be utilized as supports that can complement sophisticated detection techniques such as mass spectrometry. The enzymes can be bound to the internal walls of the columns. The advantages with using such a system are numerous. Small amounts of enzyme and reagents are required. This is ideal when examining the catecholamine synthesizing enzymes such as TH. Large amounts of TH are difficult to isolate and purify therefore the small amount required with the open-tubular procedure is ideal. In addition numerous compounds can be screened utilizing this approach and the coupling to sophisticated detection systems allows for quantitative determinations at extremely low concentrations. These modifications of the immobilized enzyme systems are currently under investigation (Appendix F).

Chapter 7

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Claims of Originality

The **general** claims in this thesis are as follows:

Multiple immobilized enzyme reactors (IMERs) can be used in a liquid chromatographic system to perform enzymatic transformations and compound synthesis. The particular aspects of the system are:

1. The system utilizes **separate** IMERs, not all of the enzymes immobilized in one column {although each IMER can contain more than one enzyme} and, therefore, the system is a **modular approach to multiple on-line syntheses.** IMERs can be added, subtracted or replaced to meet the requirements of the synthetic plan.
2. The system utilizes **on-line purification and identification** of the products after each enzymatic process in the modular system.
3. The system permits the **recycling** of unreacted starting materials to optimize their use.
4. The system can be used for **designed** synthetic pathways or to mimic **natural biosynthetic** processes.
5. The system as a **whole** can be used for **discovery and characterization** of new drug candidates, and **each IMER** can be used separately for the discovery and characterization of new drug candidates.

The **particular** claims in this thesis are as follows:

1. The synthesis of IMERs containing dopamine beta-hydroxylase and phenylethanolamine N-methyltransferase by **covalent immobilization and hydrophobic entrapment** on silica-based liquid chromatographic supports has been accomplished. Each IMER is a new entity.

2. The activity of the immobilized enzymes **reflects** the non-immobilized enzymes.

3. Each IMER can be used individually **to identify new drugs.**

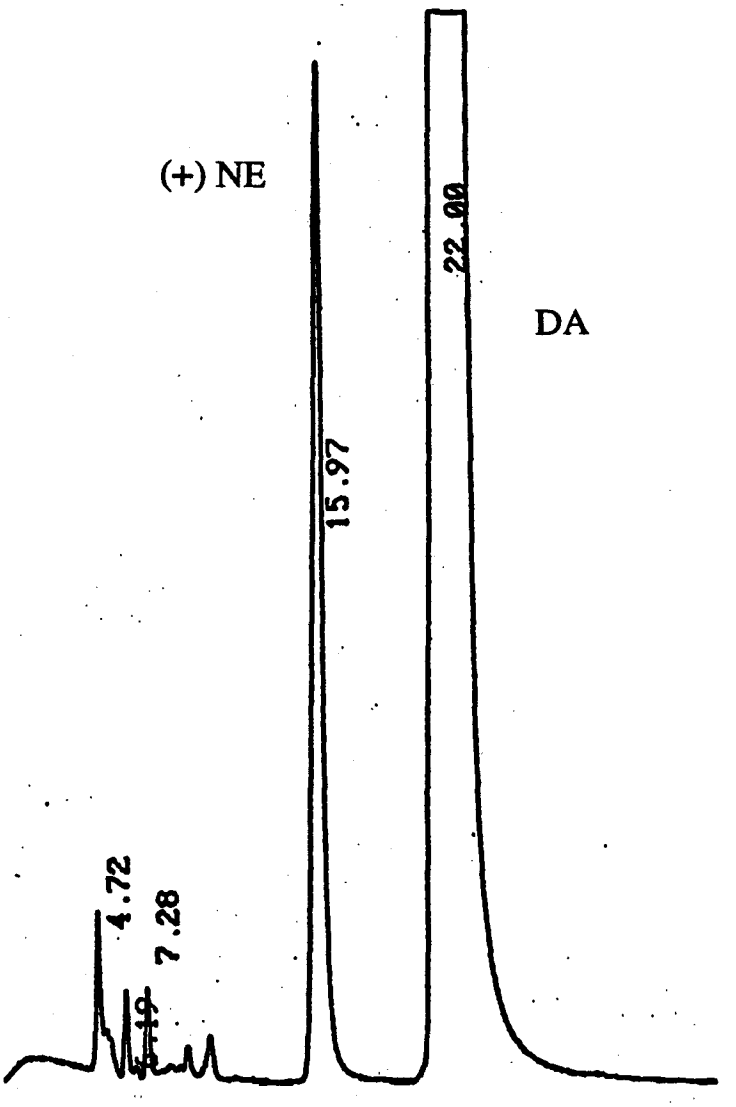
4. The on-line coupling of these systems to produce a **modular** liquid chromatographic system for the two-step synthesis of epinephrine from dopamine with on-line separation and purification of products and substrates has been accomplished.

