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DEVELOPMENT OF METHODOLOGY FOR THE DETERMINATION OF CATECHOLAMINES, INDOLEAMINES, AND RELATED HYDROXYLATING ENZYMES

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DEVELOPMENT OF METHODOLOGY FOR THE

DETERMINATION OF CATECHOLAMINES,

INDOLEAMINES, AND RELATED

HYDROXYLATING ENZYMES

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

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DOCTOR OF PHILOSOPHY

By

PETER KWEI-YEN WONG

Norman, Oklahoma

1980

DEVELOPMENT OF METHODOLOGY FOR THE

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APPROVED BY

DISSERTATION COMMITTEE

ACKNOWLEDGEMENTS

Finally, the author wishes to dedicate this to the memory of his late parents who helped to lay the foundation and motivation which made the completion of this long educational journal possible.

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CHAPTER 1

GENERAL INTRODUCTION

I. Tyrosine Hydroxylase and Tryptophan Hydroxylase

A. Background. During the past few decades, a large number of studies have been undertaken in an attempt to more fully understand the adrenergic and serotonergic nervous systems in the mammalian brain. These systems utilize the catecholamines, namely, dopamine (DA) and norepinephrine (NE), and the indoleamine, 5-hydroxytryptamine (5-HT, serotonin), as neurotransmitters. The development of highly specific histochemical techniques for observation of these species have placed these transmitters in a very unique position in neurobiology. Such techniques have allowed a detailed mapping of their respective pathways in the central nervous system. In conjunction with a variety of biochemical and pharmacological investigations, specific pathways have been demonstrated to have a definite relationship to both animal and human behavioral responses. Other studies have very strongly implicated the involvement of these biogenic amines and their associated pathways in the disordered mental states.

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The biosynthesis of these neurochemical transmitters, as shown in Figure 1.1, exhibits many common features among the two, separate pathways. Both pathways indicate hydroxylation of a dietary amino acid (1), followed by decarboxylation. The appropriate enzymes are specifically located in the catecholaminergic and serotonergic cells, respectively. Additionally, noradrenergic cells contain dopamine-β-hydroxylase, the enzyme which catalyzes the conversion of dopamine into norepinephrine. The decarboxylation in both pathways is commonly assumed to be affected by the same, non-specific aromatic amino acid decarboxylase. However, decarboxylation may be effected by separate enzymes as indicated in Figure 1.1. The conversions of L-tyrosine to L-dopa and L-tryptophan to 5-hydroxytryptophan by their respective hydroxylating enzymes are universally accepted to represent the rate limiting steps in the biosynthesis of these transmitters (2,3). Therefore, considerable attention has been focused on these particular enzymes following their initial discovery and isolation some fifteen years ago (2). The activities of these enzymes are, relatively speaking, very low. Thus, investigations of their location and properties demand utilization of highly sensitive analytical procedures.

Tyrosine hydroxylase (TH) is widely distributed in animal tissues. It is most prominent in the adrenal medulla, the spleen, the peripheral sympathetic nervous system, and the central nervous system (6,10). In the central

L-tyrosine →(TH)→ L-dopa →(DDC)→ dopamine (DA) →(DBH)→ norepinephrine (NE)

Hydroxylation
(O_2 + Reduced Pteryin)

Decarboxylation

Hydroxylation

L-tryptophan →(TPH)→ 5-hydroxytryptophan (5-HTP) →(5-HTPDC)→ serotonin (5-HT)
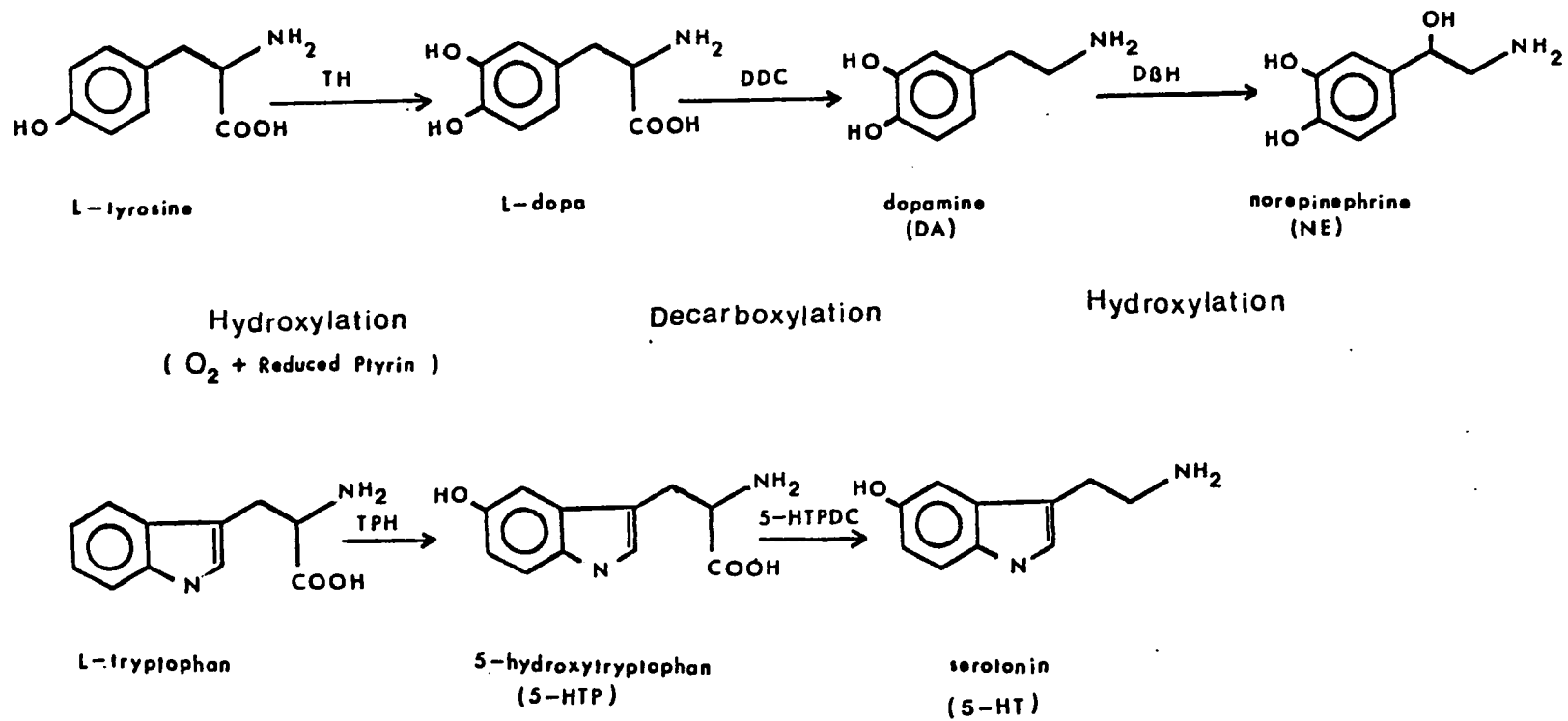
Figure 1.1 Biosynthetic Pathways for Catecholamines and Indoleamines

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nervous system, TH is not homogeneously distributed and is found to decrease in the following regional sequence: caudate nucleus>hypothalamus>thalamus>midbrain>medulla oblongata>cerebral cortex>cerebellum (1). As for sub-cellular distribution, some TH activity is found in cell bodies, but the majority is confined to the nerve terminals or varicosities of catecholamine-containing cells. There is also considerable evidence that the enzyme exists in two distinct physical forms, soluble and membrane-bound (7,8). The soluble form is present largely in cell bodies while the membrane-bound form is localized primarily in the nerve terminal regions (16).

Tryptophan hydroxylase (TPH) is not as widely dis-tributed in mammals as is TH. It has been found, however, in fairly large concentrations in the gastrointestinal tract (smooth muscle), the pineal gland, and the central nervous system. As with most cerebral enzymes, there are marked differences in the regional distribution of tryptophan hydroxylase in the central nervous system. The highest activities were reported for the hypothalamic and thalamic regions, with the midbrain and medulla exhibiting considerable, but lesser activities (17,18). The cortices and cerebellum show very low levels of tryptophan hydroxylation. Most of the enzyme activity from brain stem is recoverable in the 30,000xg supernatant following homogenization in a hypotonic, 0.05 M tris-acetate buffer (pH 7.4-7.6), but

not in an isotonic sucrose (0.32 M) supernate (19). As with TH, the existence of two distinct forms of TPH has also been suggested (20). TPH from the brain appears to be predominately soluble (19), although Knapp and Mandell have reported that a small portion of the enzyme is found in a particulate fraction. The ratio of particulate to soluble TPH varies from region to region, with the highest value being obtained in those areas with the greatest number of serotonergic nerve endings (20).

Neither TPH nor TH have been isolated as homogeneous entities. The best preparation of tyrosine hydroxylase has been obtained from adrenal medulla (11). It is about 40% pure and appears to be at least similar to, if not identical with, the TH found in catecholaminergic neurons (12). TPH has been obtained from a number of different tissues, but has not yet been extensively purified. Several attempts at purifying this enzyme from central nervous system tissues have yielded only partial success (13,14).

B. Kinetic properties. Much of the mechanistic and kinetic information obtained for TH and TPH has been preceded and greatly aided by the work of Kaufman and coworkers (21) on a related hepatic enzyme, phenylalanine hydroxylase. These workers have clearly established phenylalanine hydroxylase as a mixed function oxidase requiring both molecular oxygen and a reduced pterin, later identified as tetrahydro-

biopterin. It now appears that both tyrosine and tryptophan hydroxylation proceed by similar mechanisms. The reduced pterin cofactor for both tyrosine and tryptophan hydroxylation in vivo is also believed to be tetrahydrobiopterin (22). However, 6,7-dimethyl-5,6,7,8-tetrahydropterin (DMPH$_4$) and 6-methyl-5,6,7,8-tetrahydropterin (6-MPH$_4$) are commonly used for in vitro hydroxylation due to ease of handling and commercial availability. Kinetic data for tyrosine hydroxylation are consistent with the following individual steps (3,9):

1. Reduction of an oxidized form of the enzyme by the reduced pterin cofactor and dissociation of the oxidized pterin,

2. Formation of a complex with the substrate,

3. Aerobic oxidation of the enzyme-bound tyrosine producing 3,4-dihydroxyphenylalanine (dopa) and the oxidized formed of the enzyme.

This can be summarized as:

$$E + DMPH_4 \rightarrow EH_2 + DMPH_2$$

$$EH_2 + Tyr \rightarrow [EH_2 \cdot Tyr] \xrightarrow{O_2} E + dopa + H_2O$$
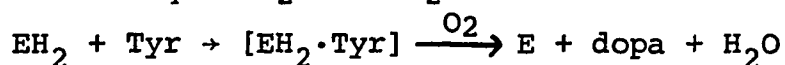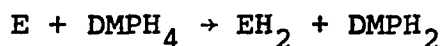
Metal ions, particularly iron(II), may play an important role in these hydroxylation reactions. A wide variety of assay conditions used by different workers have alternately demonstrated both dependence and lack of dependence upon added ferrous ion for optimal in vitro activity. Stimulation of TH or TPH activity by ferrous ion appears

to be dependent on the degree of purification of the enzymes, the nature of the pterin cofactor used (2), and the source of the enzyme preparations (4). The role of ferrous ion as a possible cofactor in either of these hydroxylation reactions is, thus, currently a controversial topic. One possible explanation of the observed stimulation by Fe^{+2} is the known ability of this species to destroy H_2O_2 believed to be generated during nonenzymatic oxidation of the reduced pterin cofactor (5). This explanation is supported by the fact that iron can often be replaced by catalase, an enzyme which rapidly decomposes H_2O_2.

Completely valid observations on the kinetic properties of enzymes can, of course, be obtained only from pure preparations. However, much valuable information has been obtained from the partially purified preparations of TH and TPH. The work of Kaufman and his associates (5,14, 15) has shown a marked interdependence of the cosubstrates (amino acid, reduced pterin and oxygen) with regard to their kinetic constants. For example, with brain tryptophan hydroxylase, the K_m value for tryptophan is seen to shift from $3x10^{-4}$ $\underline{M}$ to $5x10^{-5}$ $\underline{M}$ as one changes the cofactor from 6,7-dimethyl-5,6,7,8-tetrahydropterin to tetrahydrobiopterin.

Based upon multiple investigations of the physical properties of these hydroxylating enzymes, we are now able to conclude that mammalian tissues contain three distinct, but related enzymes. These three systems, summarized

in Table 1.1, are involved in the hydroxylations of, re-
spectively, phenylalanine, tyrosine, and tryptophan.
With the exception of tryptophan hydroxylase, none of these
enzymes is capable of exhibiting complete substrate specifi-
city with respect to the other two substrates.

C. Enzyme preparations. Depending on the specific
nature of the investigation to be undertaken, there are
two major different approaches one may select in preparing
either TH or TPH. By employing an isotonic homogenizing
medium and a glass/Teflon homogenizing apparatus with a
moderate clearance, typically 0.01 mm, the nerve cell
endings will be first separated from the cell bodies and
then their membranes reseal to form synaptosomes. This
synaptosomal preparation preserves the architectural in-
tegrity of the tissue to be investigated and contains a
relatively intact hydroxylating system. Harris et al. (35)
used such an approach in a study of tyrosine hydroxylase.
They claimed that this preparation was relatively simple
to use since it does not require the addition of exogenous
cofactor. Presumably, the natural cofactor is contained
within the synaptosomes. Being more like the in vivo con-
dition than a more purified preparation, synaptosomes have
also been claimed to offer greater insight into the
physiological functioning of these enzyme systems. Notable
attributes of such preparations include stereoselectivity,

TABLE 1.1

Substrate Specificity of Mammalian Hydroxylating Enzymes*

Enzyme Source	Substrate		
	Tyrosine	Tryptophan	Phenylalanine
Liver (Phenylalanine Hydroxylase)	-	+	+
Noradrenergic cell of brain (Tyrosine Hydroxylase)	+	-	+
Serotonergic cell of brain (Tryptophan Hydroxylase)	-	+	-

*A + in the table indicates the hydroxylation of the substrate is catalyzed by the corresponding enzyme system.

low substrate K_m values, nearly neutral pH optima, inhibition
by low concentrations of "extracellular" catechols in the
case of TH (<u>vide</u> <u>infra</u>), and loss cf activity following
selective destruction of the associated nerve endings.
Citing these attributes, Davis (37) recently used synapto-
somal preparations to examine the affinity of tyrosine
hydroxylase for oxygen.

However, hydroxylation by synaptosomes is a very com-
plex process. It necessitates uptake of tyrosine or trypto-
phan and oxygen into the subcellular particles before the
enzymatic hydroxylation can occur. The measured enzyme
activity is, thus, generally limited in such preparations.
To avoid these problems and to obtain higher absolute
activities, most investigators employ a hypotonic homogenizing
medium. Such hypotonic solutions lead to extensive lysis
of cellular and subcellular membranes. Thus, the enzyme
becomes completely exposed to the homogenizing medium.

Whether one chose hypotonic or isotonic homogenization,
the preparation of tyrosine or tryptophan hydroxylase, es-
pecially from brain regions, has been historically difficult.
Even the best preparations cannot presumably avoid eliciting
alteration in the relevant physical and kinetic properties
of these enzymes (38). The chemical composition of the sur-
rounding solution is certainly modified in going from the
<u>in</u> <u>vivo</u> to the homogenized condition. Important regulatory
factors may also be present in the tissue milieu. Thus,

many workers prefer the use of crude tissue homogenates
as the enzyme source to minimize the effects of such altera-
tions from the in vivo state.

D. Regulation and Control of Tyrosine and Tryptophan
Hydroxylase. A detailed understanding of the functional
utilization of catecholamines and indoleamines quite
naturally focuses upon the enzymes representing the rate
limiting step in their biosynthesis. The current under-
standing of the regulation of these processes is known to
involve the following important factors:

1. Availability of substrate and cofactors.

2. Presence of product (for TH) or feedback inhibition.

3. Conformational changes in the protein.

4. Synthesis or degradation of enzyme molecules.

5. Possible existence of endogenous inhibitors and
   activators.

Manipulation of these regulating factors by pharmacological
or other means will certainly play a major role in correcting
any associated defects occurring via their possible im-
balance in diseased states.

The simplest method for intervening in these regula-
tory processes is to incorporate an exogenous inhibitor
into the system. Thus, a great deal of attention has been
paid to possible inhibitors of both TH and TPH.

Two major groups of inhibitors have been identified

for TH (26): tyrosine analogs and catechol-containing

compounds. The latter group is represented by dopa, NE,

DA and 3,4-dihydroxyphenylpropylacetamide (H22/54) (27).

Inhibition by these compounds is known to be competitive

with the reduced pterin cofactor and uncompetitive with

tyrosine (26,28). Such inhibition, while typically in-

volving K_i values on the order of only 10^{-3} $\underline{M}$, do provide

an effective product inhibition at the high levels of

transmitters found within the nerve varicosities.

The tyrosine analogs are much more potent inhibitors

of TH (26). Those showing the greatest inhibition of TH

typically have an α-methyl or 3-halogen substituent. Both

of these groups of compounds exhibit competitive inhibition

with the substrate tyrosine (26). Included in the latter

group are 3-iodotyrosine and 3,5-diiodotyrosine, two species

which are endogenous precursors of thyroxine in the thyroid

gland. The former group is best represented by α-methyl-p-

tyrosine. This species has been used to lower the catechol-

amine levels in guinea pigs (23) and other animals to less

than 1% of control values. This leads to the noticeable

impairment of motor activity and mild sedation. α-Methyl-

p-tyrosine has also been used to measure turnover rates

for catecholamines (24). One simply measures the rate of

decline of the catecholamine levels following the injection

of this blocking agent (24). Finally, this agent has also

seen some limited clinical use in the treatment of hyper-

tension (25).

In an attempt to find comparable inhibitors for tryptophan hydroxylase, McGeer and Peters (30) screened some 700 compounds. They notably added 6-halotryptophans and α-methyldopa to the previously known inhibitors, the 4-halophenylalanines (29).

A better understanding and associated manipulations of these hydroxylating enzymes will certainly necessitate employment of highly selective procedures for their determinations. Advances in microdissection techniques (31-34) already can provide reproducible tissue samples in the μg range. Thus, we have attempted to apply the highly selective method of liquid chromatography with electrochemical detection to the measurement of these enzymatic activities (vide infra).

E. Analytical Procedures. Existing techniques for the determination of TH activities are all based upon the conversion of L-tyrosine to L-dopa. These methods include measurement of the dopa produced by spectrophotometry (5), fluorometry following intracyclization of dopa according to the trihydroxyindole procedure (40-44), various radiochemical procedures (45-48), and liquid chromatography with electrochemical detection (LC/EC) (49). Since TH activity is very low in most cases, only the radioassays and LC/EC can routinely provide adequate detection limits

and selectivity.

One popular radioassay involves the use of L-tyrosine-3,5-^{3}H (45). This is converted to L-dopa-5-^{3}H during the hydroxylation reaction with the release of tritiated water. The tritiated water is separated from the rest of the radioactive materials by cation exchange chromatography and subsequently measured by liquid scintillation counting. A second radioassay procedure (6) makes use of the conversion of L-tyrosine-^{14}C to L-dopa-^{14}C. The L-dopa-^{14}C is isolated by adsorption onto an alumina column and then determined by liquid scintillation counting.

In the method of Waymire and coworkers (47), L-tyrosine-1-^{14}C is converted to L-dopa-1-^{14}C. Dopa decarboxylase, which has been added to the incubation mixture then leads directly to the formation of dopamine and ^{14}CO$_2$ (gas). The latter is trapped in a separate vial containing a highly basic solution and then subjected to liquid scintillation counting. A modification of this procedure has been applied to the measurement of labelled DA resulting from the use of a substrate which is not labelled on the carboxyl group; the DA is isolated by extraction into 3-heptanone after complexation with sodium tetraphenylboron (48).

The determination of tyrosine hydroxylase activity has also been reported (49) using liquid chromatography with electrochemical detection. This report, however, was somewhat limited in scope. It employed a less efficient

column, packed with relatively large particles, rather than the highly efficient microparticulate column reported here. It also focused on the synaptosomal preparation rather than hypotonically homogenized samples. This restricted its detection limit considerably by yielding lower activities when expressed on a per gram basis.

Assay techniques for tryptophan hydroxylase (TPH) are generally based upon the conversion of L-tryptophan to L-5-hydroxytryptophan (5-HTP). Several of the methods reported employ fluorometric detection of the product. In these methods, the condensation product of 5-hydroxytryptophan with o-phthalaldehyde is measured (51). Radiometric assays are more commonly used for TPH due to their higher sensitivity and greater selectivity. Of these, the tritium-release procedure (52) seems to be the most frequently employed. In this method, L-tryptophan-5-^{3}H is enzymatically converted to 5-hydroxytryptophan-4-^{3}H. Tritium in the 4-position of 5-hydroxytryptophan, however, exchanges readily with water under acidic conditions. The resulting product is, thus, acidified and the tritiated water separated from the substrate by ion exchange chromatography. Quantitation is, again, achieved with liquid scintillation counting. In the method of Renson (53) the same starting material is used, but the product 5-hydroxytryptophan-4-^{3}H is directly separated from substrate by ion exchange chromatography and measured without acid treatment.

Ichiyama et al. (54) reported the use of L-tryptophan-1-^{14}C as a substrate for tryptophan hydroxylase. The product 5-hydroxytryptophan-1-^{14}C is further converted to 5-HT and $^{14}CO_2$ by added aromatic amino acid decarboxylase. The gaseous $^{14}CO_2$ is trapped in a basic solution and subsequently quantitated. Tryptophan hydroxylase activity can also be measured through the multiple enzyme procedure of Kizer et al. (56). 5-Hydroxytryptophan, the immediate product of hydroxylation of tryptophan is enzymatically converted to 5-hydroxytryptamine by added aromatic amino acid decarboxylase. This is then converted to N-acetylserotonin by serotonin-N-acetyltransferase. Finally, a radioisotope label is introduced by the enzymatic methylation of N-acetylserotonin in the presence of ^{3}H-methyl-S-adenosylmethionine and hydroxyindole-O-methyltransferase (HIOMT). The ^{3}H-melatonin thus formed is isolated by solvent extraction and counted.

In addition to these radiometric assays, there is a method reported for TPH using liquid chromatography with fluorescence detection (57). Experiencing considerable noise levels, this procedure does not, unfortunately, possess a sensitivity comparable to the radiochemical procedures.

Besides the in vitro procedures for TH and TPH reported above, there have been many investigations concerned with the in vivo measurement of activities for these enzymes. These procedures, pioneered by Carlsson et al.

(58,59) measure the buildup of dopa and/or 5-hydroxy-tryptophan after _in_ _vivo_ blockade of aromatic amino acid decarboxylase. Since the endogenous levels of these compounds are very low, measurement of their levels at a specific time after injection of the blocking agent yields a direct determination of the rate of their formation.

Assuming the existence of a steady state (1) for the associated transmitters (rate of synthesis = rate of degradation), these measurements also directly indicate the utilization, or so-called turnover, rate of these amines. The initially developed method for _in_ _vitro_ TH described in this thesis was, thus, also modified to allow determinations of _in_ _vivo_ activities.

II. 5-Hydroxytryptamine (5-HT, Serotonin)

A. General Background. Serotonin was first identified in biological materials when it was discovered to be the factor in serum which stimulated smooth muscle (69, 70). Since this discovery, it has been shown to be widely distributed in nature. In man, the highest concentrations occur in the pineal gland, the gastrointestinal tract, and the brain. Serotonin is not homogeneously distributed in the brain. The highest levels of this transmitter are found in hypothalamus, brain stem, neostriatum, and specific areas of the limbic system (71-73).

Investigations of the regional localization of

serotonin in the central nervous system have been greatly aided by histochemical techniques. These procedures have been used to identify serotonin-containing cell bodies in the raphe nuclei of the midbrain, pons and medulla (74). Serotonergic nerve terminals have been identified in hypothalamus, the limbic system, and other forebrain and brain stem structures (75).

As previously indicated, serotonin is synthesized from tryptophan by enzymatic hydroxylation and decarboxylation in order. The principal route of serotinin degradation involves oxidation by monoamine oxidase to the aldehyde. Further oxidation to 5-hydroxyindole-3-acetic acid (5-HIAA) by aldehyde dehydrogenase or reduction to the alcohol by aldehyde reductase is eventually followed by urinary excretion.

The role of serotonin as a neurotransmitter has been well established. It has also been found to be related to certain physiological aspects such as blood pressure (76), temperature control (77), and sleep (78). Defects in the function and metabolism of serotonin have been postulated to be intimately involved in the affective disorders, mania and depression (79-81).