5. The resulting system **mimics a known biosynthetic pathway** demonstrating the ability to construct these systems.

Appendices

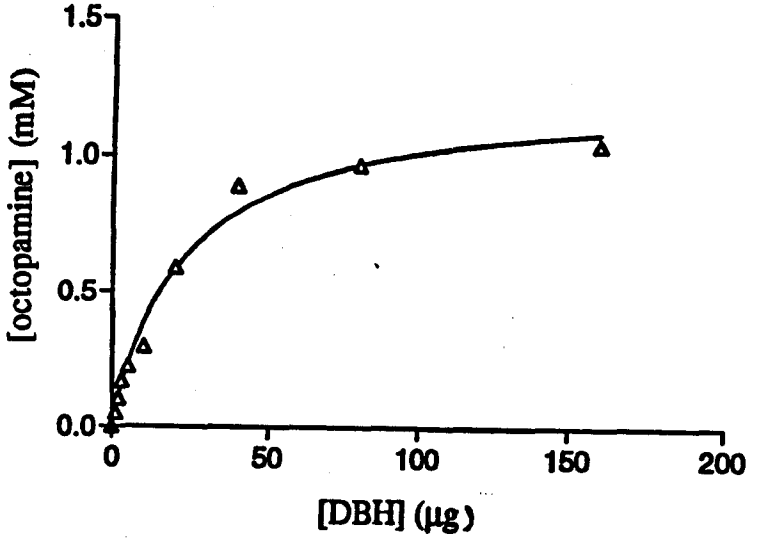
Appendix A: Representative chromatograms from DBH assay analyzed under chiral conditions: immobilized on the Glut-P interphase.

Immobilized DBH onto Glut-P chromatographic support.

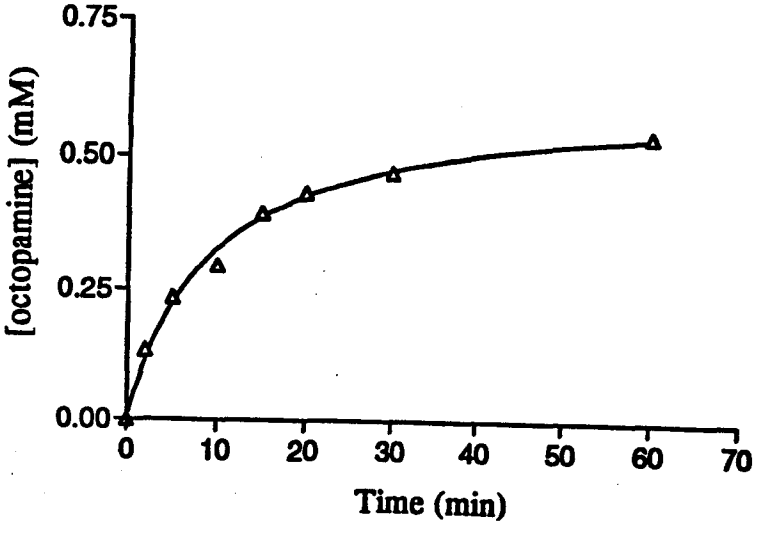


Appendix B: Effect of incubation and enzyme concentration on dopamine beta-hydroxylase (DBH) activity.

A: Effect of enzyme concentration

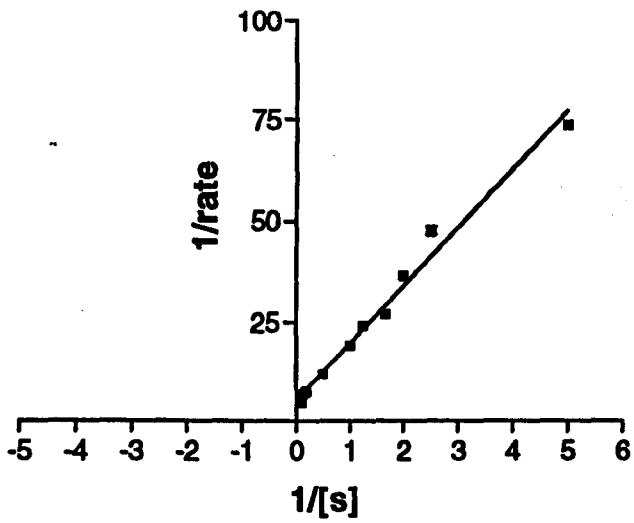


B: Effect of incubation time

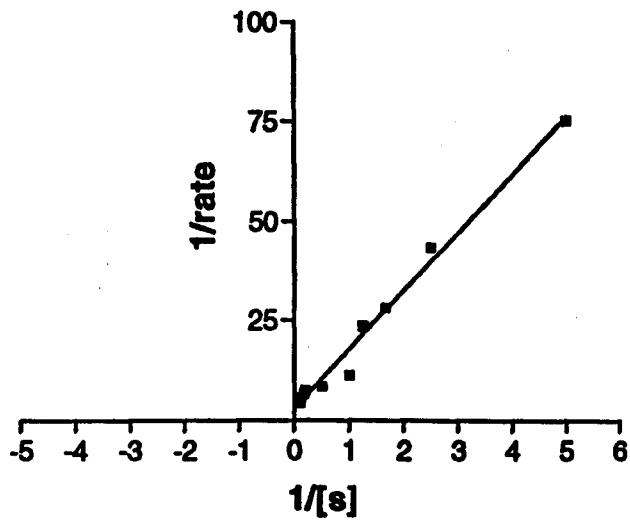


Appendix C: Lineweaver-Burke plots of immobilized and non- immobilized dopamine beta-hydroxylase

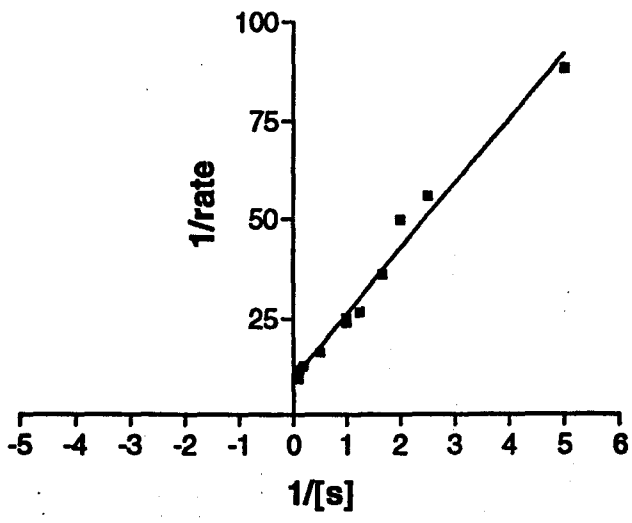
A: Effect of Tyramine on non-immobilized DBH



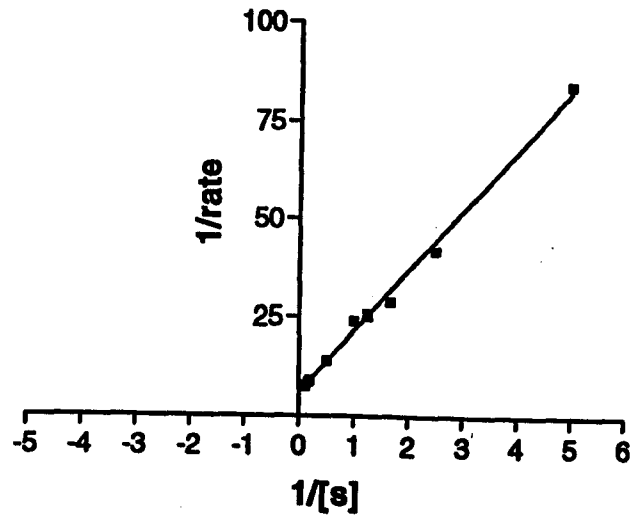
B: Effect of Dopamine on non-immobilized DBH



C: Effect of Tyramine on immobilized DBH (DBH-Glut-P)



D: Effect of Dopamine on immobilized DBH (DBH-Glut-P)



Appendix D: Effect of contact time on phenylethanolamine N-methyltransferase immobilized enzyme reactor (PNMT-IMER) activity (at a fixed flow rate or 0.2ml/min).