In order to thoroughly examine the function and regulatory mechanisms associated with this transmitter, precise quantitation of serotonin levels and changes in its metabolism will have to be accomplished in vanishingly

small samples. This will encompass highly reproducible microdissection techniques as well as assay procedures with great selectivity and low detection limits.

B. Analytical Determination. There are many analytical techniques currently employed for the determination of serotonin. Among these, the fluorometric procedures have experienced the most widespread utilization. These procedures have used native fluorescence in strong acid (82) or, for enhanced sensitivity, prior derivatization with o-phthalaldehyde (83) or ninhydrin (84). The sensitivity obtained by fluorescence methodology, however, is not typically adequate for small (μg) tissue samples. Additionally, the selectivity of these procedures is highly questionable when applied to tissues containing compounds structurally similar to 5-HT. A recently reported determination of 5-HT using liquid chromatography with fluorometric detection may alleviate some of these specificity problems (88). This method also has a detection limit of 1 pmol for serotonin.

Gas chromatography-mass spectrometric (GC-MS) (85) and radio-enzymatic procedures (86,87) can provide determinations of serotonin in the 0.3-0.5 pmol range. The GC-MS procedures, while being virtually specific, require chemical derivatization of 5-HT prior to analysis. These procedures also necessitate the use of very expensive

instrumentation leading to a large cost per sample. Radio-
enzymatic methods for serotonin typically incorporate two
separate enzymatic conversions. The enzymes involved, un-
fortunately, are not totally specific and the final, labelled
product is isolated through a nonspecific solvent/solvent
extraction. Thus, these methods may also suffer a loss in
specificity due to the presence of other 5-HT related com-
pounds in the tissue samples.

Since the initial employment of liquid chromatography
with electrochemical detection (LC/EC) for the determination
of catecholamines by Adams and coworkers (61,92), this
technique has been demonstrated to possess a high degree
of selectivity and very low detection limits. It is also
quite rapid, relatively inexpensive, and quite easy to use
on a routine basis. Several papers have appeared during
recent years exhibiting the applicability of this technique
to the determination of serotonin (89,90,91,93,94). These
procedures generally maintain all the advantages listed
above for the LC/EC determinations of catecholamines. They
typically provide detection limits of 0.1 pmol (20 pg)
for 5-HT. However, all these methods incorporate some
sample clean-up process in addition to protein precipitation.
This may be either a solvent extraction step or isolation
and subsequent elution from an ion exchange column. The
sample processing time would, thus, be considerably
shortened for routine applications if these intermediate

steps could be eliminated. The LC/EC procedure reported herein provides for greater sample throughput by just such an elimination, while retaining almost all the advantages of LC/EC.

III. Liquid Chromatography with Electrochemical Detection

Recent advances in liquid chromatography (LC) have made this technique a very efficient and powerful tool for the separation of structurally similar components. It has become increasingly important in the analysis of nonvolatile, heat-labile, and biologically active compounds. However, its application to very low level (pmol) quantitative work had to await the development of highly sensitive detectors. Included in the group of these detectors is the electro-chemical device introduced in 1973 (61). This detector is simply a thin-layer carbon paste electrode, placed at the outlet of the chromatographic column. The electrode is maintained at a fixed, predetermined potential with respect to a reference electrode, where it may oxidize (or reduce) electroactive compounds passing by its surface. A conventional, three electrode potentiostat is used to control the predetermined, constant potential of the working electrode and to measure the limiting current resulting from the oxidation (or reduction) under these convective flow conditions. As little as a few femtomoles of certain com-pounds can be detected by this method (62). Additionally,

this system has been shown (63) to have a linear dynamic range of $\underline{ca}$. 10^5 to 10^6.

The column packing materials originally used for the determination using liquid chromatography with electrochemical detection were typically the pellicular strong cation exchange resin, Zipax SCX, or the anion exchange resin, Zipax SAX (61,62,64). These two ion exchange resins are fluoropolymer based packings with active $-SO_3^-$ or $-NR_4^+$ functional groups and a particle size of 30-40 μm (65). The interaction of these packings with solute molecules typically involves both dispersion forces and ion exchange mechanisms, although they rarely conform to the behavior one expects from a classical ion exchange system. Zipax ion exchangers are unique in having this fluoropolymer backbone. Most others, such as Vydac column packings (65), incorporate a polystyrene-divinylbenzene backbone. Compounds with an aromatic character usually interact very strongly with the backbone resulting in considerably larger capacity factors (k' values) and retention times. The desired separation of a particular set of compounds can usually be achieved by simple manipulation of appropriate chromatographic parameters. These parameters include the ionic strength of the mobile phase, the pH of the mobile phase, the flow rate of the mobile phase, the length of the column, the temperature of the column and the particle size of the packing material. The major disadvantage of

using these packing materials lies in the fact that ion
exchange chromatography is applicable only to the separation
of ionic sample components. Neutral molecules of interest
are not retained and thus appear in the so-called solvent
peak. Moreover, the relatively large particle size (30-40
μm) of these packing materials greatly hinders their re-
solving capabilities by yielding rather large values for
the HETP (height equivalent to a theoretical plate). Large
HETP values also lower the peak capacity of the system,
thus severely limiting the number of components which can
be separated in a single chromatogram. As a result, these
packing materials are gradually becoming obsolete as the
more recently introduced microparticulate reverse-phase
columns have emerged in the market place. These micro-
particulate columns are composed of 5-10 μm silica beads
with chemically bonded organic groups serving as the active
component of the stationary phase. The small particle size
leads to a considerable lowering of the HETP and, thus, a
better resolving power. The most widely used bonded phases
are those composed of hydrocarbon moeities such as the
ethyl, octyl, and octadecyl groups. The octadecyl group
is most frequently used at the present time. Because the
eluant is generally more polar than the stationary phase,
the term reverse-phase chromatography, originally coined
by Howard and Martin (66) is used to describe this liquid
chromatographic technique. The versatility of these

chemically bonded materials is further enhanced by the fact that they can be operated in either the normal reverse-phase mode or a dynamic ion exchange mode (67). In the ion exchange mode, polar lipids bearing various functional groups (68) are added to the mobile phase. These lipids are typically moderate-to long-chain alkane sulfonic acids or quaternary amines. The non-polar portion of these modifiers are strongly attracted to the stationary phase through dispersion forces and then provide the column with characteristics resembling chemically-bound ion exchangers. When these modifiers are added to the mobile phase, the technique is generally referred to as reverse phase ion pair chromatography or, more simply, soap chromatography. The alkyl sulfates or sulfonates increase the capacity factor for cations, whereas the tetraalkylammonium salts do likewise for anions. This procedure provides highly efficient separation for many ionic species, including the catecholamines (39,46,60). The ionic strength of the mobile phase plays a role in soap chromatography completely analogous to what one would expect from ion exchange chromatography; increased ionic strength leads to lower k' values and shortened retention times.

When small amounts (or none) of these modifiers are added to the mobile phase, the bound stationary phase can still interact with neutral and zwitterionic species through hydrophobic or dispersion forces (36). This

leads to the possibility of using a single column for the simultaneous separation of a wide variety of molecular species in a single chromatographic run (11). Using reverse-phase chromatography in this way, we can easily adjust the individual capacity factors, as needed, through the adjustment of the following parameters: carbon content, or coverage, of packing material; chain length of the bonded stationary phase; pH of the mobile phase; ionic strength of the mobile phase; solvent composition of the mobile phase; and the proper selection of an appropriate stationary phase modifier.

Considering these recent and widereaching improvements in reverse-phase liquid chromatography as well as the low detection limits, wide dynamic range, and great selectivity of the electrochemical detector, it is of little wonder that the combination of these two units represents the currently preferred methodology for small, electroactive molecules from tissue or tissue homogenates.

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CHAPTER 2

DETERMINATION OF TYROSINE HYDROXYLASE ACTIVITY BY

LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL

DETECTION

I. Assay Procedure for in vitro Tyrosine Hydroxylase

Activity

A. Introduction

The need for a rapid and sensitive technique for

the determination of tyrosine hydroxylase (TH) activity has

been amply demonstrated (vide infra). This need was par-

tially fulfilled in 1976, when this laboratory introduced

a procedure (1) employing liquid chromatography with electro-

chemical detection(LC/EC). While offering some distinct

advantages over competitive methods, this procedure cur-

rently has only a limited applicability. This situation

is due to a number of instrumental and theoretical advance-

ments that have taken place in the intervening time. The

present report may, therefore, be considered an extension

of the previous work with emphasis upon modifications in

the following specific areas: (1) the use of a micro-

particulate reverse-phase column to achieve better resolu-

tion, higher sensitivity and shorter analysis times; (2) the
use of a hypotonic tissue homogenate in the incubation to
increase the measured activity over the previously employed
synaptosomal preparations; (3) the use of 6-MPH$_4$ (6-methyl-
tetrahydropterin) as a cofactor to replace DMPH$_4$ (6,7-
dimethyl-tetrahydropterin); (4) the reassessment of optimal
conditions for other experimental parameters, (pH, temperature,
etc.) obtained after these changes were added to the pro-
cedure; and (5) the careful examination and minimization
of blank values (noise levels) in order to improve the
detection limit of the procedure.

B. Development of the Assay Procedure

1. *Overview*. *In vitro* TH assays may be conveniently
divided into the following general steps: (1) incubation
of the enzyme preparation with the substrate, cofactors,
a dope decarboxylase inhibitor, and associated chemicals;
(2) isolation of the hydroxylation product, dopa (3,4-di-
hydroxyphenylalanine), from the incubation mixture; and
(3) separation and quantitation of the dopa formed. The
development of the present assay procedure quite naturally
examined each of these steps in reverse order. A method
for quantitation was required before the isolation procedure
could be investigated, and both quantitation and isolation
were required before incubation could be investigated.
After outlining the materials and methods appropriate to

the entire assay, we will go back and discuss the development of each of these steps as they occurred in the present investigation.

2. Materials and Methods.

a. Apparatus:

Liquid Chromatography. The liquid chromatograph with an electrochemical detector has been adequately discussed in general terms in Chapter 1. For some of the initial work, we used a system identical with that described by Blank and Pike (1). However, this was quickly discarded during an investigation of the chromatographic conditions. The present system is composed of the following components:

Pump: Milton Roy Minipump, Model No. 13906-1,

5,000 p.s.i. maximum pressure.

Damping Coil: Li-Chroma-Damp from Hardy & Harman

Tube Co., Norristown, PA, 2,000 p.s.i.

maximum operating pressure.

Connecting Tubing: Stainless Steel (SS 316),

0.009 in i.d.

Injector: Rheodyne Model 7120 Sample Injector.

Detector: Homemade, as previously described (1).

Detector Electronics: The potential controller/

current monitoring instrument was identical

to that previously described (1).

Column: 4.6x150 mm Ultrasphere ODS, 5 μm particle

size from Beckman.

Pressure Limiter: Electronic device attached to

pressure gauge to shut off pump when pressure

>2,000 p.s.i. This unit was constructed by

Peter Lin in our laboratory to prevent damage

to the damping coil.

Chromatographic Conditions:

Mobile Phase: A solution containing 0.025 $\underline{M}$

citric acid, 0.050 $\underline{M}$ sodium acetate,

0.060 $\underline{M}$ NaOH, and 0.019 $\underline{M}$ acetic acid

was passed through a 0.4 μm Millipore

filter prior to use.

Flow Rate: 0.3 ml/min.

Detector Potential: 0.65 V vs. Ag/AgCl.

Tissue Homogenizer: A Potter-Elvehjem glass/Teflon

homogenizer from Kontes Glass was used. The

Teflon pestle was driven by a Fisher model 43

Dynamix motor, connected _via_ a piece of rubber

tubing.

Centrifuge: Sorvall RC2-B refrigerated, high-speed

centrifuge equipped with an SM-24 rotor.

Shaker: An Eberbach, Model No. 6010 shaker was

operated at either 280 cycle/min or 180

cycle/min.

Vortex Mixer: VORTEX-GENIE, Scientific Industries,

Inc., Springfield, MA.

Incubator: Sherer, Controlled Environment Chamber,

Model CEL 37-14.

b.   Animals:

Adult male mice, typically 6-12 weeks old, of the Sprague-Dawley ARS-HA/ICR (albino) strain were obtained from Gibson Laboratories of Madison, WI. The animals were maintained on a 12 hr. light/12 hr. dark cycle (lights on from 6 a.m. to 6 p.m.) and allowed access to food and water ad libitum. No mice were used in any experiments until they had been allowed one week to become accustomed to the local conditions. In the typical assay, the animals were sacrificed between 10 and 12 a.m. by cervical dislocation.

c.   Reagent Sources:

3,4-Dihydroxyphenylalanine (dopa)-

Aldrich Chemical Co., Milwaukee, WI.

Dopamine hydrochloride (DA·HCl)-Aldrich.

Norepinephrine hydrochloride (NE·HCl) -

Aldrich.

3,4-Dihydroxybenzylamine hydrobromide

(DHBA·HBr) - Aldrich.

6,7-Dimethyl-5,6,7,8-tetrahydropterin

($DMPH_4$) - Aldrich.

6-Methyl-5,6,7,8-tetrahydropterin (6-MPH_4) -

Sigma Chemical Co., St. Louis, MO.

NSD-1034 [N'-Methyl-N'-(3-hydroxybenzyl)

hydrazinium dihydrogen phosphate] -

Sandev Research, Gilston Park, Hartlow,

England.

NSD-1015 [3-hydroxybenzylhydrazine] - Sandev.

L-Tyrosine - Sigma.

3-Iodotyrosine - Aldrich.

Catalase - Sigma.

All other chemicals were purchased from commercially available sources in the highest available purity and used without further purification. Water used for preparation of all solutions was double-distilled.

    d. Stock Standard Solutions:

Dopa (500 $\mu\underline{M}$) - prepared by dissolving 9.84 mg dopa in 100 ml of a 1×10^{-3} $\underline{M}$ ascorbic acid solution. The resultant solution was always used within 2 weeks.

NE/DA (NE-600 $\mu\underline{M}$, DA-800 $\mu\underline{M}$) - prepared by dissolving 14.98 mg NE·HCl and 19.33 mg DA·HCl in 100 ml of 10^{-3} $\underline{M}$ ascorbic acid. The resultant solution was never kept over 2 weeks.

    e. Working Standard Solution:

On the day of use, a 1.00 ml aliquot of the dopa stock standard and a 1.00 ml aliquot of the NE/DA stock standard were transferred to a 100 ml volumetric flask and diluted to the mark with a 1×10^{-3} $\underline{M}$ ascorbic acid solution.

f. Internal Standard Solution:

A solution of 8×10^{-4} $\underline{M}$ DHBA was prepared by

dissolving 17.6 mg of DHBA·HBr in 100 ml of

1×10^{-3} $\underline{M}$ ascorbic acid solution. On the day

of use, a 1:10 dilution of this solution

is made with a 1×10^{-3} $\underline{M}$ ascorbic acid

solution.

g. Blank solution:

On the day of analysis, a 1.00 ml aliquot

of the NE/DA stock solution was diluted to

100 ml with the 10^{-3} $\underline{M}$ ascorbic acid

solution.

h. Ascorbic Acid Solution:

Prepared immediately before use, the 1×10^{-3}

$\underline{M}$ ascorbic acid solution was obtained by

dissolving 40 mg of ascorbic acid in 250 ml

of distilled water.

i. Acid washed alumina was prepared as described

by Anton and Sayre (2).

j. Alumina Wash Solution:

Contained 100 μl of 0.1 $\underline{M}$ EDTA, 100 μl of

1 $\underline{M}$ NaHSO$_3$, and 1 ml of Tris buffer, (0.5 $\underline{M}$,

pH 8.60) per 100 ml of distilled water.

k. Dopa Elution Solvent:

On the day of analysis, 50 ml of 0.03 $\underline{M}$

HClO$_4$ containing 0.025 $\underline{M}$ KClO$_4$ was deaerated

for 15 minutes.   20 µl of a 1 $\underline{M}$ NaHSO$_3$
was added, and the solution was thoroughly
mixed.

1.   The Standard TH Assay Procedure:

The procedure was started by sacrificing the
animals.   The brains were removed as quickly as possible,
weighed and stored on ice.   Homogenization, effected with
the glass/Teflon homogenizer, incorporated the following
materials in the listed proportions:

1 brain/2.00 ml H$_2$O/200 µl DHBA internal standard
solution

The incubation mixture was prepared by mixing the
following components to yield a total volume of 500 µl.
The concentrations of the components in the final mixture
are given in parentheses: 100 µl of a 1.0 $\underline{M}$ acetate buffer,
(0.20 $\underline{M}$) containing 0.05 $\underline{M}$ phosphate (0.01 $\underline{M}$) and having a
pH of 6.1; 50 µl of a solution which is 4.0 m$\underline{M}$ in DMPH$_4$
(0.4 m$\underline{M}$), 2.0 $\underline{M}$ in 2-mercaptoethanol (0.2 $\underline{M}$), and 4.0 m$\underline{M}$
in benzyloxyamine (0.4 m$\underline{M}$); 50 µl of a 10 m$\underline{M}$ ferrous sulfate
solution (1.0 m$\underline{M}$) containing 10.0 unit/µl (1.0 unit/µl)
catalase; 50 µl of 1.0 $\underline{M}$ L-tyrosine (0.10 m$\underline{M}$) in 0.010 $\underline{M}$
HCl (0.001 $\underline{M}$); and, 250 µl of the brain homogenate.   All
these solutions, except the L-tyrosine and the brain homo-
genate were preincubated after mixing at 37°C for 30 minutes.
The calculation of the final concentrations of the individual
components in the incubation mixture, incidentally, assume

the homogenate is an aqueous solution with negligible amounts of particulate matter.

Standard 'homogenates', containing a known concentration of dopa, were prepared by mixing 500 µl of the dopa working standard with 2.00 ml of distilled water and 200 µl of the DHBA internal standard solution. 250 µl of this solution replaced the brain homogenate in the above procedure to provide a standard incubation mixture.

Since tyrosine solutions contain dopa as an impurity and dopa is produced by nonenzymatic routes from tyrosine (5), it is important to run blanks for each assay. This is accomplished by replacing the brain homogenate in the incubation mixture with 250 µl of a solution prepared by mixing the following components: 500 µl NE/DA blank solution, 2.00 ml H_2O, and 200 µl of the DHBA internal standard.

The incubation mixture was placed in a capped centrifuge tube, and the incubation was carried out at 37°C in an air bath for 30 minutes. The tubes were positioned horizontally in the shaker with the long axis parallel to the direction of shaking. The shaker was set on the high speed setting. The reaction was terminated with the addition of 200 µl of 1 $\underline{M}$ $HClO_4$. 50 µl of a solution containing 0.30 $\underline{M}$ $NaHSO_3$ and 0.030 $\underline{M}$ EDTA was also added to inhibit oxidation of the catechols. The samples were then centrifuged at 16,000xg and 4°C for 15 minutes with storage on ice before

and after.

500 μl of the supernatant was placed in a small vial containing 20 mg of acid-washed alumina and 2.00 ml of a 0.50 M Tris buffer at pH 8.60. The buffer was previously deaerated for 15 minutes with O_2-scrubbed N_2 (6). The vials were shaken at room temperature for 15 minutes. Upon removal from the shaker, the alumina was allowed to settle. The supernatant solution was removed with a Pasteur pipette connected to a vacuum aspirator. The alumina was washed three times (shaking for 3-5 seconds on a vortex mixer, settling, and aspiration) with 2.00 ml of deaerated wash solution. The compounds of interest were eluted from the alumina by addition of 200 μl of elution solvent. The vials were thoroughly mixed (3-5 seconds) by use of a vortex mixer to insure homogeneity.

About 5 μl of the alumina solvent was finally injected into the LC/EC for the separation and quantitation of dopa.

m. Calculations:

The amount of dopa formed by the hydroxy-lation reaction per unit time per unit weight is a direct measure of the TH activity. The calculation of this activity, thus, incorporated the ratio (R) of peak heights for dopa and DHBA for the sample, the standards, and the blanks. A typical calculation is given as:

Dopa formed (nmol/g/hr) =

$$\left[\frac{R_{sample} - R_{blank}}{R_{std} - R_{blank}}\right] \left[\frac{nmol\ dopa\ in\ a\ standard\ incubation\ mixture}{(tissue,\ wt,\ g)\ (incubation\ time,\ hr)}\right]$$

$$\text{where } R = \frac{\text{peak height, dopa}}{\text{peak height, DHBA}}$$

The R_{std} and R_{blank} values represent the average ratios for all standards and blanks run during a particular analysis.

Each result in this chapter, which is expressed with an estimate of uncertainty represents the mean ± the standard error of the mean (S.E.M.) for at least four separate determinations. Each experiment was also typically repeated to confirm its validity. Statistical comparisons of data sets were performed with the Null Hypothesis using Student's t-distribution (7).

3. Chromatographic Conditions

The LC/EC procedure previously developed for the TH assay employed a column with very low resolution. This column also provided separations rather exclusively for cations. Consequently, a mobile phase with a very low pH had to be employed to convert the zwitterionic amino acid, dopa, into its positively charged form. Unfortunately, under these conditions small amounts of ferrous ion suddenly become visible in the chromatogram. The source of this Fe^{+2} is not presently known. And, although Fe^{+2} is well separated from the other compounds of interest, its relatively large retention time significantly increases the time required for each analysis (see Figure 2.1). Unsuccessful attempts were made to solve this problem by adding complexing agents ($C_2O_4^=$, EDTA, and o-phenanthroline) to the mobile
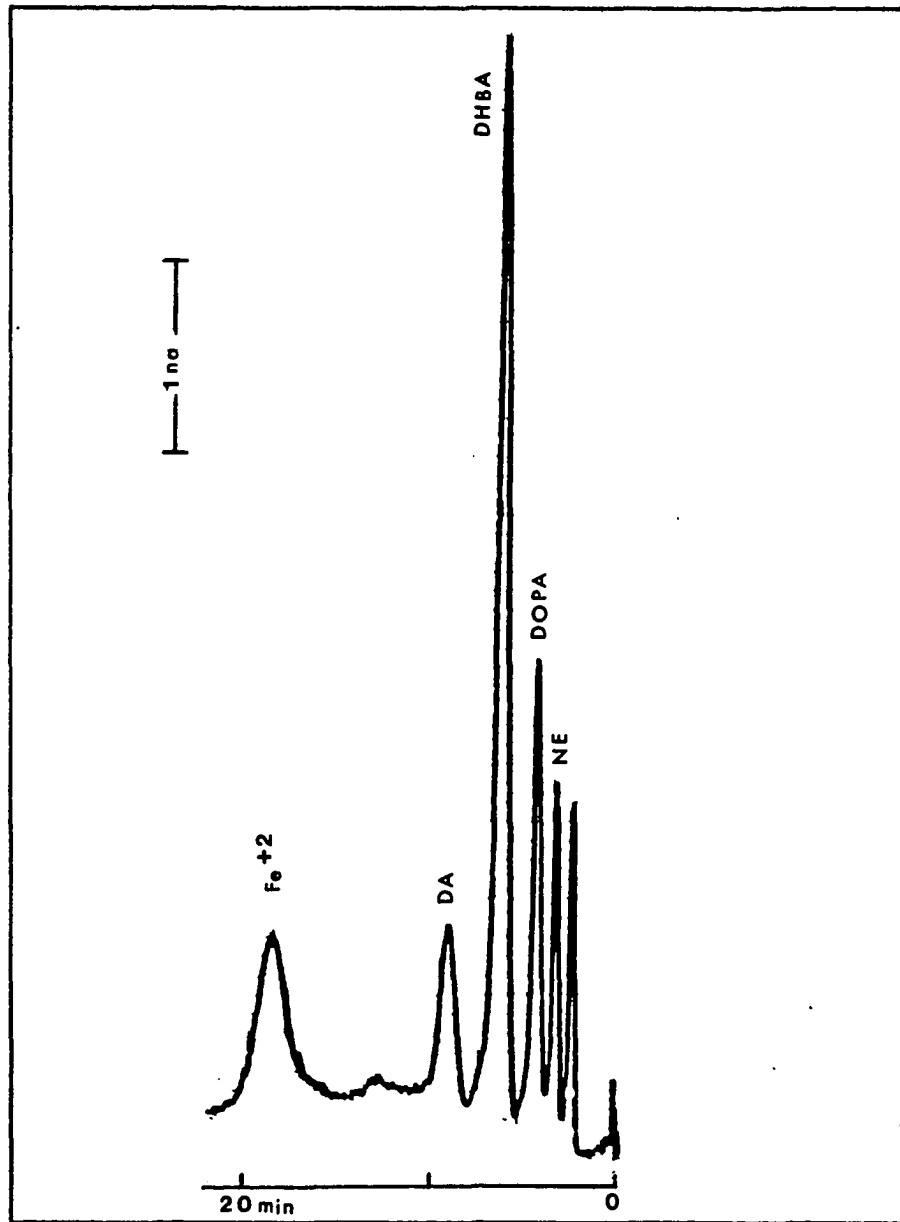
Figure 2.1 Low Resolution Chromatogram Used in Previous
            TH Assay.
                Column: Zipax SCX, 750x3 mm
                Mobile Phase: 0.025 $\underline{M}$ $KClO_4$, 0.030 $\underline{M}$ $HClO_4$
                Flow Rate: 0.7 ml/min

phase and by adjusting the pH of the mobile phase (3).
At a pH $\geq$ 4, however, Fe^{+2} no longer appears in the chromato-
grams. But, dopa is not retained at this pH.

We, thus, clearly welcomed the recent commercial
availability of microparticulate reverse-phase columns.
Operated in the normal reverse-phase mode, the pH of the
mobile phase can be significantly raised for these columns
without eliminating the retention of dopa. After trying
several mobile phases, including different molarities and
pH values for citrate/phosphate and citrate/acetate buffers,
we found that the citrate/acetate buffer, described in the
Materials and Methods section of this chapter, provided the
desired separation for all the compounds involved in the TH
assay (see Figure 2.2). The elimination of the Fe^{+2} inter-
ference, coupled with the high efficiency of the micro-
particulate column, effectively cut the analysis time in
half. It should be noted that the LC/EC must adequately
separate norepinephrine (NE), dopamine (DA), 3,4-dihydroxy-
phenylacetic acid (DOPAC) and 3,4-dihydroxybenzylamine
(DHBA) in addition to dopa. NE, DA and DOPAC are endogenous
components of brain, while DHBA is the added internal stan-
dard. And, all four of these species will be isolated from
the incubation mixtures using the alumina procedure des-
cribed below.
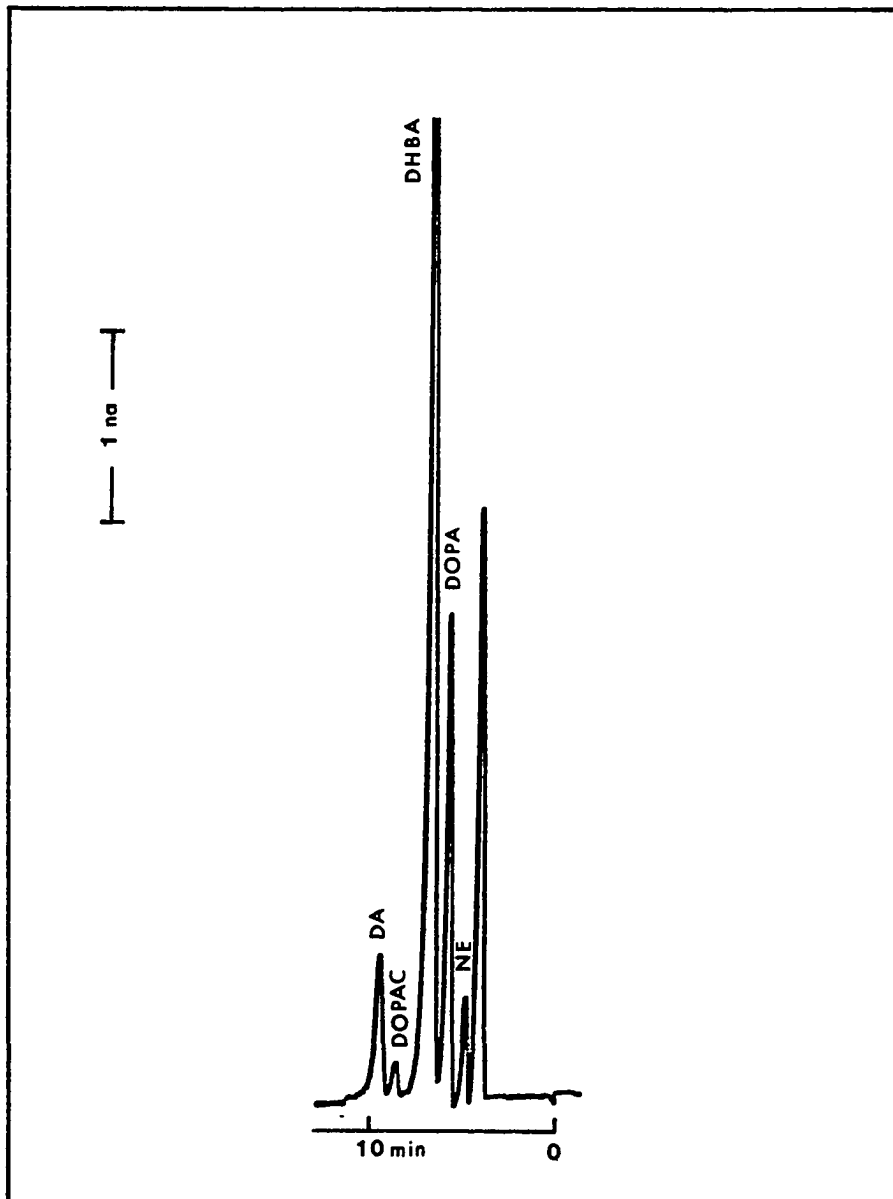
Figure 2.2   High Resolution Chromatogram Used in Current
             TH Assay
                Column: Ultrasphere ODS, 150x4.6 mm
                Mobile Phase: Citrate/acetate, pH 5.1
                Flow Rate: 0.3 ml/min

4. Isolation Procedure

The isolation of dopa from the incubation mixture is most efficiently accomplished by the alumina adsorption procedure of Antone and Sayre (2). This is a highly pH dependent process, with selective adsorption onto Al_2O_3 being obtained for compounds containing a catechol moiety at pH 8.6. After washing the Al_2O_3-bound species, their elution is achieved by simply lowering the pH of the contacting solvent.

Being concerned about the applicability of this Al_2O_3 isolation procedure to the present incubation mixtures, we decided to accurately determine the efficiency of the process in the presence and absence of tissue homogenates. This study incorporated incubation mixtures with brain homogenates (samples) and incubation mixtures without brain homogenates (blanks). Varying amounts of dopa solutions and fixed amounts of DHBA were added to these mixtures. Some of the standard dopa and DHBA solutions were subjected to LC/EC quantitation at this point. The remaining samples and blanks were then carried through the alumina adsorption procedure. After elution from alumina, a precisely measured amount of the eluate was injected into the LC/EC system. The observed peak heights for both DHBA and dopa, after correcting for dilution, were compared with those from the original standard solutions to obtain the percent recovery for each species. Typical results are listed in Table 2.1.

TABLE 2.1

Percentage Recovery of Dopa and DHBA After Alumina Adsorption

Compounds	Percentage Recovery	
	In the absence of brain	In the presence of brain
DOPA	84.0±2.6	42.8±0.4
DHBA	83.9±0.9	47.4±0.6

The linearity of the peak height ratio of dopa and DHBA (H_{Dopa}/H_{DHBA}) vs. the amount of dopa added during the adsorption process was also obtained from these studies. The results for the samples containing tissue homogenates are shown in Figure 2.3. As can be seen, excellent linearity is obtained for the usual values of dopa encountered in routine TH assays.

5. Optimization of Incubation Parameters

The initial incubation conditions are presented in the Materials and Methods section of this chapter. These conditions represent a carefully selected combination of a number of existing TH procedures. But, even a careful selection does not necessarily provide optimal conditions. Thus, we decided to optimize these incubation parameters. Appropriate ranges of each parameter were examined while holding all others constant. On occasion, appropriate ranges of alternate reagents were also examined. The sequence of the parameters investigated, however, is important. The optimum for a given parameter, once determined, was incorporated into the procedure for all subsequent optimization studies. It should also be noted that all concentrations refer to the final incubation mixture.

a. Molarity of Acetate Buffer. The concentration of the buffer system used in enzymatic assays frequently effects the measured activities. The results of
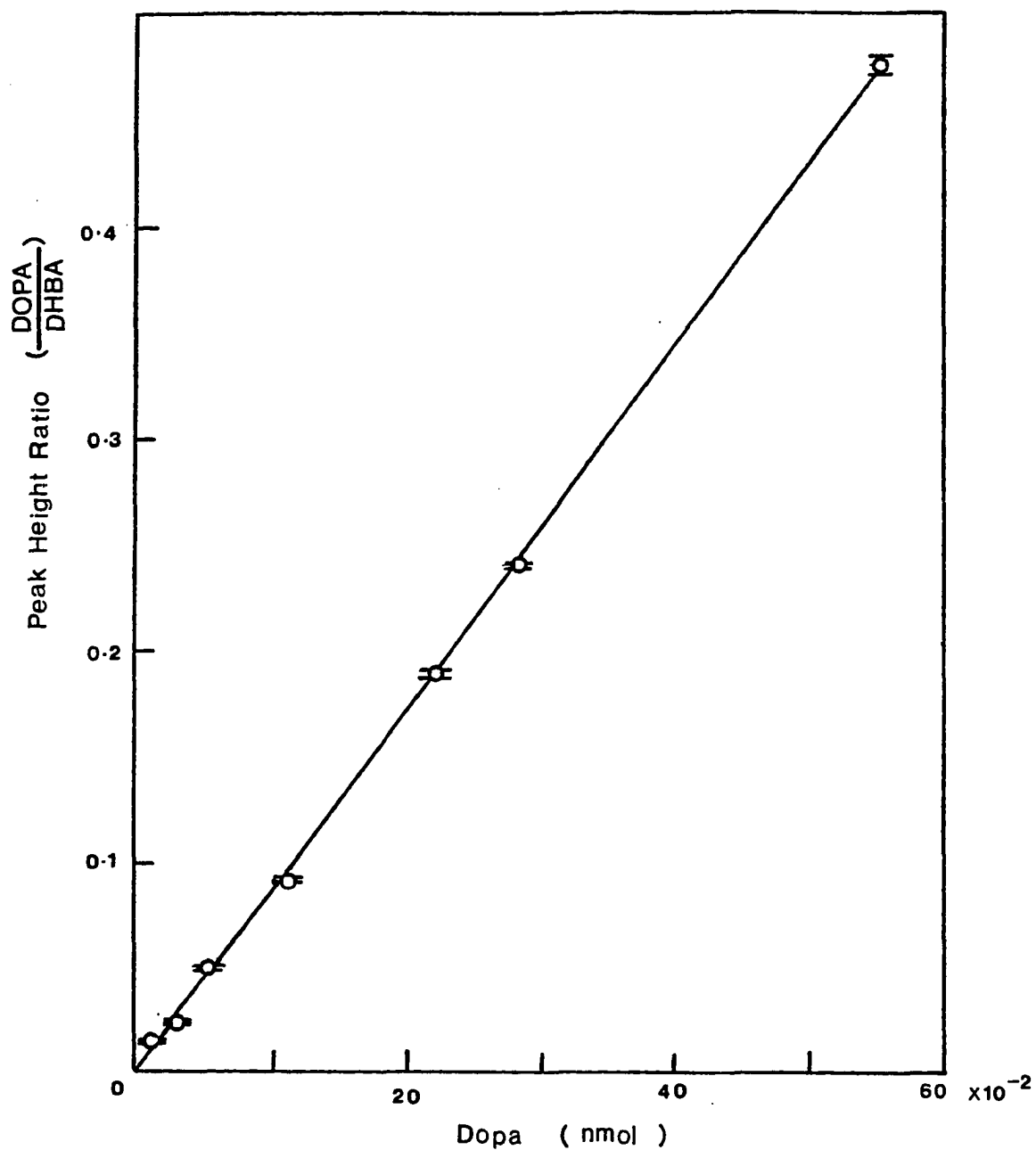
Figure 2.3 Relationship Between the Peak Height Ratio of
Dopa and DHBA and the Amount of Dopa Added
in the Alumina Adsorption Procedure.

The amount of dopa added was varied between
0.014 nmol and 0.56 nmol. The amount of DHBA
was fixed at 0.98 nmol.

TABLE 2.2

The Effect of Concentration of Acetate Buffer on

TH Activity

Acetate Concentration ($\underline{M}$)	TH Activity (nmol/g/hr)
0	10.5±1.5
0.01	9.1±0.7
0.02	9.1±0.2
0.05	8.7±1.1
0.10	12.3±0.7
0.20	22.4±0.6
0.50	25.0±0.1

the investigation of the molarity of the acetate buffer in
the TH assay are presented in Table 2.2 As can be seen,
the TH activity reached a reasonably high value at a buffer
concentration of 0.20 $\underline{M}$. While the higher concentration,
0.50 $\underline{M}$, yielded a slightly higher activity, it is incon-
venient to employ in routine investigations. Its concen-
trated form often provides solid formation upon slight
evaporation. This solid material leads to pipet clogging
and associated difficulties. Thus, the 0.20 $\underline{M}$ buffer was
used for all subsequent determinations.

b. pH of Acetate Buffer. Previous studies
have indicated that TH activity is highly pH dependent,
As shown in Figure 2.4, the optimum of the current assay
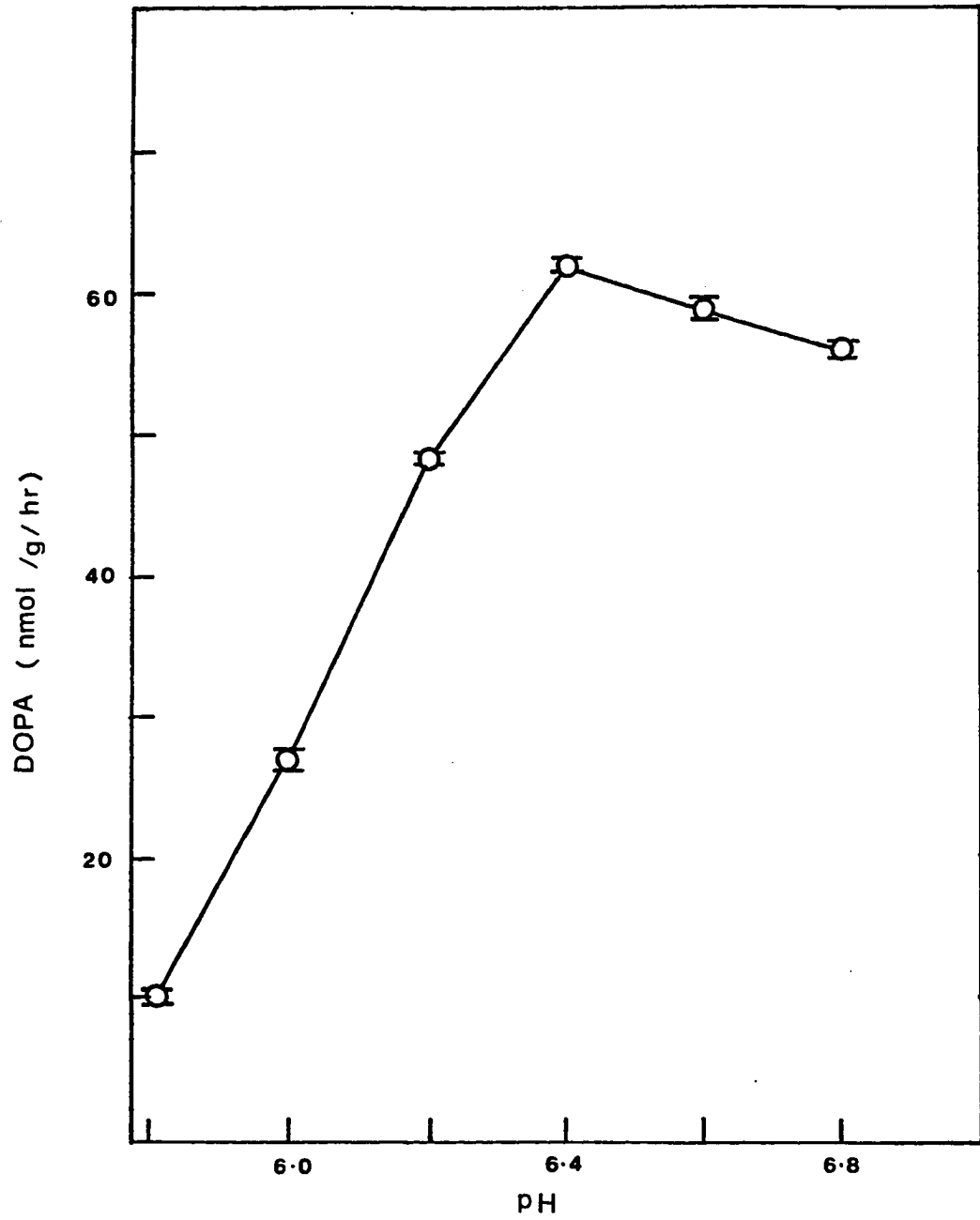
Figure 2.4   The Effect of Buffer pH on TH Activity.

occurs at a pH of 6.4. This value is a bit higher than the value of 6.0 previously used by a number of workers (8,9). The multiple differences (enzyme source, animal species, incubation parameters, etc.) between the current and previous investigations, however, preclude a direct assessment of this apparent pH shift. All the following studies used a pH of 6.4.

c. Phosphate Concentration. Some reports of TH assay procedures (8) have claimed that phosphate, added to the incubation mixture, provides greater measured activity levels. In the present system, as indicated in Figure 2.5, the addition of phosphate caused a decrease in the measured activity, with higher concentrations eliciting lower activities. Therefore, phosphate was eliminated from subsequent determinations.

d. Catalase. Catalase has been reported by some to enhance TH activity. The results of the present investigation, however, do not support these reports (see Table 2.3). The smallest concentration (0.01 unit/μl) tested appears to provide a minimal increase in activity. But, this result is not significantly different from the case where no catalase was added. Thus, catalase was omitted from all subsequent assays.

e. Ferrous Ion. As indicated in Chapter 1, the role of the ferrous ion in TH assays is still somewhat controversial (5). The results of the current investigation
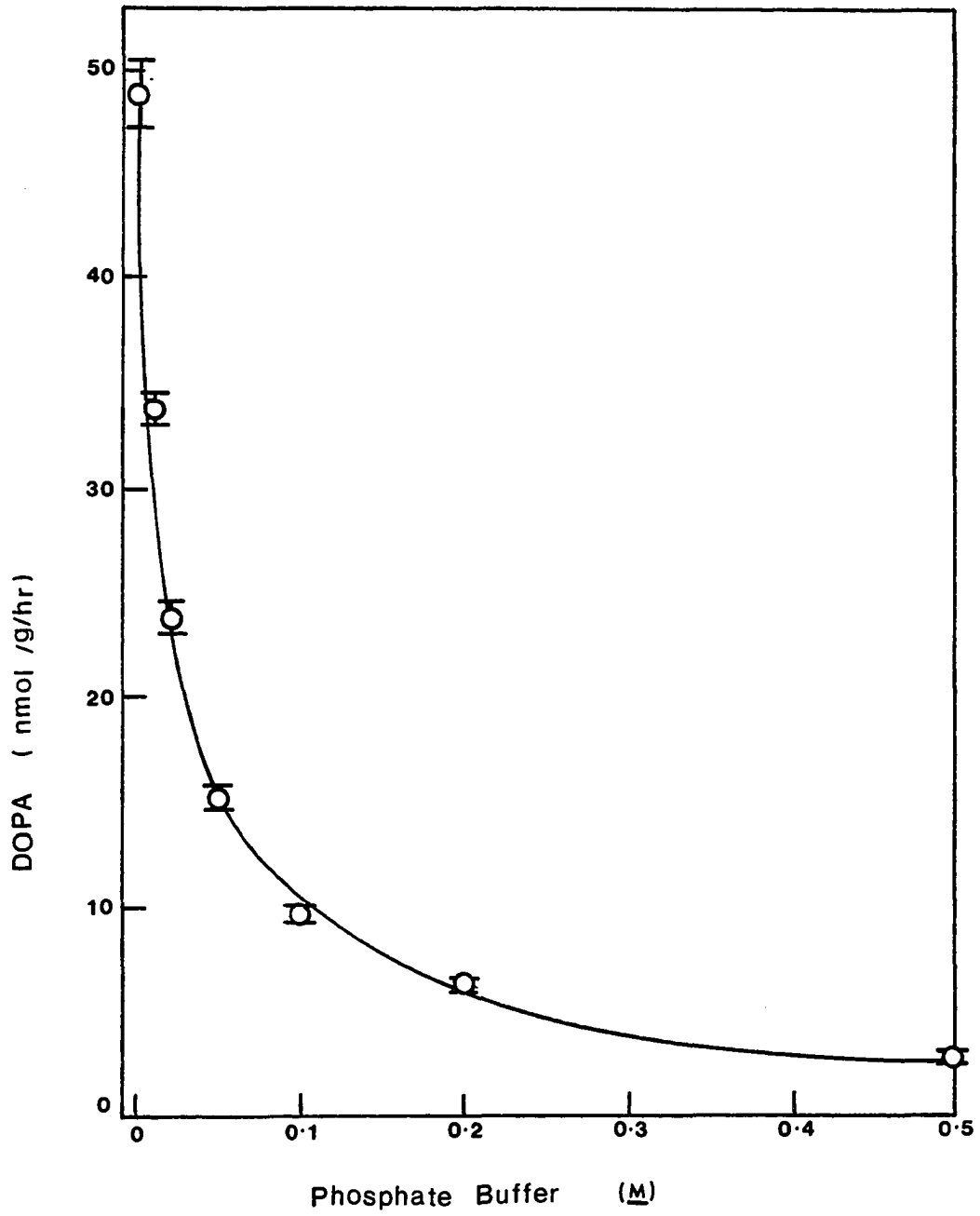
Figure 2.5 The Effect of Various Phosphate Concentration on TH Activity

TABLE 2.3

The Effect of Catalase on TH Activity

Amount of Catalase (unit/μl)*	TH Activity (nmol dopa/g/hr)
0	45.4±2.1
0.01	49.7±7.1**
0.1	37.4±6.9
0.4	31.6±4.3
1.0	28.6±5.9
4.0	20.9±5.7
10.0	15.1±2.5

*1 unit of catalase decomposed 1 μmol of H_2O_2 per minute at pH 7.0 and 25°C while the H_2O_2 concentration in the reaction mixture falls from 10.3 to 9.2 mol per ml. The solid material used as the source of catalase in this experiment exhibited 3,000 units/mg.