Contact time (min)	Recovery of NM (%)	[M] (μM)
5	45	4.11 ± 0.12
10	87	4.24 ± 0.32
15	95	4.58 ± 0.19
20	94	4.93 ± 0.22
30	96	4.95 ± 0.11

NM: Normetanephrine

M: Metanephrine

Appendix E: The effect of temperature on immobilized phenylethanolamine N-methyltransferase (PNMT-SP) in non-flow format (i.e. batchwise) and immobilized PNMT in a column on the on-line system (PNMT-IMER) (n=3).

Temperature (°C)	[Metanephrine] (μM)	
	PNMT-SP	**PNMT-IMER**
25	5.12 ± 0.17*	7.44 ± 0.02*
30	5.48 ± 0.09*	8.33 ± 0.14*
37	8.82 ± 0.06	9.57 ± 0.29
45	6.36 ± 0.18	7.45 ± 0.28
60	7.10 ± 0.04	7.06 ± 0.35

* P values < 0.05

Appendix F:

The feasibility of immobilizing PNMT onto open-tubular columns was investigated. The following section describes the preliminary results obtained by work carried out with Dr.Ruin Moaddel.

Introduction

Numerous methods for immobilizing enzymes have been reported. The ideal method of choice is contingent upon numerous variables. The immobilized enzyme allows for its reuse and investigation into substrate/inhibitor interactions. The development of on-line systems that can carryout quantitative and qualitative determinations are important for the rapid discovery of drug candidates for various diseases. Miniaturization of these processes will result in a fast and effective method of screening thousands of compounds that are developed by combinatorial chemists.

Based upon the results obtained from previous work (Markoglou and Wainer, 2001), PNMT has been immobilized onto an open tubular column utilizing a modified procedure described by Yang et al. (Yang et al.,1998). The open tubular column containing immobilized PNMT was coupled to a mass spectrometer for analysis. Preliminary results have confirmed that with immobilization of PNMT onto the capillary the enzyme has remained active and retained its enzymatic characteristics.

Materials

Phenylethanolamine-N-methyltransferase, DL-metanephrine, S-adenosyl-L-methionine, DL-normetanephrine hydrochloride, 3-amino propyl trimethoxysilane, sodium hydroxide, glutaric dialdehyde, tris-hydrochloride and other chemicals unless otherwise stated were obtained from Sigma Chemical Co. (St.Louis, MO, USA). An open tubular capillary (50 cm x100 μm ID) was purchased from Polymicron Technologies (USA).

Procedure

An open tubular capillary (50 cm x 100 μm ID) was attached to a vacuum through a 200 μl pipet tip and parafilm. The capillary was cleaned with 0.5 N NaOH by passing it via vacuum suction for 1 hour at room temperature The process was repeated with distilled deionized water for an additional 30 minutes. The water was removed by vacuum suction and the capillary was then placed in a GC 5890 oven at 95°C for 1 hour.

A solution of APTS (3-amino propyl triethoxysilane) 10 parts and 90 parts water was passed through by vacuum suction at room temperature for 10 minutes and then dried at 95 °C for 30 minutes. This was repeated twice. Subsequently, a gluteraldehyde solution (1% v/v) in 50 mM PBS pH 7.0 was passed through the capillary for 1 hour at room temperature. A PNMT solution of 1mg/mL (25 mM phosphate buffer, pH 8.4) was applied to the capillary with vacuum suction for 1 hour at room temperature. Both ends of the capillary were then placed in the remaining enzyme solution and incubated overnight.

The following day a solution of 0.5 M Tris-HCl buffer pH 7.5 was passed through the capillary for 30 minutes at room temperature followed by the 25 mM Phospate buffer solution pH 8.5 for 1 hr. The capillary was stored in the cold room with both ends immersed in the phosphate buffer solution.

Assay conditions for PNMT activity on capillary

The activity of immobilized PNMT on the capillay was assayed as follows: A solution containing the substrate, normetanephrine (2.25 mM) and the cofactor, S-adenosyl-L-methionine (25μM) was prepared. This solution (20 μl) was pumped through the capillary for 30 seconds at a flow rate of 0.1 mL/min, and incubated for 30 minutes. Subsequently, the capillary was connected to the PE- SCI-EX API-100 MS and was run in negative ion mode.