**Not significantly different from the result for 0 unit/μl (P>0.05).

TABLE 2.4

The Effect of Iron(II) on TH Activity

Fe^{+2} Concentration (m$\underline{M}$)	R_{blank}	TH Activity (nmol dopa/g/hr)
0.0	0.035±0.005	45.0±0.6
0.05	0.065±0.000	58.5±0.3
0.10	0.067±0.009	65.5±0.5
0.40	0.094±0.005	62.0±2.8
1.00	0.131±0.003	48.8±11.7

of the effect of Fe^{+2} are listed in Table 2.4. These results clearly demonstrate that the ferrous ion has a significant effect on TH activity in the present procedure. It has also been reported, however, that the ferrous ion contributes to the nonenzymatic hydroxylation process (25). Thus, blanks were simultaneously run for each of the investigated values of Fe^{+2} concentration. The choice of an "optimal" value under these conditions can be appropriately interpreted in two distinct ways. One optimum would simply be that which yields the greatest measured TH activity. An alternative optimum is that which yields the highest ratio of tissue/blank values. Both of these viewpoints were considered in selecting 0.10 $\underline{M}$ Fe^{+2} as the overall optimum value. While yielding the maximum measured activity, however, it should be noted that this concentration does not represent the maximum signal/noise ratio. The maximum ratio occurs with no added Fe^{+2}. Nonetheless, 0.10 $\underline{M}$ Fe^{+2} was employed in all subsequent assays.

f. Dopa Decarboxylase Inhibitors. The previous assay for TH originating from this laboratory had selected benzyloxyamine (BOA) as the dopa decarboxylase inhibitor primarily due to its commercial availability (1). However, this agent exhibited some noticeable inhibitory properties. Thus, we decided to include NSD-1015 and NSD-1034 in the present investigation. As shown in Figure 2.6, none of these agents are ideal. Each exhibits inhibition of TH
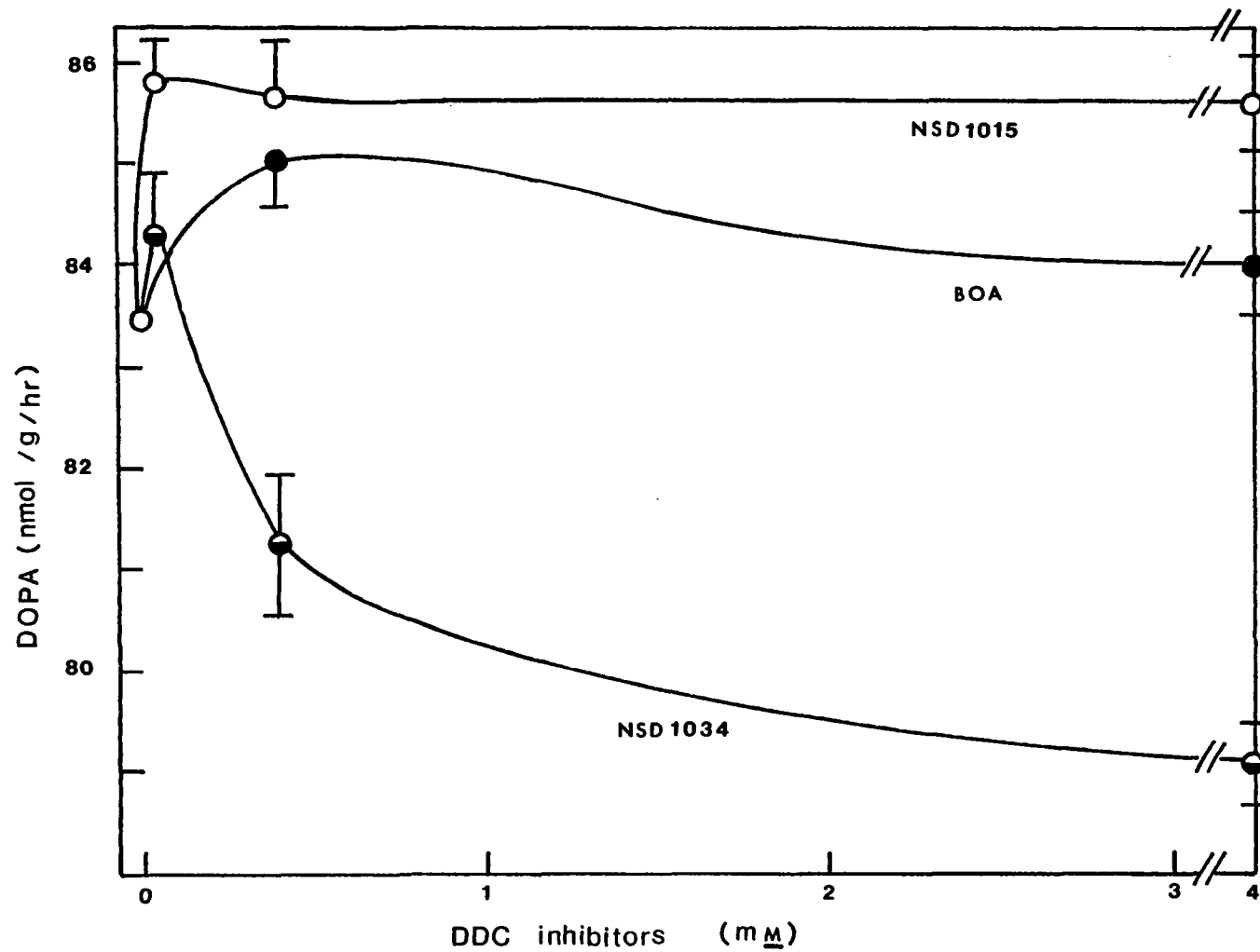
Figure 2.6 The Effect of Different Dopa Decarboxylase Inhibitors on TH Activity.

at higher concentrations. Based upon the results, however, 0.040 $\underline{M}$ NSD-1015 was selected as the optimal value for the following assays.

g. Pterin Cofactor. The effect of two different cofactors, $DMPH_4$ and $6\text{-}MPH_4$, on the rate of formation of dopa were also investigated. The results are presented in Tables 2.5 and 2.6. The two data sets are not strictly comparable since each cofactor investigation was run on a separate day and employed different brain homogenates. Nonetheless, some general comments are appropriate.

Both of the cofactors tested have a significant effect on the production of dopa, and $6\text{-}MPH_4$ appears to be slightly more effective. A concentration of 1.5-2.25 $\underline{mM}$ was required for $6\text{-}MPH_4$ to provide an approximately maximal activity, while 4.0 $\underline{mM}$ was required for $DMPH_4$. This is in agreement with a previous report (12) that $6\text{-}MPH_4$ is approximately twice as efficient as $DMPH_4$ in its role as cofactor.

A kinetic analysis of the data for $6\text{-}MPH_4$ is shown in Figure 2.7. The double reciprocal plot in this figure was used to determine the K_m (0.31 $\underline{mM}$) and V_{max} (97.6 nmol/ g/hr) values for this cofactor. Subsequent assays employed 2.0 $\underline{mM}$ $6\text{-}MPH_4$.

h. 2-Mercaptoethanol. This compound is frequently employed in enzyme assays as a protein activator (11). It can also serve as a reducing agent to inhibit the non-enzymatic oxidation of the pterin cofactor. Employment
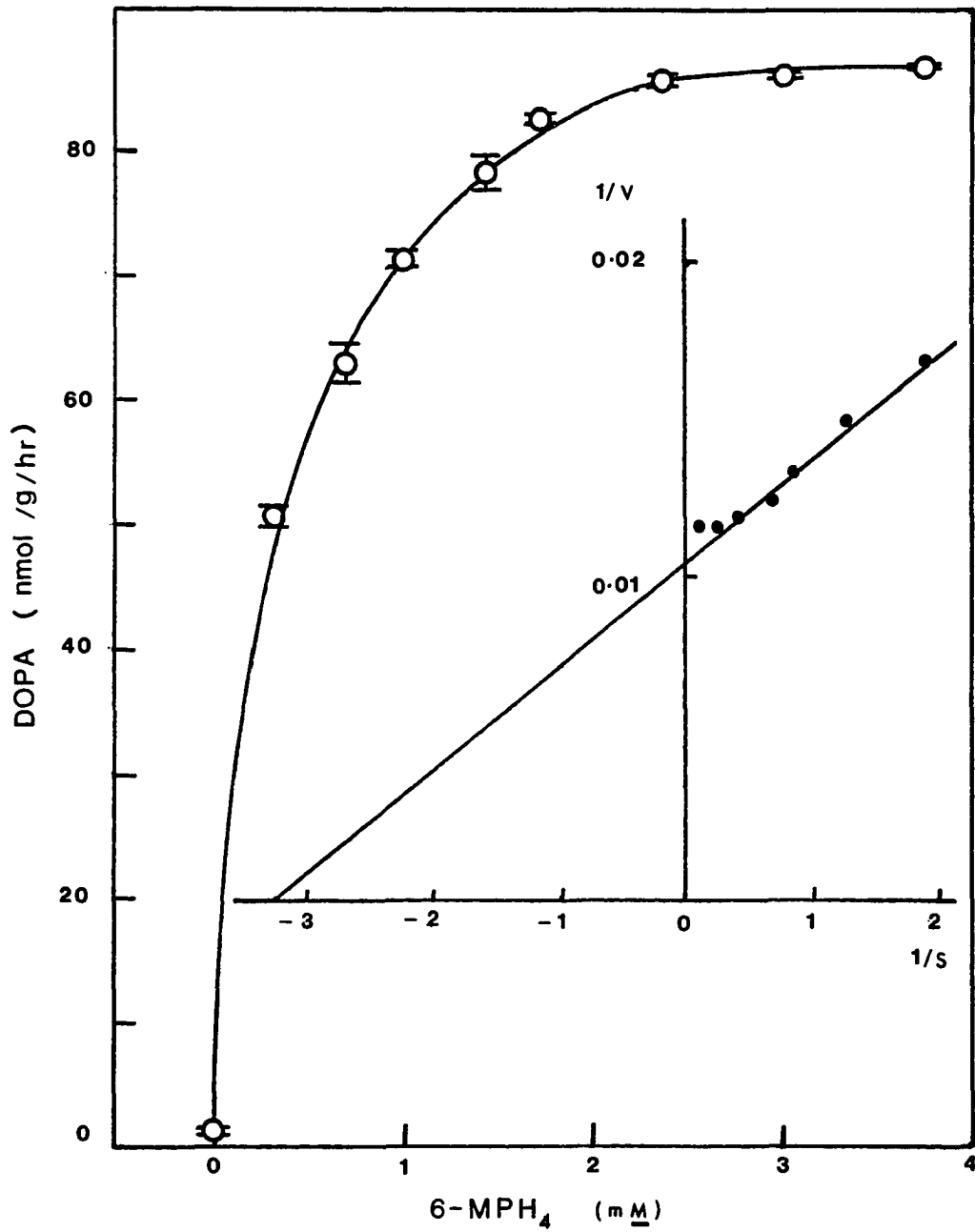
Figure 2.7   The Effect of 6-MPH$_4$ on the Rate of Formation of Dopa.

TABLE 2.5

The Effect of $DMPH_4$ on TH Activity

$DMPH_4$ (m$\underline{M}$)	TH Activity (nmol dopa/g/hr)
0.00	0.9±0.7
0.04	12.6±0.5
0.10	19.1±0.2
0.40	36.8±0.2
1.00	51.6±0.2
4.00	70.7±0.5
10.00	62.4±1.4

TABLE 2.6

The Effect of $6-MPH_4$ on TH Activity

$6-MPH_4$ (m$\underline{M}$)	TH Activity (nmol dopa/g/hr)
0.00	0.40±0.1
0.30	49.3±0.9
0.60	62.8±2.0
0.90	71.3±0.7
1.20	77.4±1.6
1.50	83.3±0.5
3.25	85.8±0.6
3.00	86.0±0.4
3.75	85.2±0.2

of 2-mercaptoethanol in the present assay, as seen in Figure

2.8, appears to both stimulate and inhibit TH activity. The

optimal value of 0.050 $\underline{M}$ was selected for subsequent assays.

     i. Incubation Temperature. Previous studies

of TH have fairly uniformly employed 37°C as the incubation

temperature. We briefly examined the applicability of this

temperature by comparing it to higher (40°C) and lower (34°C)

values. As seen in Table 2.7, 37°C does, indeed, yield the

optimal TH activity.

     j. Shaking Parameters. These parameters include

the shaking rate during incubation and orientation of the

centrifuge tubes containing the incubation mixtures. The

results are listed in Table 2.8. For these studies, it

should be noted, the direction of shaking was horizontal.

Also, a horizontal tube position means the tube was not

only laying on its side, but the long axis of the tube was

parallel to the direction of shaking.

As expected, the horizontal tube placement yields

the maximal TH activities with no significant differences

being observed for the two shaking rates. At either shaking

rate, the vertical placement results were not effected by

having the tubes capped or uncapped. But, the faster shaking

rate provided smaller activities with vertical placement.

This result cannot be completely explained at the present

time.

The horizontal placement, as mentioned, provides
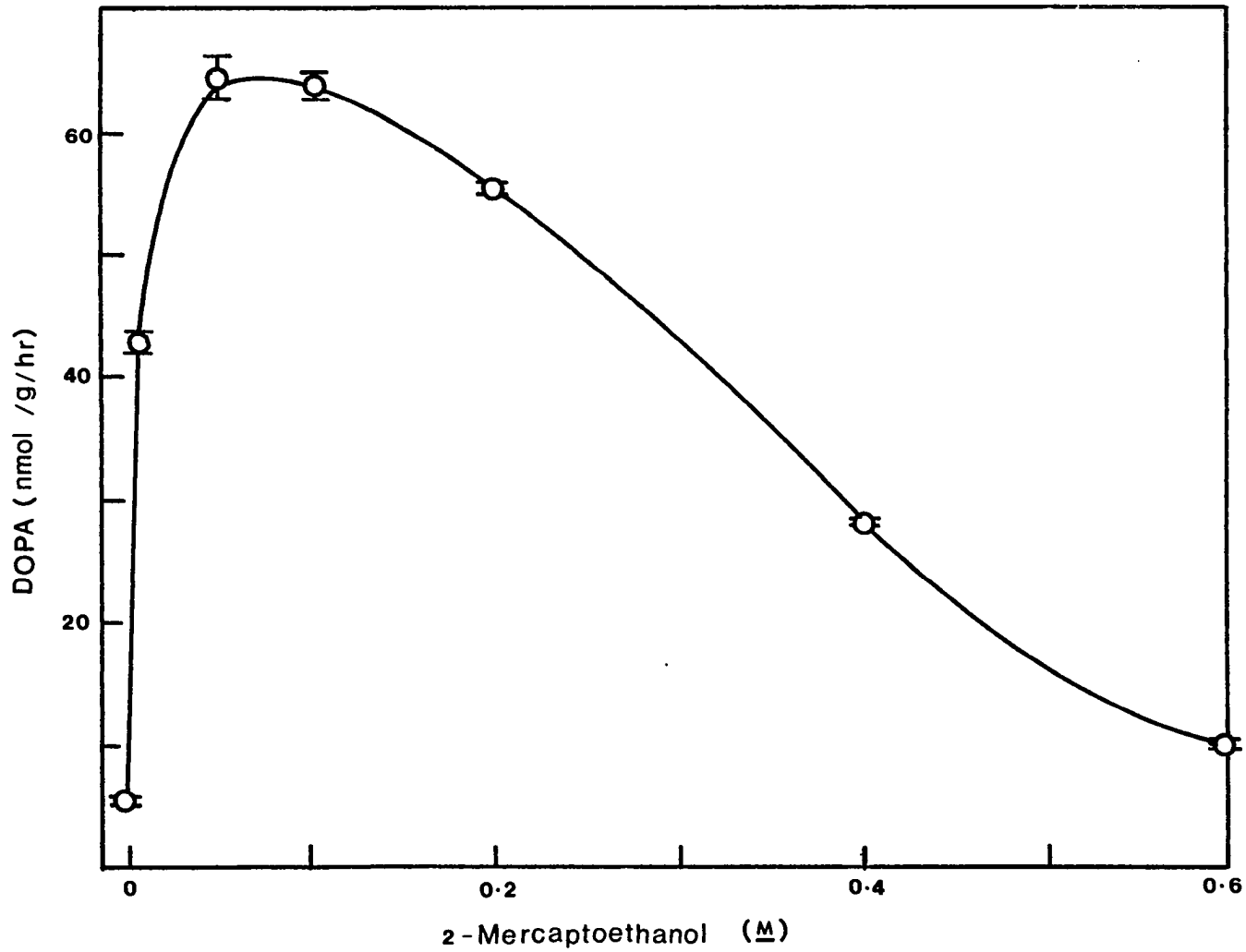
Figure 2.8   The Effect of 2-Mercaptoethanol on TH Activity.

TABLE 2.7

The Effect of Incubation Temperature on TH Activity

Incubation Temperature (°C)	TH Activity (nmol dopa/g/hr)
34	38.2±1.1*
37	55.7±2.7
40	44.3±1.8*

*Significantly lower than the result obtained at 37°C (P<0.05).

TABLE 2.8

The Effect of Shaking Rate and Position of Incubation
Tube on the Rate of Dopa Production

Shaking Rate	Position of Tube	TH Activity (nmol/g/hr)
Fast (4.7 cycle/sec)	Vertical, uncapped	82.5±2.5
	Vertical, capped	81.7±0.9
	Horizontal, capped	111.0±1.0
Slow (3.0 cycle/sec)	Vertical, uncapped	105.0±1.0
	Vertical, capped	103.0±2.0
	Horizontal, capped	109.0±2.0

the maximal TH activity. But, handling for this placement requires capping prior to incubation and uncapping after incubation for each sample. The uncapped vertical placement at a slow shaking rate, on the other hand, does not necessi- tate such inconveniences and yet yields an activity that is not significantly different from the horizontal placement. For this reason, the uncapped vertical conditions and the slow shaker rate were selected as optimal for routine application.

k. Tyrosine. The concentration of tyrosine contributes to both the enzymatic and nonenzymatic formation of dopa. As in the investigation of Fe^{+2}, therefore, an optimal value must consider both of these processes. Practically, one must select the minimal saturating value for tyrosine. The effects of varying substrate concentration on both tissue samples and blanks are presented in Table 2.9. The Michaelis-Menton kinetic plot of this data is shown in Figure 2.9. The tyrosine values used to construct Figure 2.9, it should be noted, incorporated the endogenous tissue value of 10 µg/g for the substrate (13). From this data, the derived values of K_m and V_{max} are $4.0x10^{-5}$ $\underline{M}$ and 131 nmol/g/hr, respectively. Thus, the minimal saturating value of 0.25 m$\underline{M}$ was selected for all the following assays.

l. Incubation Time. As shown in Figure 2.10, linear accumulation of dopa ceases after an incubation time of approximately 20 minutes. Thus, all subsequent determinations employed a 20 minute incubation period.
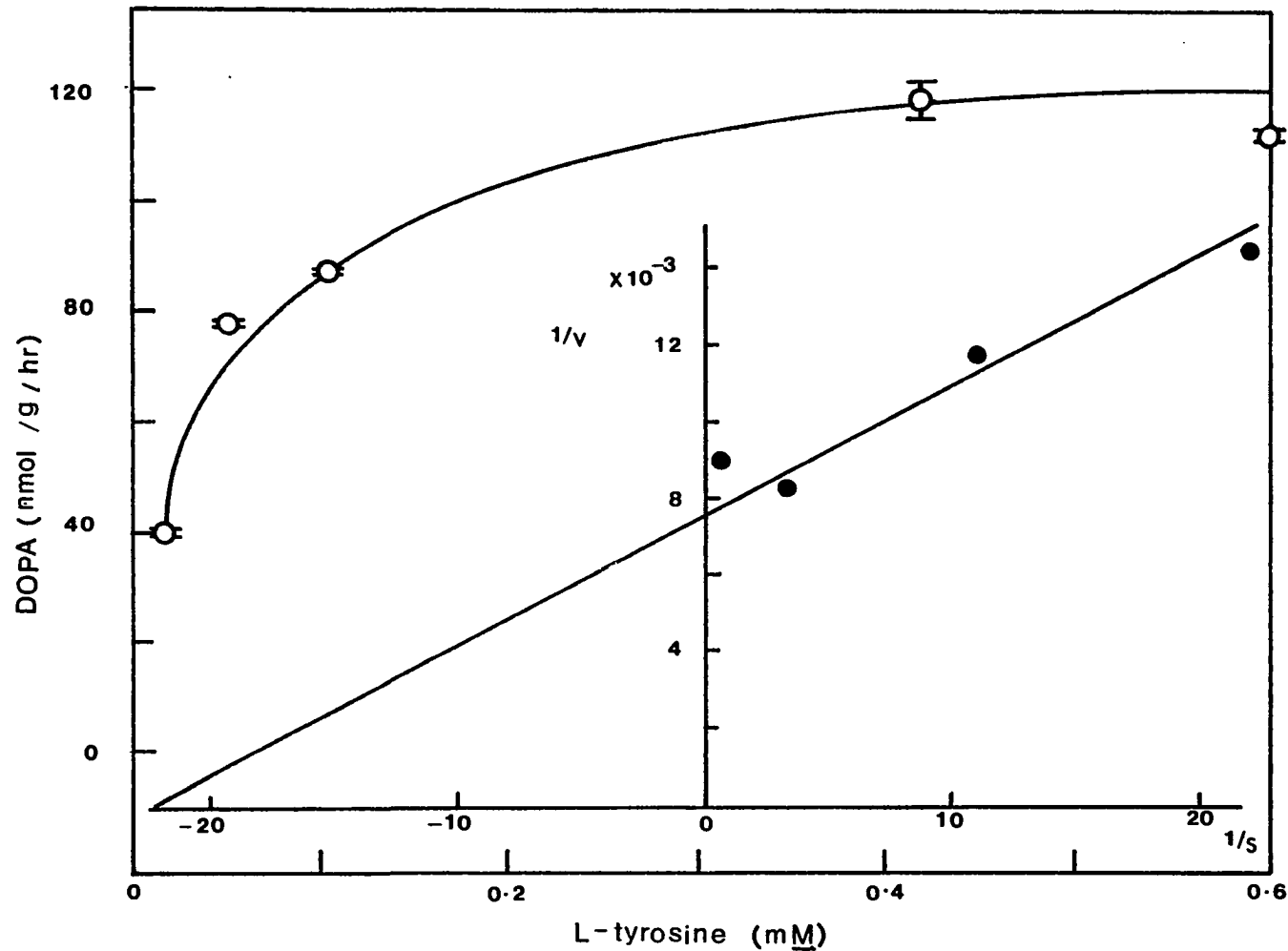
Figure 2.9 The Effect of L-tyrosine on the Rate of Dopa Formation.
The contribution of endogenous tyrosine from the tissue homogenate
is incorporated into the values used to construct these graphs.
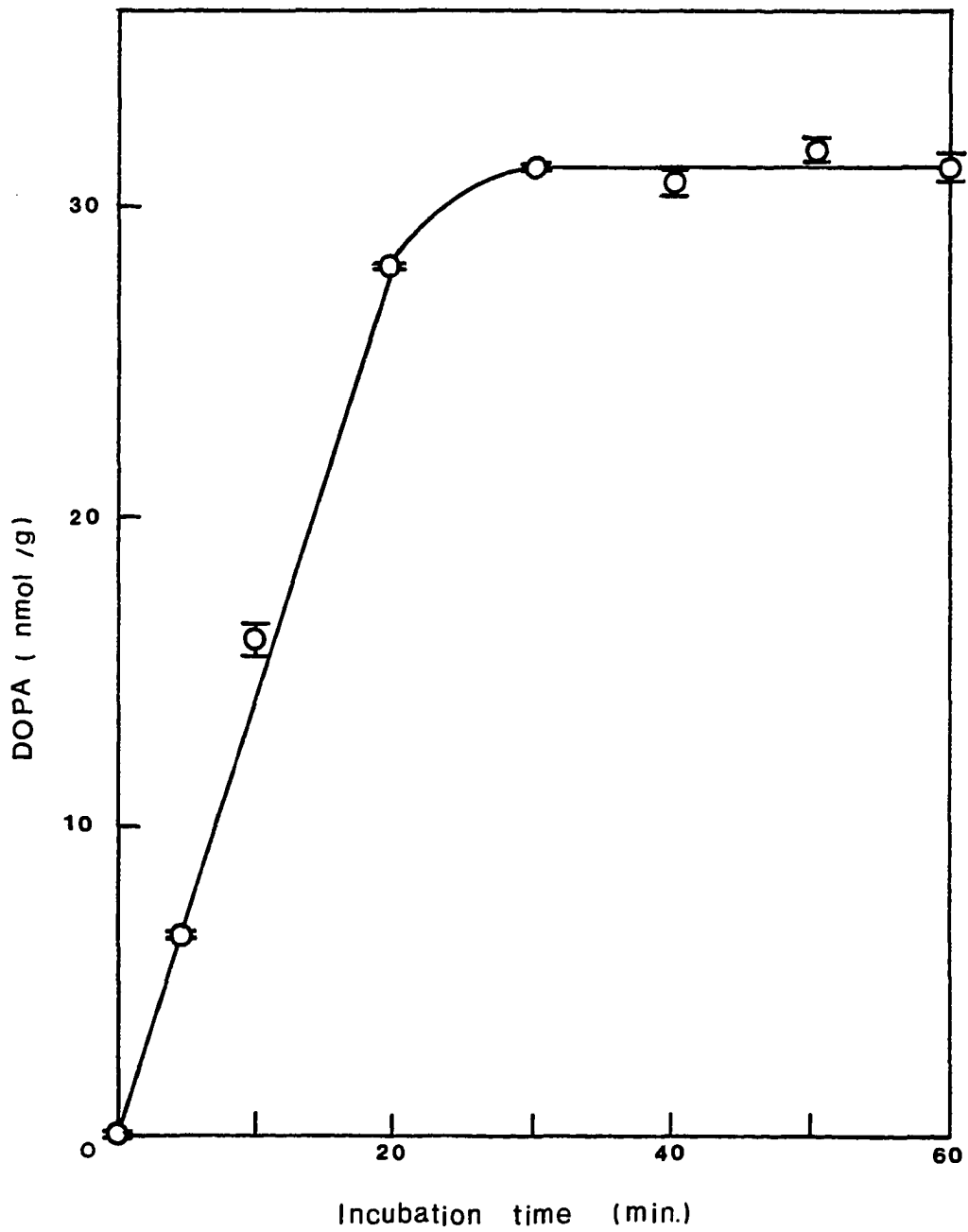
Figure 2.10   The Effect of Incubation Time on TH Activity.

TABLE 2.9

The Effect of Tyrosine Concentration on Blank

Conc. Tyrosine (m$\underline{M}$)	R_{blank}	TH Activity (nmol/g/hr)
0.01	0	43.3±1.3
0.05	0.0201±0.001	75.7±1.1
0.10	0.0337±0.007	87.0±0.7
0.40	0.160±0.023	124.0±4.0
0.60	0.215±0.006	110.0±2.0

m. Preincubation Time. Regardless of the source of enzyme, preincubation of some of the incubation components has been shown to provide greater activities. However, results for hypotonically homogenized whole mouse brain samples have not been previously reported. Thus, we decided to examine the need for preincubation in the current procedure. One group of samples was preincubated for 30 minutes before the addition of substrate and tissue homogenate while another group was processed without any preincubation. As expected, the group which had been pre-incubated provided higher activities (115±14 vs. 91±12 nmol/g/hr). Therefore, preincubation was retained in the present procedure.

n. Enzyme Concentration. Finally, the obser-
vation of TH activity as a function of the amount of added
enzyme (expressed as the volume of added brain homogenate)
was undertaken to ascertain the absence of any endogenous
inhibiting materials in the homogenate. The results for
this investigation are shown in Figure 2.11. As can be
seen, the amount of dopa formed was linearly dependent on
the amount of homogenate added to the incubation mixture.
Thus, the originally selected amount of tissue homogenate
experiences no inhibition from endogenous factors and is
completely appropriate for routine analysis.

6. Minimizing Blank (Noise) Levels.

Blank values directly affect the limit of
detection for TH assays. The signal to noise ratio obtained
by these determinations may be conveniently expressed as
the tissue/blank level. Thus, attempts to lower the detection
limit are naturally concerned with minimizing the blank
values. A major contribution to blank values is presumed
to arise from nonenzymatic hydroxylation of the substrate.
This reaction has been extensively studied for the related
Fe^{+2}/O_2/tetrahydropterin/phenylalanine system (25), where
hydroxylation is postulated to proceed by the following
mechanism:
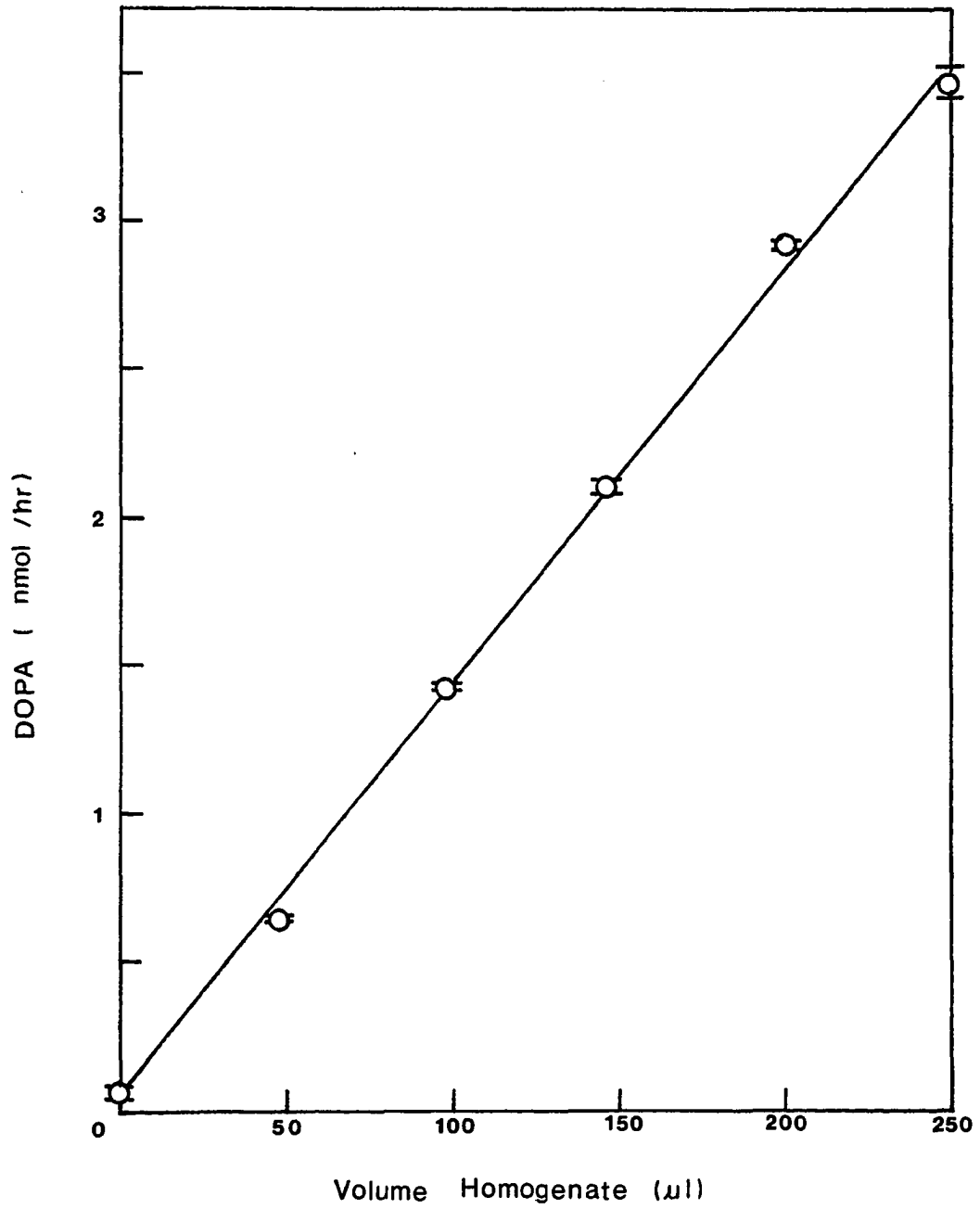
Figure 2.11   Dopa formed as a Function of Added Brain
             Homogenate.
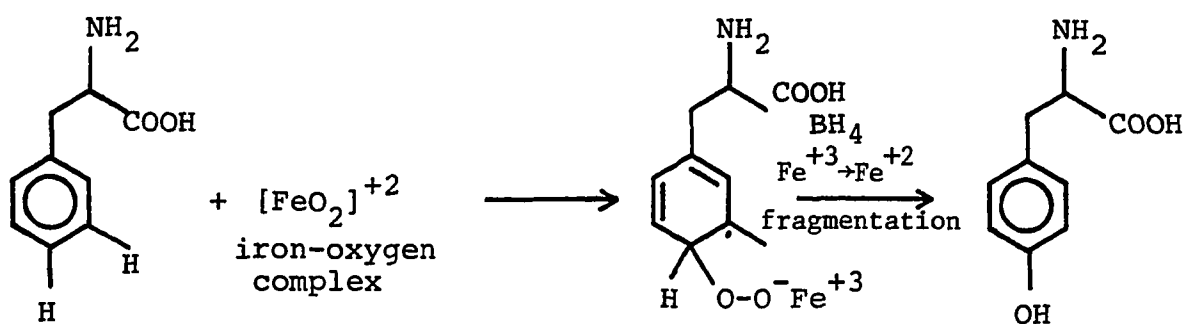
Due to the structural similarities between tyrosine and
phenylalanine, it is assumed that nonenzymatic formation
of dopa from tyrosine proceeds through a similar mechanism.
From this mechanism, it is apparent that the concentrations
of both tyrosine and Fe^{+2} would directly affect the unde-
sirable production of dopa. We, thus, decided to more
thoroughly examine the importance of these concentrations.
As seen in the upper part of Table 2.10, nonenzymatic dopa
production clearly increases with increasing Fe^{+2} concen-
trations. At 10 mM Fe^{+2}, no dopa was obtained from the
Al_2O_3 eluate. However, this was later shown to result from
the direct adsorption of Fe^{+2} leading to the loss of ad-
sorption sites for dopa. The third and fifth points in
the upper part of Table 2.10 additionally demonstrate the
seemingly greater dependence of nonenzymatic hydroxylation
upon the tyrosine concentration.

A second likely source of nonenzymatic dopa is re-
presented by the dopa impurity present in the "pure"

TABLE 2.10

The Effect of Fe^{+2} and Tyrosine on Blank Values

Conc. Fe^{+2} (m$\underline{M}$)	Incubation/Preincubation Times (min)	Conc. Tyrosine (m$\underline{M}$)	R_{blank}
0.0	20/30	0.10	0.090 ± 0.005
0.4	20/30	0.10	0.313 ± 0.005
1.0	20/30	0.10	0.440 ± 0.011
10.0	20/30	0.10	--*
1.0	20/30	0.40	1.52 ± 0.04
0.0	0/0	0.4 (purified)	0.044 ± 0.004
0.0	0/0	0.4 (unpuri-fied)	0.066 ± 0.005

*No dopa is found after Al_2O_3 adsorption. See text.

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tyrosine used as a reagent. Purification of commercial tyrosine according to the methods of Nagatsu (5) and Ikeda (7) was undertaken to investigate this possibility. The results of the comparison between this "purified" tyrosine and the unpurified, commercially obtained material is shown at the bottom of Table 2.10. No incubation or preincubation was employed for these samples so as to detect only the contribution from impurities. The purified material provided blank values only slightly lower than the unpurified material. This difference appears even less important when one considers the contribution of impurities to the total nonenzymatic dopa. Thus, purification of tyrosine was deemed unwarranted for routine determinations of TH activity. In connection with the existence of dopa impurities in commercial tyrosine sources, we should mention that a number of authors employing radioassays have promoted the use of D-tyrosine for blanks. We would suggest that this may be inappropriate. The radiolabelled D-tyrosine used in the blank is almost certain to contain a different fraction of dopa impurity than is the L-tyrosine used for sample. The dopa impurities in both the D- and L-sources should be carefully examined before they are used interchangeably.

It is also conceivable that NE and DA, normally added to blanks, could be inhibiting the nonenzymatic production of dopa. This is very reasonable since both of these compounds are aromatic and both are relatively

easily oxidized. The additions of these compounds to blanks is completely justified since they are normal components of the tissue homogenates. Nonetheless, we decided to examine the effect of added NE and DA on blanks just to satisfy our curiosity. As seen in Table 2.11, these compounds do, indeed, inhibit the nonenzymatic production of dopa.

Our investigations of blanks can be summarized as follows:

a. The major contribution to blank values is represented by the nonenzymatic production of dopa. This source of dopa is very strongly affected by the concentration of tyrosine in the incubation mixture. It is also affected by the Fe^{+2} concentration.

b. Dopa impurities in commercially obtained L-tyrosine

TABLE 2.11

The Effect of Added NE and DA on Blank Values

Addition of NE & DA	R_{blank}
No	0.140 ± 0.008
Yes	0.063 ± 0.007

are a second source of dopa found in blanks.
Although normally representing only a small
part of the total nonenzymatic dopa, this source
should be examined if blank values become unex-
pectedly high in routine applications.

c. NE and DA additions to blanks inhibit the non-
enzymatic production of dopa. These substances
should, thus, always be added to blanks and
standards at concentrations comparable to that
contained in the tissue samples.

7. Summary of Results

The final, optimal conditions for the assay
of in vitro TH activity are presented in Table 2.12. The
optimal assay is simply obtained by replacing the initial
conditions in the Materials and Methods section of this
chapter with the optimal values listed in this table.

Employing the optimal conditions, a typical assay
for in vitro TH activity produced a value of 117±4 nmol/g/hr
for whole mouse brain. Since there is no other data avail-
able in the literature for TH from hypotonically-homogenized
whole mouse brain, a comparison with values from rat brains
was undertaken. Cicero et al., for example, reported
a value of 94.5±4.2 nmol/g/hr for whole rat brain (15),
while values of 120±4 nmol/g/hr can be calculated from the
data of Waymire et al. (16). Our values for mouse, thus,

TABLE 2.12

Summary of Optimization of the TH Assay

Parameter	Initial Condition	Optimization Range	Optimized Values
Conc. acetate buffer	0.2 M	0-0.5 M	0.4 M
pH of acetate buffer	pH 6.1	pH 5.8-7.2	pH 6.4
Conc. phosphate buffer	0.01 M	0-0.5 M	none
Catalase	1.0 unit/µl	0-10.0 unit/µl	none
Ferrous ions	1 mM	0-1.0 mM	0.1 mM
DDC inhibitors:			
BOA	0.4 mM	0-4 mM	
NSD-1015		0-4 mM	0.04 mM
NSD-1034		0-4 mM	
Cofactor:			
6-MPH$_4$		0-3.75 mM	2.0 mM
DMPH$_4$	0.4 mM	0-10 mM	
2-Mercaptoethanol	0.2 M	0-0.6 M	0.05 M
Incubation temp.	37°C	34-40°C	37°C
Tube position	Horizontal	Vertical, Horizontal	Vertical, uncapped
Shaking rate	fast	slow & fast	slow (3.0 cycle/sec)
Tyrosine	0.1 mM	0-0.6 mM	0.25 mM
Incubation time	30 min	0-60 min	20 min
Preincubation time	30 min	0, 30 min	30 min

demonstrate a reasonable degree of agreement with those
reported for rat by these workers. The present technique
was even further validated when we used whole rat brains
as the enzyme source in our procedure. This yielded a
TH activity of 100 ± 5 nmol/g/hr, which is completely com-
parable to the previous reports.

As mentioned before, the ultimate detection limit
for the in vitro TH procedure is determined by the tissue
to blank (or signal to noise) ratio. For the present pro-
cedure, this is equivalent to:

$$(R_{sample}-R_{blank})/R_{blank}$$

The in vitro TH procedure with the highest reported tissue
to blank ratio is that of Waymire et al. (16). This pro-
cedure measures $^{14}CO_2$ produced from L-tyrosine-1-^{14}C after
hydroxylation by TH and subsequent decarboxylation of the
dopa formed. Since these authors used whole rat brain as
the enzyme source, we did the same to allow a direct com-
parison. As seen in Table 2.13, both methods provide com-
parable tissue to blank ratios.

But, we believe the LC/EC procedure is and/or should
be better than the radioassay in its ultimate detection
limit. The radioassay employed tyrosine and Fe^{+2} concen-
trations in the incubation mixture which were, respectively,
smaller and larger than those used in LC/EC procedure
(0.10 mM vs. 0.25 mM and 1.0 mM vs. 0.10 mM). The effects
of these differences on blank values should either cancel

TABLE 2.13

Comparison of Sensitivity Between LC/EC and Radioassay

Assay Method	Conc. Fe^{+2} (mM)	TH Activity (nmol/g/hr)	Activity	Blank	Activity/Blank
LC/EC (present study)	0.1	100±5	$R_{sample}-R_{blank}$ = 1.89	R_{blank} = 0.058	33
Radiometric [Waymire et al. (16)]	1.0	120±4	51,085 (dpm)	1,412 (dpm)*	36

*Incubation included 2 mM 3-iodotyrosine.

or slightly favor the LC/EC procedure if eliminated.
More importantly, the radioassay employs blanks that are
composed of normal tissue samples with added 3-iodotyrosine,
a TH inhibitor. This causes two sources of possible error
in these blanks. First, the 3-iodotyrosine is likely to
inhibit the nonenzymatic production of dopa (compare the
effect of NE & DA in the present study). Alternatively,
3-iodotyrosine may simply compete with the labelled substrate
for nonenzymatic hydroxylation. Either case will produce
an artificially low blank value for this procedure. Like-
wise, these effects will serve to artificially raise the
observed tissue to blank ratio.

The detection limit of the present assay condition,
if necessary, may be further decreased by making the fol-
lowing changes to the incubation procedure:

a. Use 'purified' tyrosine.

b. Use the horizontal position for the centrifuge
    tubes.

c. Use a lower concentration of tyrosine.

d. Use no added Fe^{+2}.

The last two of these suggestions, unfortunately, will yield
a lower value of measured TH activity along with an improve-
ment in the tissue to blank ratio. Other factors that
may be important in this regard are the concentration of
the cofactor (6-MPH_4), the concentration of the 2-mercapto-
ethanol, and the possible replacement of Fe^{+2} with catalase.

The concentration of 6-MPH$_4$ and 2-mercaptoethanol were optimized for maximal TH activity in the present investigation, but not for maximum tissue to blank ratios. The catalase, tested in the presence of Fe^{+2}, was also maximized only with regard to observed activity.

C. Application of the Developed Procedure for in vitro Tyrosine Hydroxylase Activity. Determination of TH Activity in Mouse Brain Striatum After Sacrifice by Microwave Irradiation

1. Background. Rapid post mortem degradation of endogenous transmitters and related metabolites has been an area of great concern to persons employing neurochemical measurements. In an investigation undertaken by this laboratory, it was found that dopamine (DA) might be experiencing just such a problem in the striatum (22). However, the method employed for rapid inactivation, microwave irradiation, is only presumed to elicit heat-induced denaturation of the relevant biosynthetic and biodegradative enzymes involved. Using the procedure just developed, we decided to validate the presumed inactivation for TH. Simultaneously, the activities of dopa decarboxylase, monoamine oxidase and catechol-O-methyltransferase were examined by appropriate procedures.

Excessive heat application through microwave irradiation can lead to both heat related destruction of the transmitters and pressure-induced spreading of the tissues

involved. This latter problem elicits a virtual loss

of regional integrity. On the other hand, insufficient

heat will not provide the essential loss of enzymatic

activity. The present study, thus, examined the loss

of enzyme activity as a function of the amount of micro-

wave irradiation employed.

2. Procedure. The mice were sacrificed by expo-

sure to microwave irradiation (2450 MHz, 7.5 kW) concen-

trated on the head. This technique has been adequately

described by Stavinoha et al. (23). The time of irradiation

varied between 0 and 250 msec, with the animals receiving

no irradiation being sacrificed by decapitation

with a guillotine. The brains were removed immediately

after sacrificing and dissected. The striata were weighed

and stored on dry ice. Determination of the TH activity

was performed, typically, within 24 hrs.

The optimized in vitro TH assay for whole brains in

the previous section had to be slightly modified for these

samples to accommodate the smaller tissue size and the

higher activity levels. The brain homogenate was prepared

by homogenizing the samples with a micro tissue grinder

(Kontes Glass Co.), which was operated manually. The fol-

lowing ratios were employed: one striatum/250 μl H_2O/

25 μl DHBA (4×10^{-5} M in 1×10^{-3} M ascorbic acid solution).

As in the whole brain procedure, 250 μl of the homogenate

was used for each sample. For standard 'homogenates',

the following ratios were employed: 25 µl dopa working standard/250 µl H_2O/25 µl DHBA ($4x10^{-5}$ $\underline{M}$ in $1x10^{-3}$ $\underline{M}$ ascorbic acid solution). The dopa working standard was prepared as a 4:100 dilution of the stock standard solution, described above for the whole brain procedure, with $1x10^{-3}$ $\underline{M}$ ascorbic acid. 250 µl of this solution was used in each standard as a replacement for the brain homogenate. For the blank 'homogenates', the following ratios were employed: 25 µl of $1x10^{-3}$ $\underline{M}$ ascorbic acid/250 µl H_2O/25 µl DHBA ($4x10^{-5}$ $\underline{M}$ in $1x10^{-3}$ $\underline{M}$ ascorbic acid). 250 µl of this solution was used in each blank as a replacement for the brain homogenate. The remainder of the procedure was identical to that described for whole brains.

3. <u>Results and Discussion</u>. The measured <u>in</u> <u>vitro</u> TH activity after various times of microwave exposure are presented in Figure 2.12. The individual values are calculated as a percent of the decapitated results (245±14 nmol/g/hr) for ease of comparison. As can be seen, the TH activity decreases as the time of microwave irradiation increases. This is in complete agreement with what was expected. At or beyond 250 msec of irradiation, the activity is less than 2% of the decapitated values. But, at 300 msec or more, pressure-induced spreading is observed. Thus, 250-275 msec irradiation is recommended for routine studies on <u>post</u> <u>mortem</u> degradation in this brain region.
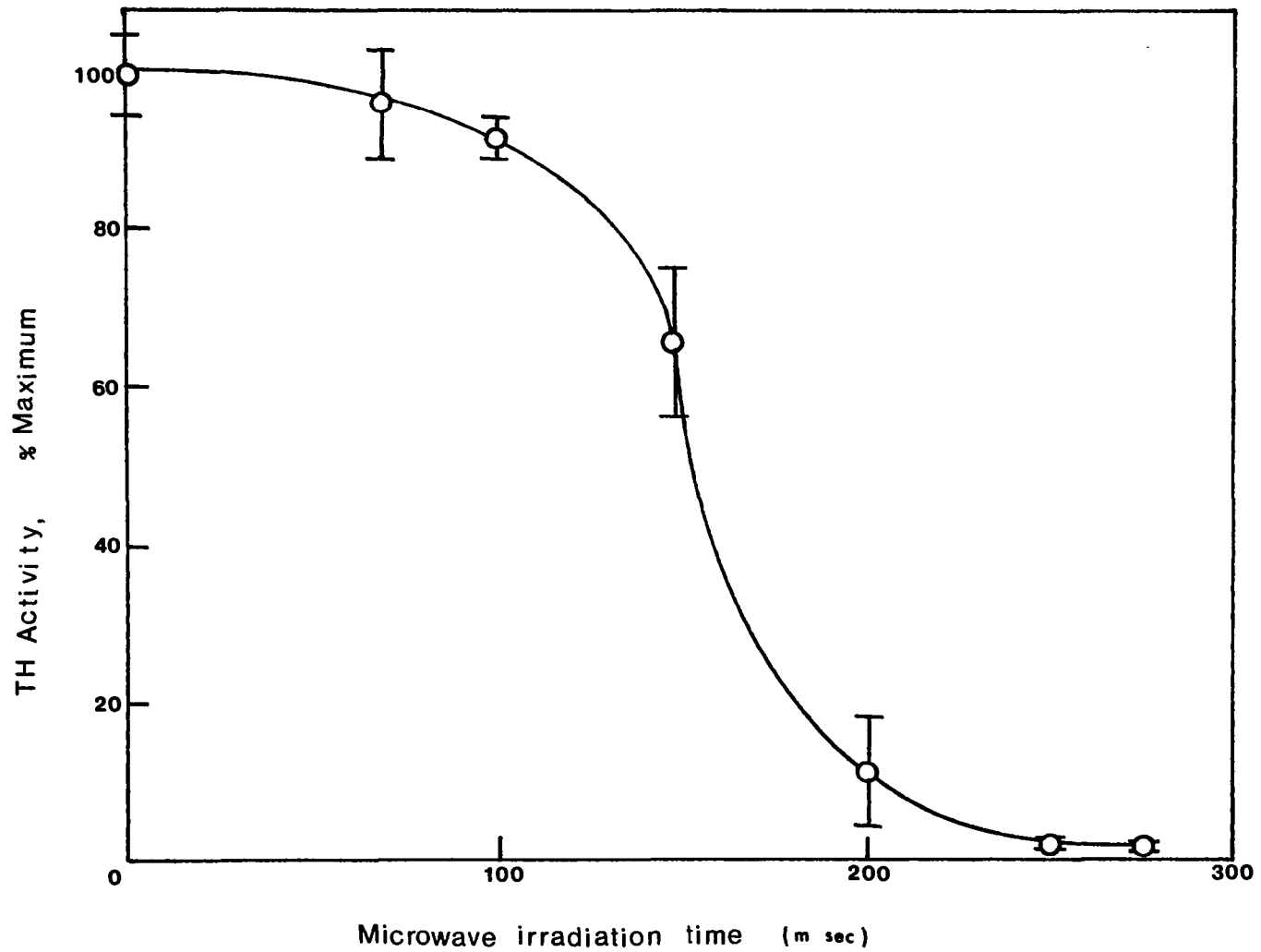
Figure 2.12   TH Activity as a Function of Microwave Irradiation Time.

II. Simultaneous Determination of <u>in vivo</u> TH Activity

and Endogenous Catecholamines

A. Introduction

The importance of the catecholamines in patho-
physiological conditions (18), Parkinson's disease (19),
and the affective disorders (20) has been adequately dis-
cussed above. However, under even normal conditions, these
transmitters are being constantly synthesized, released
and metabolized. Measurements of this utilization rate,
or turnover, are, thus, potentially as important as those
of the endogenous levels. Since tyrosine hydroxylase re-
presents the rate limiting step in the biosynthesis of
these transmitters, measurement of its <u>in vivo</u> rate can pro-
vide us with just such knowledge.

The present procedure determines the <u>in vivo</u> TH
activity by a method similar to that proposed by Carlsson
and coworkers (13,21). In this technique, a centrally
active dopa decarboxylase inhibitor is administered to the
living animals, and the dopa formed by the action of TH
subsequently accumulates. Since the endogenous level of
dopa is very low, the dopa measured at a specific time after
inhibition of the decarboxylase provides a direct determin-
ation of the <u>in vivo</u> TH activity. But, the low detection
limit of the LC/EC allows detection and quantitation of
very small amounts of dopa. This permits the employment

of very short times between administration of the blocking agent and sacrifice. And, these short time periods provide no detectable change in the endogenous levels of NE and DA (<u>vide</u> <u>infra</u>). Thus, we can simultaneously measure <u>in</u> <u>vivo</u> TH activity, NE and DA.

B. <u>Materials and Methods</u>

The reagents used, along with their preparation and/or source, are as follows:

α-MT (α-methyltyrosine) - This was obtained from Sigma Chemical Co., St. Louis, MO. The solution used for injections, containing 20 mg/ml, was prepared in deaerated isotonic saline (0.15 $\underline{M}$ NaCl) on the day of use.

MT (methyl ester of α-methyl tyrosine) - This was also obtained from Sigma. Solutions used for injections, containing 25 mg/ml, were prepared in deaerated isotonic saline (0.15 $\underline{M}$ NaCl) on the day of use.