Results

Single ion recordings were carried out for two molecular weights: 219.7 (Normetanephrine) and 233.7 (Metanephrine) in negative ion mode. The reaction mixture (substrate with cofactor) was analyzed. The production of product, metanephrine, was seen at about 10% of the final concentration of substrate, normetanephrine (Figure 1A).

A control solution (substrate without cofactor) was also analyzed (Figure 1B). In this case, no production of the product metanephrine was seen. These results confirmed the successful immobilization of active PNMT onto the surface of the open tubular capillary.

Reference

Yang, Q., Liu, X-Y., Miyake, J. (1998) Self-assembly and immobilization of liposomes in fused-silica capillary by avidin-biotin binding. Supramolecular Science 5: 769-772.

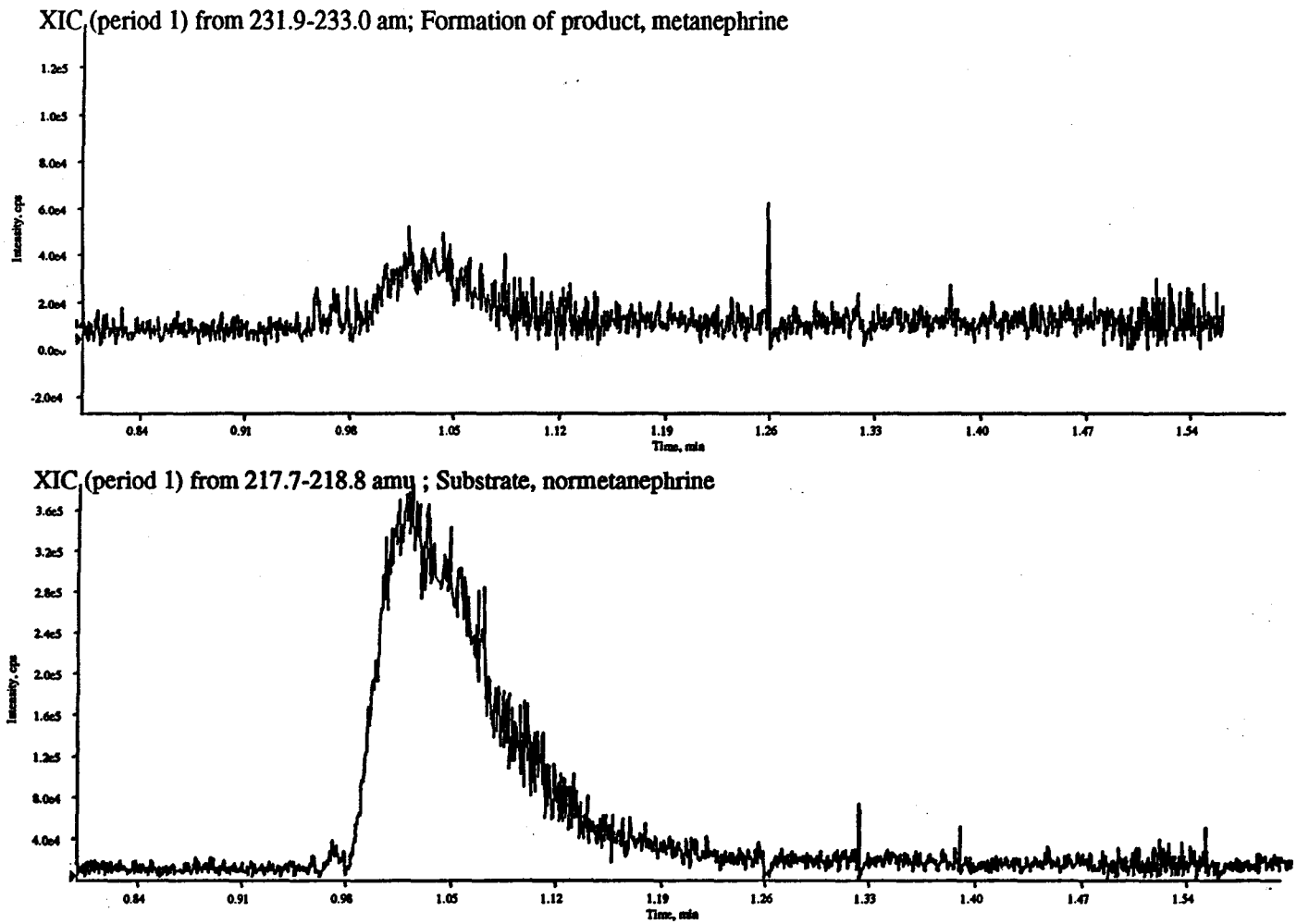


Figure 1A: Open Tubular, PNMT immobilized, reaction mixture (substrate with cofactor).