Stock standard solution of dopa, NE and DA - This was prepared by dissolving dopa (8.0 mg), NE·HCl (8.5 mg) and DA·HCl (8.0 mg) in 100 ml of 0.030 $\underline{M}$ $HClO_4$. This solution was never kept over 2 weeks.

The working standard solution - This was prepared on the day of use by making a 1:100 dilution

of the stock standard solution with 0.03

$\underline{M}$ $HClO_4$. One ml of working standard, thus,

contained 80 ng dopa, 70 ng NE and 65 ng

DA. The concentrations of NE and DA are

expressed here as the free base.

DHBA solution - We first prepared a 1.0×10^{-4}

$\underline{M}$ (4.4 ng DHBA·HBr/100 ml H_2O) stock solution.

On the day of use, a 1:10 dilution of the

stock solution was made. This second solu-

tion (1×10^{-5} $\underline{M}$) was added to tissue samples

prior to homogenization.

Other chemicals - These were as described above

for the in vitro procedure.

Male mice, treated as described previously for the

in vitro assay procedure, were employed in all experiments.

NSD-1034 (200 mg/kg) was injected intravenously a few min-

utes before sacrifice by cervical dislocation. Brains were

removed as quickly as possible, weighed, frozen with liquid

nitrogen and stored on dry ice. Each brain was then homog-

enized by either ground glass or ultrasonic homogenization

in a solution composed of 2.0 ml of 0.050 $\underline{M}$ $HClO_4$, 100 μl

of 1.0 $\underline{M}$ $NaHSO_3$, 100 μl of 0.10 $\underline{M}$ EDTA, and 100 μl of

1.0×10^{-5} $\underline{M}$ DHBA. For standards, a 1.00 ml aliquot of the

working standard solution replaced the brain in the above

mixture. After homogenization, the samples were centri-

fuged at 16,000xg, and 0°C for 15 minutes with storage

on ice before and after. Following centrifugation, the dopa, NE, DA and DHBA in the samples were isolated by alumina adsorption and quantitated with LC/EC as previously described for the in vitro procedure. The dosage of NSD-1034 used here, incidentally, was selected from the previous work of Pike (3) and is also in agreement with the studies using NSD-1015 by Carlsson et al. (13).

Since no blanks are needed for the in vivo procedure, the calculation of TH activity becomes a little simpler:

$$\text{Dopa formed (nmol/g/hr)} = \frac{(R_{sample})\,(\text{nmol dopa in std})}{(R_{std})\,(\text{tissue wt. g})\,(\text{time, hr})}$$

where

> time = time between injection of inhibitor
> and sacrifice.

The catecholamine levels are calculated as follows (e.g. for NE):

$$NE(ng/g) = \frac{(R_{sample})\,(\text{ng NE in std})}{(R_{std})\,(\text{tissue wt, g})}$$

All results for multiple determinations are expressed as the mean± the standard error of the mean (SEM) for at least four separate determinations. Statistical comparisons are made by using Student's t-distribution.

C. Results and Discussion

The accumulation of dopa for the first six minutes after administration of NSD-1034 (200 mg/kg) is shown in Figure 2.13. The accumulation appears to be
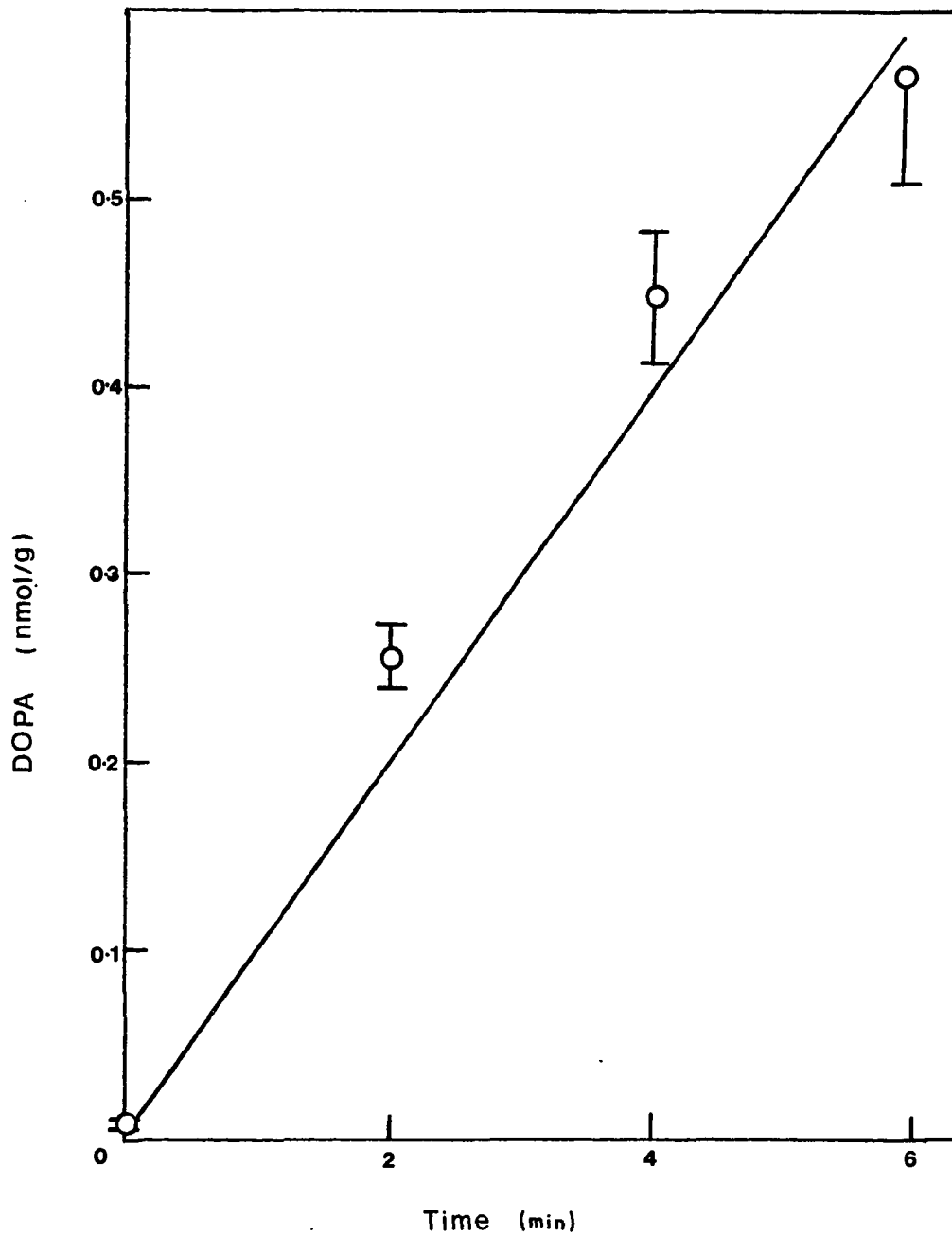
Figure 2.13   Time Course of Dopa Accumulation in Mouse
Brain After Treatment with NSD-1034.

quite linear during this time period. Assuming this repre-
sents the true rate of dopa synthesis, an in vivo TH
activity of 5.6 nmol/g/hr can be obtained from the slope
of the graph.

In order that the measurement of dopa accumulation
be a valid indication of the in vivo TH activity, the
dopa should not only be accumulated in a linear manner,
but it should also be stable. To test the stability of
the accumulated dopa, two TH inhibitors were used to block
dopa production after the previous administration of the
dopa decarboxylase blocker. The TH inhibitors used were
MT (H44/68, methyl-ester of α-methylparatyrosine) and
α-MT (α-methylparatyrosine). In these experiments, two
groups of animals were employed. Both groups were given
an i.p. injection of the dopa decarboxylase blocking agent
(NSD-1034 or NSD-1015) at time zero. After a specified
time interval (5 or 20 minutes), the first group was sacri-
ficed and the second group was given an i.p. injection of
the TH blocking agent (α-MT or MT). The results are pre-
sented in Tables 2.14 to 2.18; the results from Tables 2.14
and 2.17 have also been presented in Figure 2.14. The inter-
pretation of these results is independent of either the dopa
decarboxylase or TH inhibitor employed. Using the 5 minute
interval, a significant increase in dopa is observed after TH
blockade. Using the 20 minute interval, a significant

TABLE 2.14

Stability of Accumulated Dopa after NSD-1034 and MT.

Group	Time Schedule (min)			Dopa formed (nmol/g)
	0	5	10	
1	NSD-1034	Killed		0.38±0.03
2	NSD-1034	MT	Killed	0.53±0.03

*Different from group 1 (P<0.01)

TABLE 2.15

Stability of Accumulated Dopa after NSD-1034 and α-MT.

Group	Time Schedule (min)			Dopa formed (nmol/g)
	0	5	10	
1	NSD-1034	Killed		0.47±0.03
2	NSD-1034	α-MT	Killed	0.64±0.06

*Different from group 1 (P<0.01)

Stability of Accumulated Dopa after NSD-1034 and α-MT.

Group	Time Schedule (min)			Dopa formed (nmol/g)
	0	20	40	
1	NSD-1034	Killed		1.70±0.23
2	NSD-1034	α-MT	Killed	0.66±0.17

*Different from group 1 (P<0.001)

TABLE 2.17

Stability of Accumulated Dopa after NSD-1015 and MT.

Group	Time Schedule (min)			Dopa formed (nmol/g)
	0	5	10	
1	NSD-1015	Killed		0.31±0.02
2	NSD-1015	MT	Killed	0.39±0.02

*Different from group 1 (P<0.05)

TABLE 2.18

Stability of Accumulated Dopa after NSD-1015 and MT

Group	Time Schedule (min)			Dopa formed (nmol/g)
	0	20	40	
1	NSD-1015	Killed		0.98±0.02
2	NSD-1015	MT	Killed	0.68±0.06*

*Different from group 1 (P<0.02)

decrease in dopa is observed after TH blockade. Neither

of these represent the desired result, which would have

indicated no change in dopa regardless of the time interval.

Nonetheless, these results may be practically explained as

follows. At the 5 minute interval, the TH blocking agent,

possibly, does not penetrate the nervous system rapidly

enough. This means its effective blockade is very small

at very short times after injection (<1 min), reaching

virtually 100% blockade only towards the end of the 5 minute

period. The decline in dopa observed at the 20 minute

interval indicates that dopa is, indeed, being metabolized

and/or transported out of the brain at very long times.

Assuming these explanations are fairly accurate, the 5

minute time for measurement of dopa accumulation still

appears to be a reasonable one.
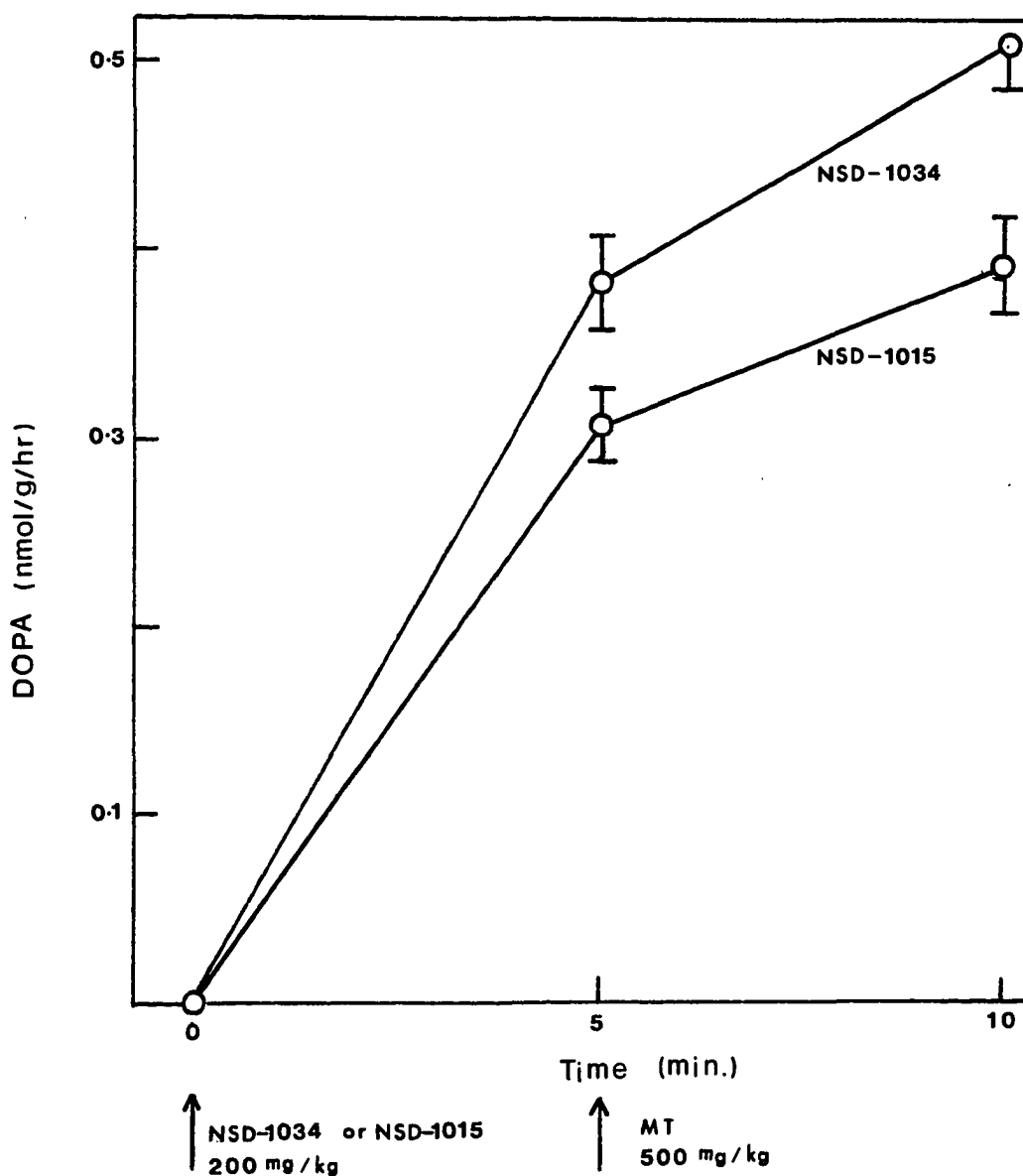
Figure 2.14   The Effect of TH Inhibition on Dopa Accumulated
              After NSD-1034 (or NSD-1015) Treatment.
              NSD-1034 (or NSD-1015) was injected i.p. at
              zero time to two groups of animals. One group
              was killed after 5 minutes. Simultaneously,
              the second group received MT (TH inhibitor).
              The second group of mice were killed after
              another 5 minutes. Dopa content was then
              measured for both groups.

Determining catecholamine levels at the same time as measuring in vivo TH activity demands that these levels not change while dopa is being accumulated. Of course, over a long period of time this is impossible. The decarboxylase agent is blocking the synthesis of the catecholamines, while catabolism and transport is not being affected. But, in the very short times, it may be possible to see no significant decrease in catacholamine levels. Thus, we decided to examine the endogenous levels of these amines at times up to 6 minutes after blockade of dopa decarboxylase. As seen in Table 2.19, there is no significant decline in NE or DA during the first six minutes after NSD-1034 administration. This means we can use the blockade of dopa decarboxylase at times up to six minutes for the simultaneous determination of in vivo TH activity, NE, and DA.

We would not recommend this procedure for any definitive measurements of NE and DA in any particular investigations. But, it should be completely reliable for in vivo TH measurements. And, it should be generally applicable as a routine procedure for the simultaneous assessment of in vivo TH activity, NE, and DA.

TABLE 2.19

Catecholamine Levels in Mouse Brain at Various Times

After NSD-1034

Interval (min)	Dopa (ng/g) (nmol/g)		DA (ng/g)	NE (ng/g)
0*	0.01	0.00	1063±81	330±18
2	0.28	0.02	917±96**	328±14**
4	0.45	0.04	1042±52**	320±13**
6	0.55	0.06	1096±49**	335±26**

*For zero time, mice were given an injection of isotonic saline and sacrificed 4 minutes later.

**There are no significant differences ($P > 0.05$) between the NE and DA levels at 0 minute when these are compared to the results obtained at the other times shown in the table.

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CHAPTER 3

DETERMINATION OF TRYPTOPHAN HYDROXYLASE in vitro

BY LIQUID CHROMATOGRAPHY WITH ELECTROCHEMI-

CAL DETECTION

I. Introduction

Tryptophan hydroxylase (TPH) exhibits a number of
similarities to the previously investigated tyrosine
hydroxylase (TH). Notably, both of these enzymes represent
the rate limiting step in the biosynthesis of their asso-
ciated transmitters. Their activities are, thus, pre-
dictably low in mammalian tissues. This means that only
highly sensitive analytical methodologies will be capable
of quantitating these activities. As with TH, TPH activity
measurements have relied heavily upon fluorescence and
radiochemical procedures. The fluorescence procedures,
however, have seen much less frequent utilization in the
recent past due to their lack of selectivity and relatively
high detection limits. The radioassays, on the other
hand, are quite rapid in routine applications and offer
reasonably good detection limits. But, radioassays require
the use of labelled chemicals and can exhibit selectivity

problems. Thus, we decided to see if liquid chromatography with electrochemical detection (LC/EC) might be applicable to the determination of TPH activity.

II. Development of the Assay

The analysis of TPH activity in tissue samples can be viewed as a three step process for simplicity. After incubation of the enzyme under appropriate conditions, the product is isolated in some type of clean-up procedure. The isolated product is then quantitated using appropriate instrumentation. As is the case with most analyses, these steps were developed in reverse order. The final step must be firmly established to assess the effects of modifications in the preceding steps.

In this report, we will begin with a brief outline of the materials and the preliminary procedure. This will be followed, in order, by detailed discussions of investigations concerning the LC/EC conditions, the isolation procedure, and the optimization of incubation parameters. We will conclude with a discussion of the finally derived method for the determination of in vitro TPH activity.

A. Materials.

1. Apparatus.

a. Liquid Chromatograph - The liquid chromatograph with electrochemical detector has been adequately described in general terms

in Chapter 1. Several modifications
were attempted before the system used
in the optimization studies was selected.
This system had the following notable
characteristics:

Column: 5 μ, reverse phase, Ultrasphere.

Mobile Phase: pH 3.0 solution con-
taining 0.15 $\underline{M}$ citric acid, 0.025
$\underline{M}$ sodium citrate, 0.15 $\underline{M}$ acetic
acid, and 0.025 $\underline{M}$ sodium acetate.

Flow Rate: 0.63 ml/min.

Detector Potential: 0.65 V $\underline{vs}$. Ag/AgCl.

b. Isolation Column - The column used to
isolate the product of hydroxylation was constructed from
a Pasteur pipet. The tip was first loaded with a small
glass wool plug, which served as a bed support. A portion
of the pipet, 2 mm in diameter, was filled to a height
of $\underline{ca}$. 20 mm with Bio-Rex AG 50W-X2 cation exchange resin.
The columns were prepared for use by washing with 1.0 ml
of 0.10 $\underline{M}$ HCl.

c. Other Equipment - All other equipment is
described in Chapter 2.

2. Animals. The animals and their housing are
described in Chapter 2.

3. Chemicals and Sources.

Catalase - Grade C-30, 21,000 unit/mg, 25 mg/ml.

Obtained from Sigma Chemical Co., St. Louis, MO.

L-5-Hydroxytryptophan (5-HTP) - Aldrich Chemical Co., Milwaukee, WI.

5-Hydroxy-N$_\omega$-methyltryptamine oxalate, (NM-5-HT) - Aldrich.

L-Tryptophan - Regis Chemical Co., Morton Grove, IL.

5-Hydroxytryptamine (Serotonin Creatinine Sulphate, 5-HT) - Aldrich.

Other Chemicals - See Chapter 2.

4. Solutions.

5-HTP - A stock standard solution was prepared by dissolving 3.75 mg 5-HTP in 50 ml of deaerated 0.10 $\underline{M}$ HCl. On the day of use, a working standard was prepared by diluting 1.00 ml to 25.00 ml with 1×10^{-3} $\underline{M}$ ascorbic acid.

5-HT - A stock standard solution was prepared by dissolving 8.63 mg of serotonin creatinine sulfate in 50.00 ml of deaerated 0.10 $\underline{M}$ HCl. On the day of use, a working standard was prepared by diluting 1.00 ml to 25.00 ml with 1×10^{-3} $\underline{M}$ ascorbic acid.

NM-5-HT - A solution of NM-5-HT was prepared by dissolving 12.2 mg of the salt in 50 ml

of deaerated 0.10 M HCl.

Elution Solution - A solution containing 0.40 M

NaOH in a 50:50 mixture of ethanol and water

was prepared for elution of 5-HTP and NM-5-HT

from the cation exchange column used in the

isolation step.

B. Data Treatment. The calculation of tryptophan

hydroxylase activity is analogous to that described for

tyrosine hydroxylase in Chapter 2. All results are expressed

as the mean ± the standard error of the mean (SEM) for at

least four separate determinations. Statistical comparisons

employed Student's t-distribution (3).

C. Initial Procedure. After sacrificing the animals

by cervical dislocation, the brains were quickly removed,

weighed and stored on ice. The tissue was homogenized

in distilled water containing the internal standard in

the following proportions:

1 brain/3.00 ml H_2O/50 µl of NM-5-HT.

The incubation mixture consisted of the following

components in a total volume of 500 µl. The concentration

of the components in the final mixture is given in paren-

theses: 100 µl of pH 7.6, 2.0 M acetate (0.40 M); 50 µl

of a solution which is 2.0 mM in 6-MPH$_4$ (0.20 mM), 1.0 M

in 2-mercaptoethanol (0.10 M), 4.0 mM in NSD-1015 (0.40

mM), and which also contains 420 unit of calalase (0.84

unit/μl); 50 μl of 1.0 mM ferrous sulfate (0.10 mM); 200 μl

of the brain homogenate; 50 μl of 1.0 mM L-tryptophan

(0.10 mM); and, 50 μl of 1.0 mM ascorbic acid (0.10 mM).

For standards, 50 μl of the 5-HTP working standard

replaced the ascorbic acid solution in the above incubation

mixture. Also, 50 μl of 60% $HClO_4$ was added prior to in-

cubation. Blanks were obtained by simply adding 50 μl of

60% $HClO_4$ to the above incubation mixture prior to incubation.

The incubation mixture was placed in an uncapped,

vertically positioned centrifuge tube, and incubation was

carried out at 37°C for 30 minutes. The shaker was set

at the low shaking rate. The reaction was terminated by

the addition of 50 μl of 60% $HClO_4$ to the samples. The

reaction mixture was centrifuged at 27,750xg and 4°C for 20

minutes with storage on ice before and after. 50 μl of the

supernatant was transferred to a cation exchange isolation

column. The column was washed by treatment with 1.0 ml of

0.1 M HCl. This was followed by 250 μl of saturated KCl

in 0.1 M HCl and 200 μl of distilled water, respectively.

100 μl of elution solution was added to the column and the

eluate collected in a small vial containing 25 μl of 4.0

M HCl. 5 μl of the elute was finally injected into the

LC/EC system for quantitation of the 5-HTP produced.

D. <u>Liquid Chromatographic Conditions</u>. In the initial

stages of this investigation it was hoped that we might

be able to eliminate the isolation procedure. Incubation would be followed by only acid precipitation of the proteins, centrifugation, and injection of the supernate into the LC/EC. The high resolution (5 μ) reverse phase columns were considered, but rejected, for this purpose. It was anticipated that these columns would be clogged by multiple injections of the rather crude supernates. Instead, we selected the Vydac CX columns. Such materials have a much larger particle diameter and, are, thus, less susceptible to clogging problems. This material had previously been shown to possess considerable retentive properties for indole compounds, while exhibiting much less retention of catecholamines and virtually no retention for acidic and neutral metabolites.

Several different conditions were attempted for the separation of the compounds of interest before a 500x3 mm column with a mobile phase containing 0.050 $\underline{M}$ HCl and 0.30 $\underline{M}$ KCl was found to provide the desired separation. A chromatogram for a typical incubation mixture is shown in Figure 3.1. While adequate for routine applications, this chromatogram also reveals the unanticipated appearance of 6-MPH$_4$. The peak for 6-MPH$_4$ is completely separated from the others, but its presence adds a considerable amount of time to the quantitation of individual samples. To make these determinations more applicable to routine determinations, we decided to shorten the chromatogram
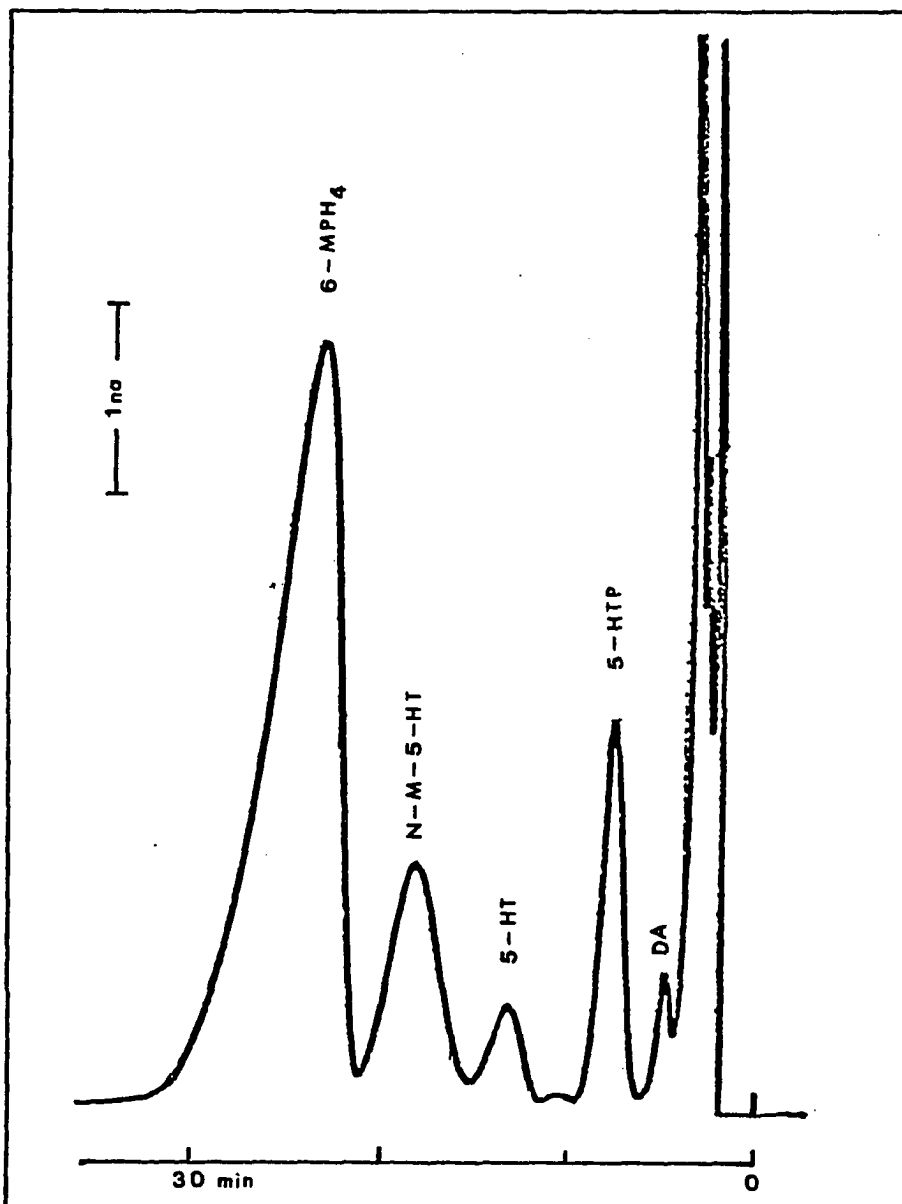
Figure 3.1   A Typical Chromatogram for a TPH Assay by
Direct Injection into a Vydac Cation Exchange
Column.

by eliminating the 6-MPH$_4$ peak. This was attempted by both chemical and instrumental means.

An investigation of the cyclic voltammetric behavior of the compounds involved indicated that the indoles are oxidized at ca. +0.6 V. vs. SCE while the cofactor, 6-MPH$_4$ is oxidized at ca. +0.3 V vs. SCE. It was, thus, thought that it might be possible to find an oxidizing agent, to be added after incubation, which might selectively oxidize only the cofactor. The agents used represented half-reactions spanning a standard reduction potential (E°) range of +0.34 to +0.69 volts and included Cu^{+2}, I$_2$, Fe(CN)$_6^{-3}$, and p-benzoquinone. These all proved to be entirely inadequate except for p-benzoquinone. This compound did, indeed, cause the disappearance of 6-MPH$_4$ from the chromatogram. Unfortunately, it was also found that the 5-HTP peak gradually deteriorated after treatment with p-benzoquinone. Thus, this approach was abandoned.

A second approach to the 6-MPH$_4$ problem employed a slightly different mobile phase and column switching. The mobile phase (0.050 M HCl containing 0.020 M KCl) coupled with a short Zipax SCX precolumn (see Figure 3.2) increased the k' value for 6-MPH$_4$ while leaving the k' values for other components virtually unchanged. This allows total isolation of the 6-MPH$_4$ peak from the rest of the peaks seen in Figure 3.1. Adequate resolution of the remaining peaks, however, still requires the full length of the
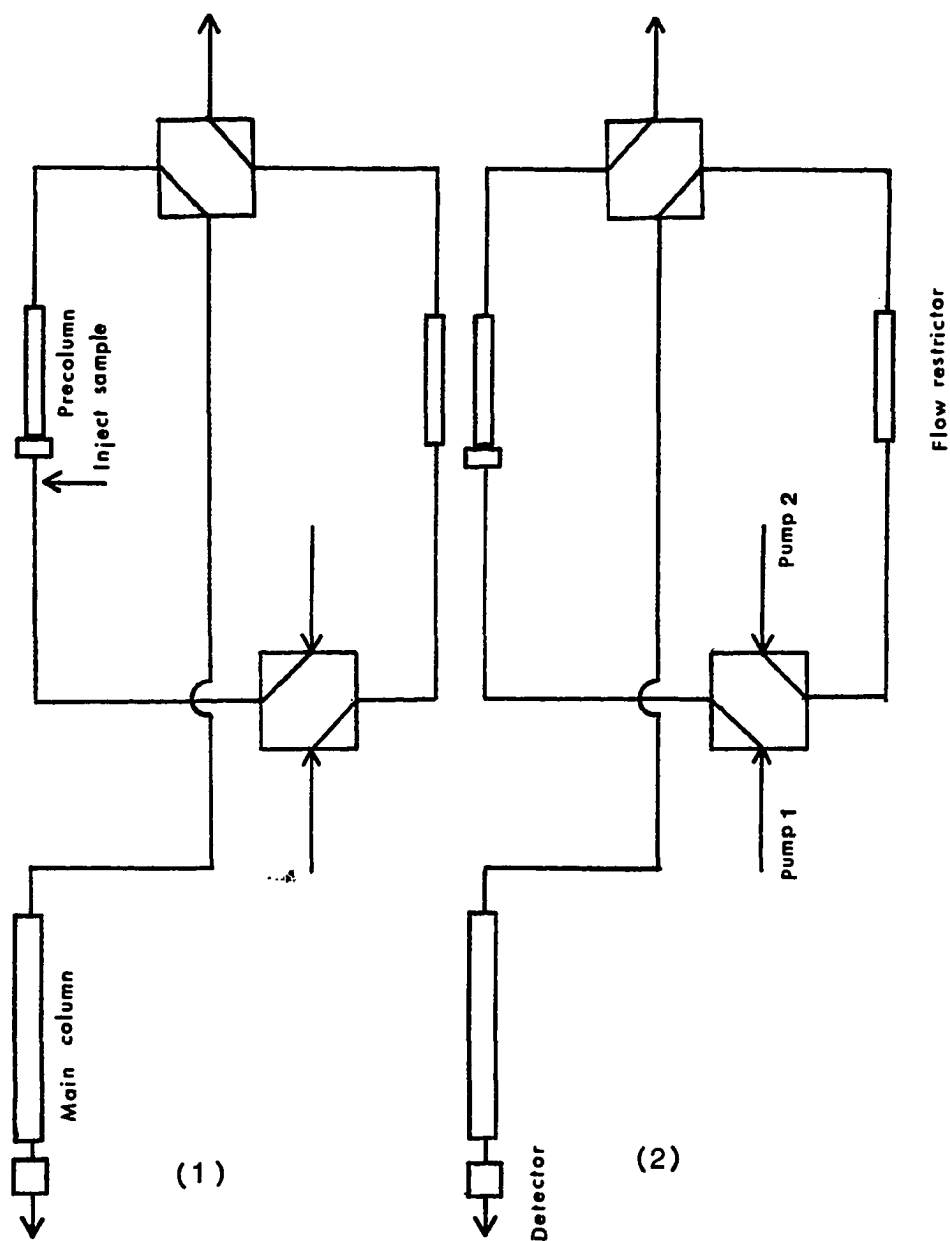
Figure 3.2   The Use of Column Switching to Separate and
Discard the 6-MPH$_4$ in a TPH Assay.

original column. This situation is advantageously coupled
with the column switching arrangement of Figure 3.2. The
sample is injected with the values set to configuration 1.
After waiting a specific amount of time, all the compounds
of interest except 6-MPH$_4$ have passed the outlet of the
precolumn. At this time, the valves are switched to con-
figuration 2. In the second position, the compounds of
interest are separated and detected by the main column,
while the 6-MPH$_4$ is simultaneously flushed from the precolumn.
This column switching approach effectively cut the individual
chromatographic times from 30 to 20 minutes. But, accurate
manual and/or automated control is essential for timing
purposes. Since we do not currently have such automatic
control, and the manual timing was difficult to reproduce,
we decided to examine another possible route to direct
injection.

The major component of interest in the present in-
vestigation, 5-HTP is an amino acid. Thus, raising the pH
of the mobile phase considerably would place this compound
in its anionic form. Employing α-methyl-5-hydroxytryptophan
as an internal standard, a 500x3 mm Zipax SAX, strong anion
exchange column packing material, and a pH 9.0 Tris buffer
(0.30 $\underline{M}$ containing 0.0010 $\underline{M}$ NH$_4$NO$_3$), we were, indeed, able
to produce a usable chromatogram. Individual injections
could be made every 20 minutes with this arrangement. Un-
fortunately, the high pH of the mobile phase caused the

resolution of the column to deteriorate in a very short period of time. Noticeable changes were seen after routine employment of the system for only one week. Another short-coming of this setup is that it produces a rather large solvent peak. This occasionally leads to difficulties in the determination of component peak heights.

Judging the above procedures for direct injection to be either incapable of or inappropriate for routine applications, we decided to try the higher resolution reverse phase column combined with an isolation procedure for sample clean-up prior to injection in the LC/EC. While recognizing that the isolation step would be adding time to the overall procedure, it was hoped that the higher resolution might also shorten the chromatographic time required for each sample. The applicable column conditions (vide supra) did just that. Individual samples could be injected every 11 minutes in the microparticulate columns.

E. Isolation Studies. There have been a number of papers which have reported the use of ion exchange beads (200 mesh) for the isolation of biogenic amines. Initially, trying to isolate 5-HTP with DOWEX 50W-X8, we found that the adsorbed compounds were very difficult to desorb due to the strong interaction between the indole ring and the styrene-divinylbenzene copolymeric substructure of this resin. Thus, we were encouraged by the reported isolation

of tryptophan from rat brain tissue on DOWEX 50W-X2 by
Koch and Kissinger (2). Using this resin, we were also
able to isolate the desired 5-HTP and NM-5-HT (internal
standard). However, our eluting solvent, 0.40 $\underline{M}$ NaOH in
a 50:50 mixture of ethanol and water, was a bit stronger
than the 0.10 $\underline{M}$ NH$_3$ reported by these authors.

Using this isolation procedure, we examined the per-
cent recovery and linearity of 5-HTP and NM-5-HT. For these
studies, incubation mixtures with various added amounts
of 5-HTP and a fixed amount of NM-5-HT were subjected to
the isolation procedure prior to LC/EC quantitation. These
samples were compared to pure solutions of 5-HTP and NM-5-HT
which were directly injected. The results are presented
in Table 3.1.

The linearity of peak height ratio ($H_{5-HTP}/H_{NM-5-HT}$)
as a function of the amount of 5-HTP added to the incubation
mixture (constant amount of added NM-5-HT) was also investi-
gated. The results are shown in Figure 3.3. As can be
seen, linearity is exhibited over the usual operating range
of 10-190 ng (47-950 pmol).

A chromatogram obtained from a routine sample of
whole mouse brain employing the usual incubation along with
the above described isolation and LC/EC condition is shown
in Figure 3.4.
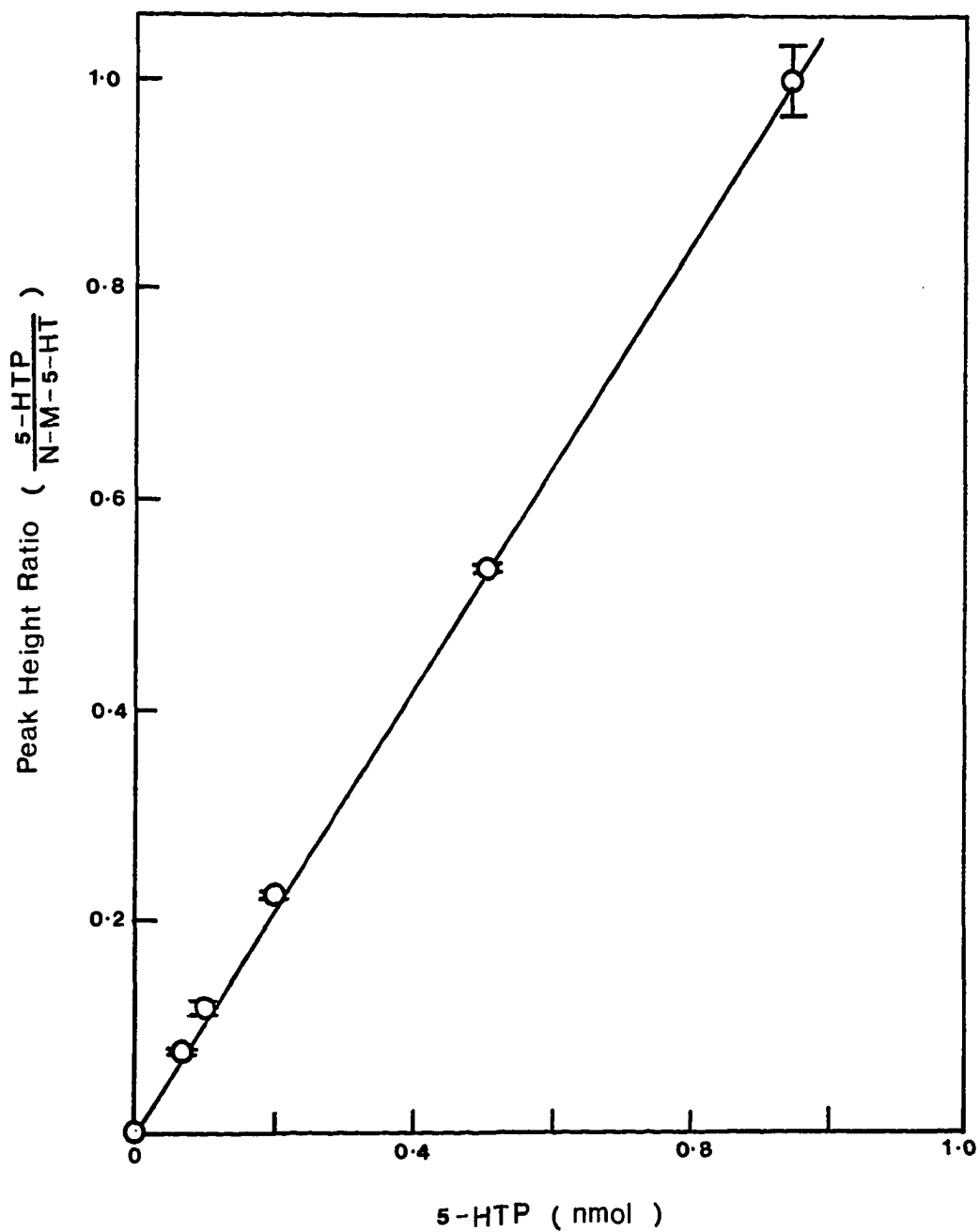
Figure 3.3   Peak Height Ratio of 5-HTP to NM-5-HT as a
             Function of the Amount of 5-HTP Added Prior
             to AG 50W-X2 Isolation.
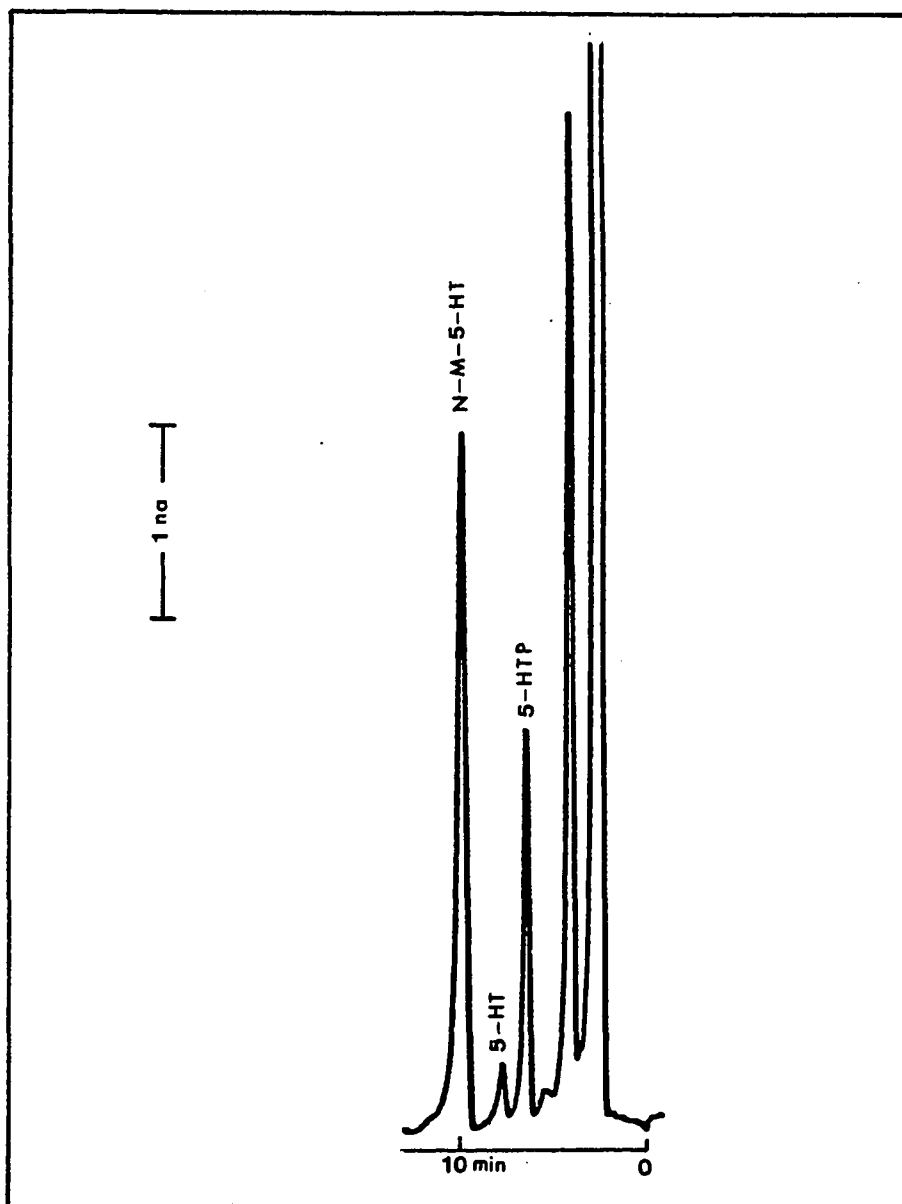
Figure 3.4 A Typical Chromatogram for the TPH Assay Using a Reverse-Phase, Microparticle Column.

TABLE 3.1

Recovery of 5-HTP & NM-5-HT from Incubation Mixtures

After Isolation with AG 50W-X2 Cation

Exchange Resin

Compound	% Recovery
5-HTP	55.6 ± 0.6
NM-5-HT	43.8 ± 1.1

F. Optimization of Incubation Parameters. The optimum condition for any single parameter can be interpreted in two ways. One is simply that condition which provides the maximum observed activity, expressed as nmol/g/hr. The second is that condition which provides the maximum signal/ noise, or tissue/blank, ratio. The parameters for the present TPH assay were primarily optimized for maximum ob- served activity, although signal/noise ratios were also monitored as appropriate. The ordering of these optimization studies, incidentally, is important. The optimal condition for a given parameter, once found, was incorporated into all succeeding investigations.

All concentrations in this section are with respect to the final incubation mixture.

1. Buffer Type. Three major buffer systems were

considered for this assay procedure: acetate, Tris and
phosphate. The latter two would provide much better buffering
in the generally accepted pH optimum range of 7.0 to 8.0.
But, previous reports have indicated that phosphate inhibits
TPH. Tris has been used by some authors (4), although
it is also reported to be inhibitory (6). Nonetheless, we
compared acetate to Tris. The results (79.1±1.2 vs.
65.1±3.4, respectively) clearly show the acetate buffer
to be better. Thus, acetate was used in all subsequent
experiments.

2. Divalent Cation. Stimulation of TPH in tissue
homogenates has been claimed in the presence of Fe^{+2} and,
more recently, in the presence of Ca^{+2} (7,8,13). The re-
sults for the present investigation of these cations is
presented in Table 3.2. Not only is the activity of TPH
higher in the presence of 10 mM Ca^{+2}, but the blank values
are noticeably lower than with Fe^{+2}. Thus, 10 mM Ca^{+2}
was selected as the optimal divalent cation concentration.
Many other studies, it should be noted, have found that
TPH activities are not greater when 100 mM Ca^{+2} is used
instead of 10 mM Ca^{+2}. Thus, higher concentrations were
not examined in the present study.

3. Buffer pH. Knapp et al. (7) reported that
TPH not only provided improved activity in the presence
of Ca^{+2}, but it also exhibited a shift in its pH maximum
when compared to Fe^{+2}. Being somewhat intrigued by this

TABLE 3.2

Effect of Fe^{+2} and Ca^{+2} on TPH Activity* and Blank Values

Ion	Concentration (m$\underline{M}$)	R_{sample}	TPH Activity (nmol/g/hr)	R_{blank}	Signal/Noise
none	0	0.417±0.010	49.5±2.1	0.062±0.003	5.7
Fe^{+2}	1	0.655±0.048	77.8±6.5	0.077±0.004	7.5
Ca^{+2}	0.1	0.404±0.010	58.7±1.9	0.065±0.002	5.2
	1	0.487±0.012	70.8±5.1	0.067±0.007	6.3
	10	0.756±0.023	102.1±3.3	0.064±0.003	10.8

*pH of incubation mixture was 7.60.

report, we proceeded to examine the pH maximum for TPH

in the presence of each of these cations independently.

As seen in Figure 3.5, our results support the previous

data. With Fe^{+2}, the maximum occurs at a pH of 7.7-7.8,

whereas with Ca^{+2} it occurs at 8.0. Thus, a pH of 8.0

was selected for all following measurements. We must also

point out, however, that the acetate buffer is virtually

devoid of buffering capacity at this value. Thus, a

search for a buffer with a greater capacity around pH 8.0,

i.e., one having a pK_a in the vicinity of 8.0, but without

the inhibitory properties of Tris or phosphate, might still

be a worthwhile venture.

4. 2-Mercaptoethanol. This reagent has been re-
ported to function as both a protein activator (9) and a

reducing agent. In the present assay, as seen in Figure

3.6, a relatively low concentration (0.020 $\underline{M}$) produced

a dramatic increase in the activity of TPH. Higher con-

centrations, however, exhibit an inhibitory effect. The

highest concentrations, i.e., greater than 0.10 $\underline{M}$, also

resulted in the appearance of several unidentified peaks

in the final chromatogram. However, at the optimal con-

centration of this reagent, no such interferences were

observed.