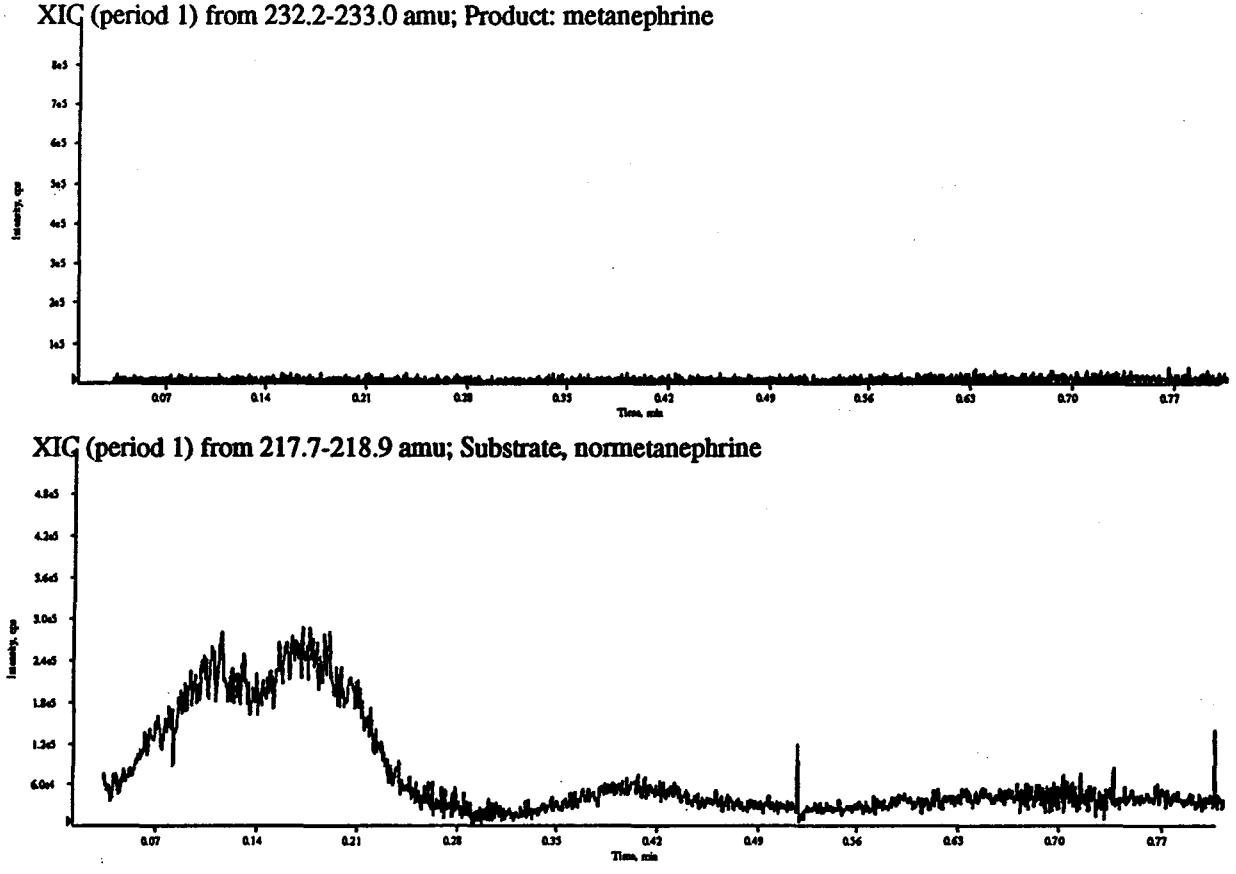


Figure 1B: Open Tubular, immobilized PNMT, control (Substrate only).