5. 5-Hydroxytryptophan Decarboxylase Inhibitor.
The decarboxylase inhibitor, NSD-1015, produces a small

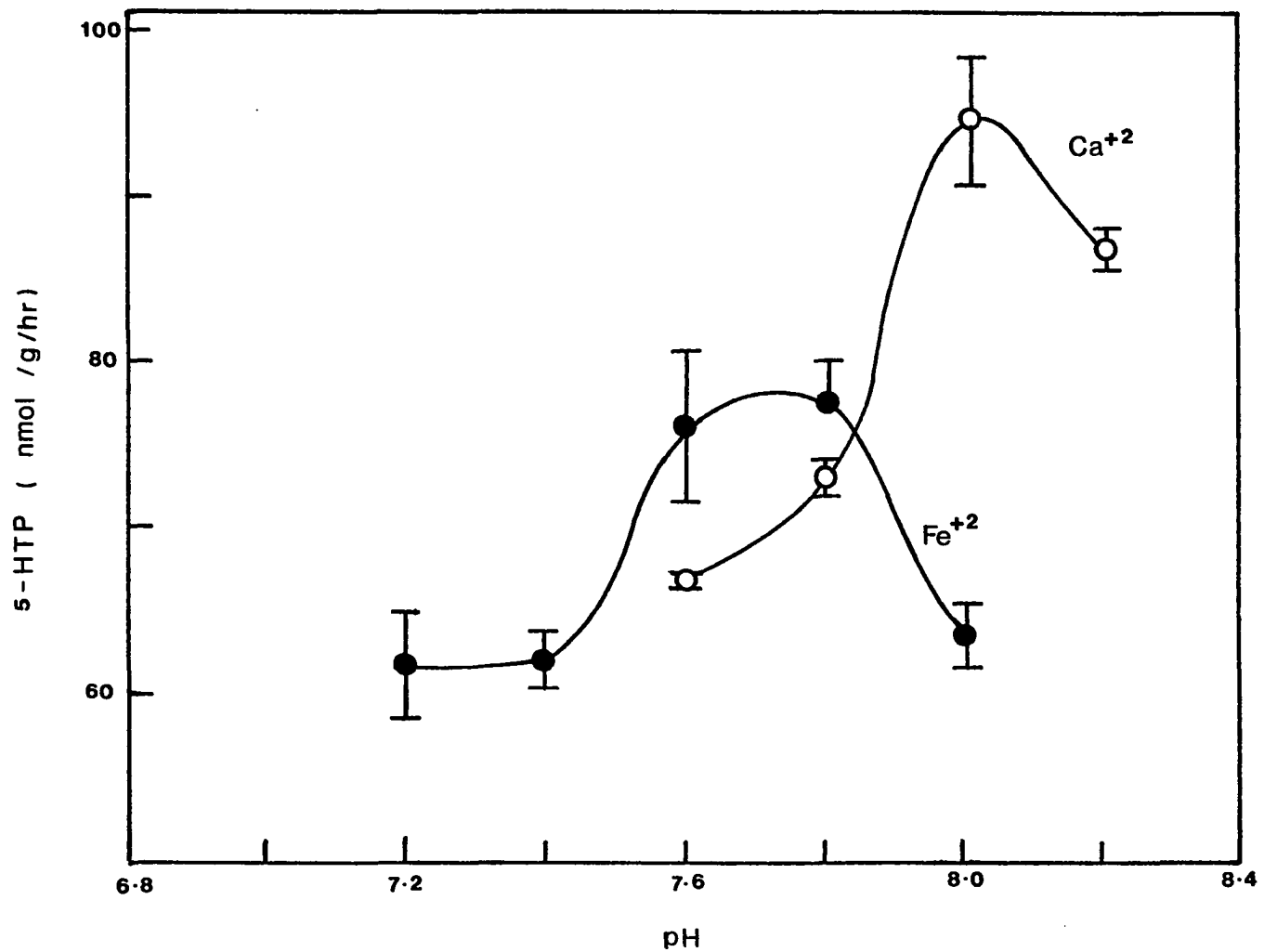increase in 5-HTP production at low concentrations.

Figure 3.5   The TPH Activity as a Function of the pH of the Incubation Medium.
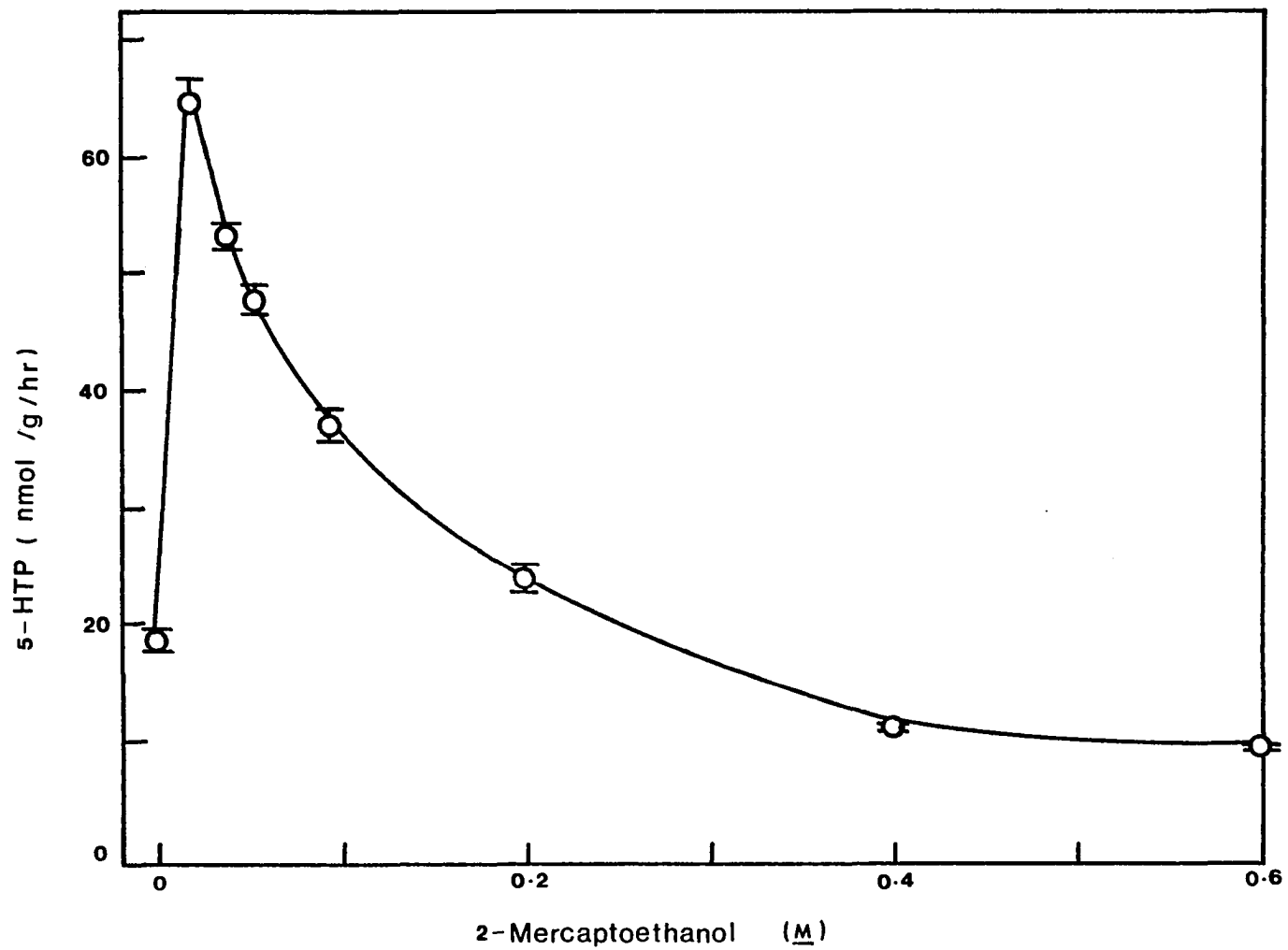
Figure 3.6   The Effect of 2-Mercaptoethanol on TPH Activity.

However, higher concentrations resulted in inhibition of TPH activity. From this effect of NSD-1015 on TPH activity, shown in Figure 3.7, 0.010 m$\underline{M}$ was chosen as the optimal value.

6. Pterin Cofactor. The effect of the pterin cofactor, 6-methyltetrahydropterin (6-MPH$_4$), on TPH activity, is shown in Figure 3.8. The inset plot of 1/V $\underline{vs}$. 1/S indicates a V$_{max}$ of 154 nmol/g/hr and a K$_m$ of 0.34 m$\underline{M}$. From these results, the minimal saturating concentration was estimated to be 0.50 m$\underline{M}$. 6,7-Dimethyltetrahydropterin (DMPH$_4$) was also investigated for use as the pterin cofactor. But, our results, in agreement with a previous report (5), showed this agent to be much less effective than 6-MPH$_4$.

7. Catalase. In contrast to the lack of effect of catalase on TH activity observed in Chapter 2, catalase definitely does exhibit a stimulating effect on TPH activity. As can be seen from Figure 3.9, catalase, at concentrations 5.25 unit/μl or greater, produce maximal activity. Therefore, 5.25 unit/μl was chosen for subsequent utilization.

8. Substrate. From the previous work on tyrosine hydroxylase, we anticipated that the concentration of L-tryptophan would affect both the rates of enzymatic and non-enzymatic 5-HTP production. We, therefore, used individual sets of samples, standards and blanks for each level of tryptophan investigated. The results are plotted in Figure 3.10. The inset in this figure, which takes
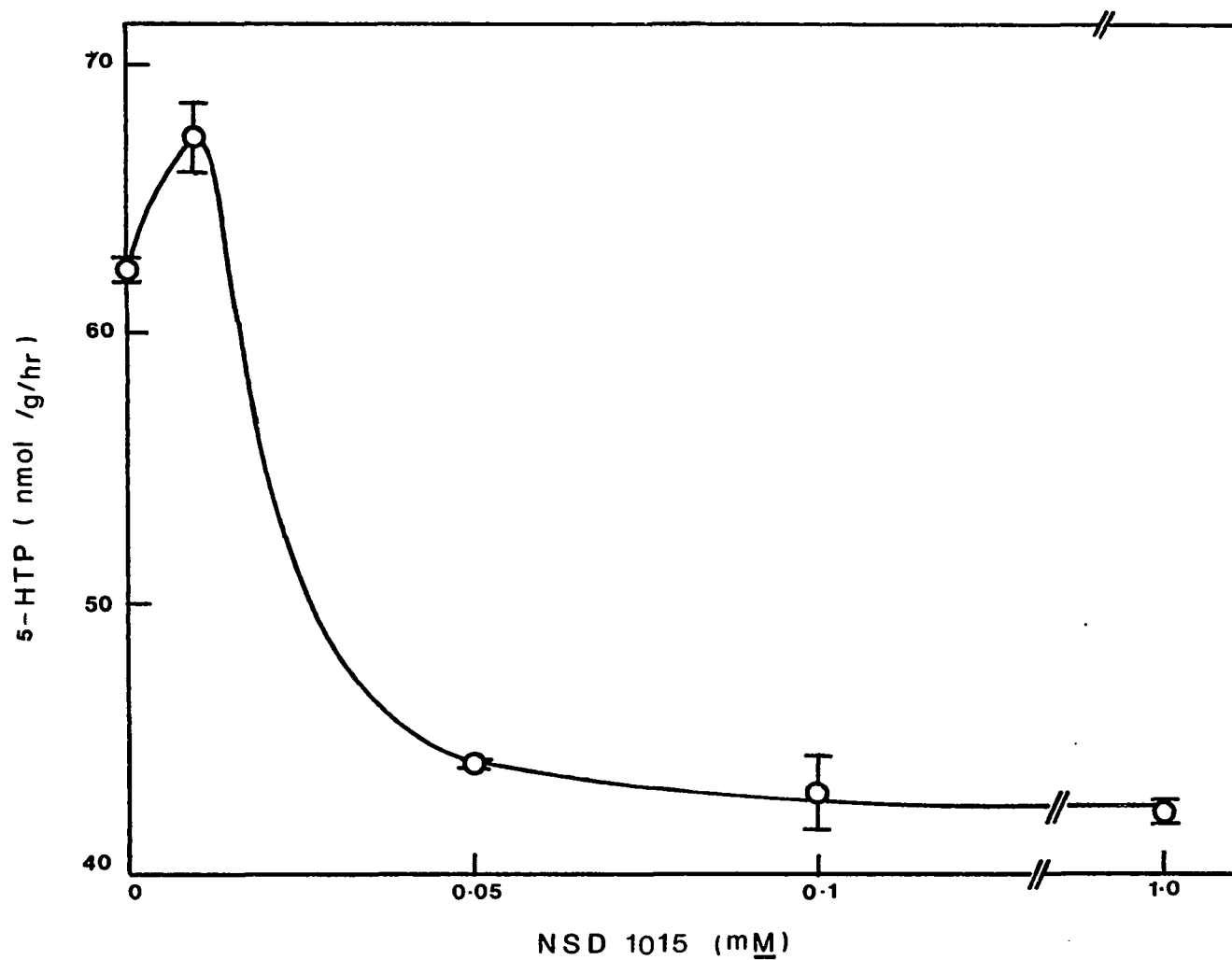
Figure 3.7   The Effect of NSD-.015 on TPH Activity.
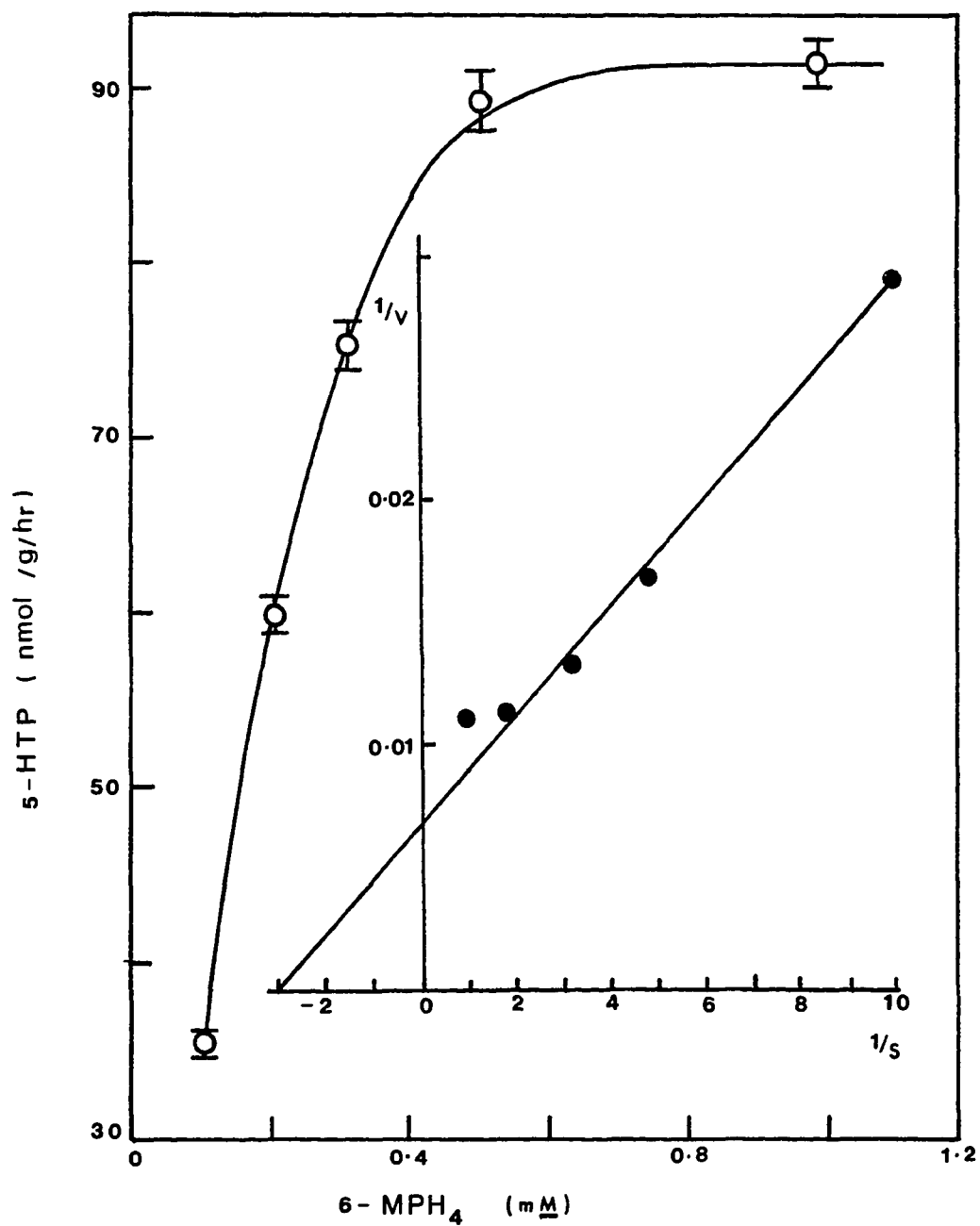
Figure 3.8   The Effect of the Cofactor on the Rate of
             5-HTP Formation.
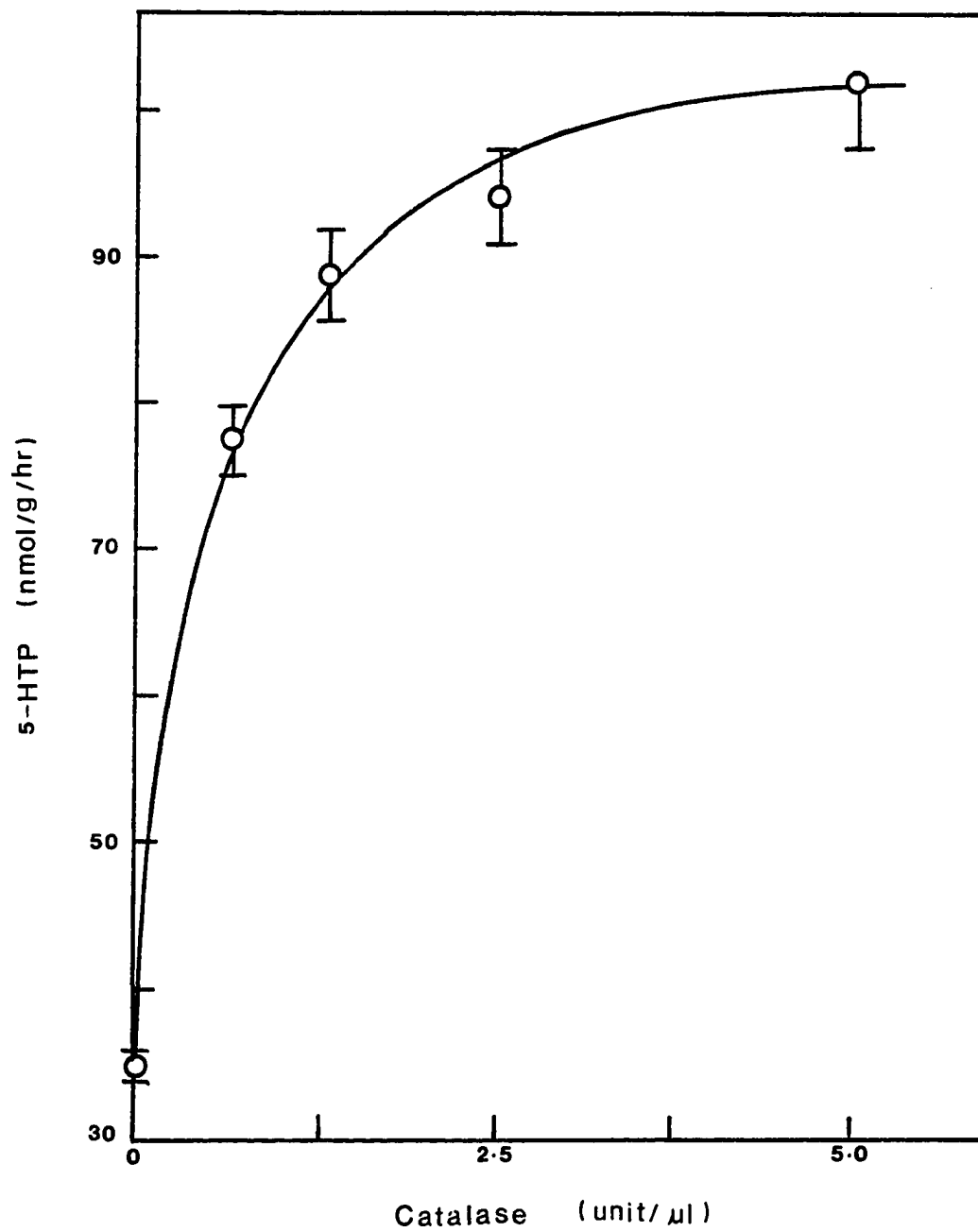
Figure 3.9   The Effect of Catalase on TPH Activity.
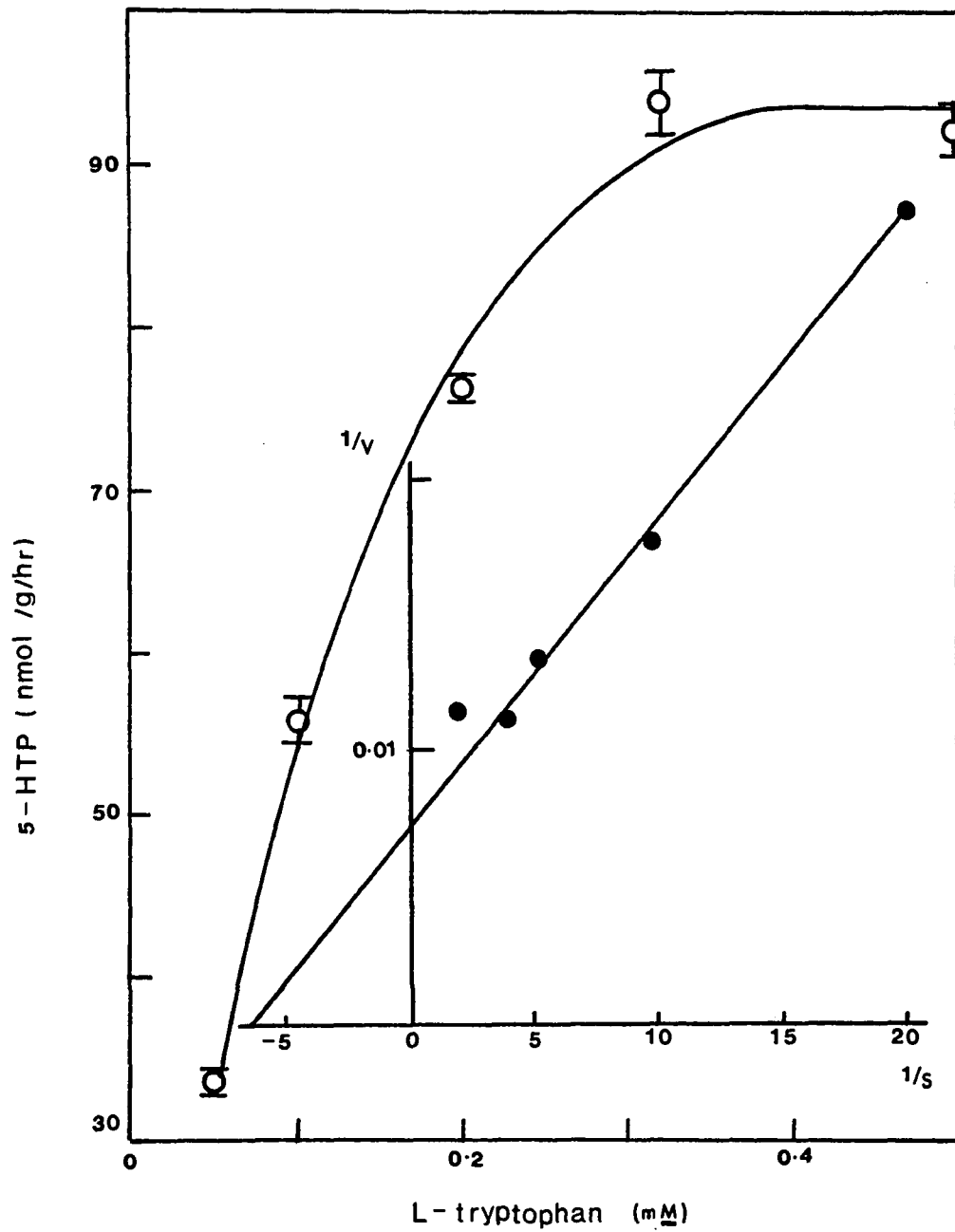
Figure 3.10   The Effect of the Substrate Concentration
on the Rate of 5-HTP Formation.

into account an endogenous tryptophan concentration of 6.1 µg/g, yields a V_{max} of 133 nmol/g/hr and a K_m of 0.15 mM. Thus, subsequent studies employed the estimated minimal saturating concentration of 0.40 mM L-tryptophan.

9. Incubation Time. The amount of 5-HTP formed as a function of the incubation time is shown in Figure 3.11. From this, an optimal value of 25 minutes was selected.

10. Preincubation Time. Preincubation of all components except the substrate has been shown to increase the observed activity for many enzymes. However, as presented in Figure 3.12, this was not the case for TPH. Therefore, no preincubation was used in the following TPH investigations.

11. Incubation Temperature. Identical groups of samples were incubated for the same period of time at different incubation temperatures. The results are shown in Figure 3.13. From this graph, it was apparent that incubation at 37°C gives the highest activity. This incubation temperature was, thus, selected as optimal.

12. Enzyme Concentration. An investigation of TPH activity as a function of the amount of added enzyme, expressed as the volume of added brain homogenate, was undertaken to ascertain the absence of endogenous inhibitors. As can be seen from Figure 3.14, the activity observed was linearly dependent on the amount of enzyme added to the incubation mixture. Thus, our originally chosen homogenate
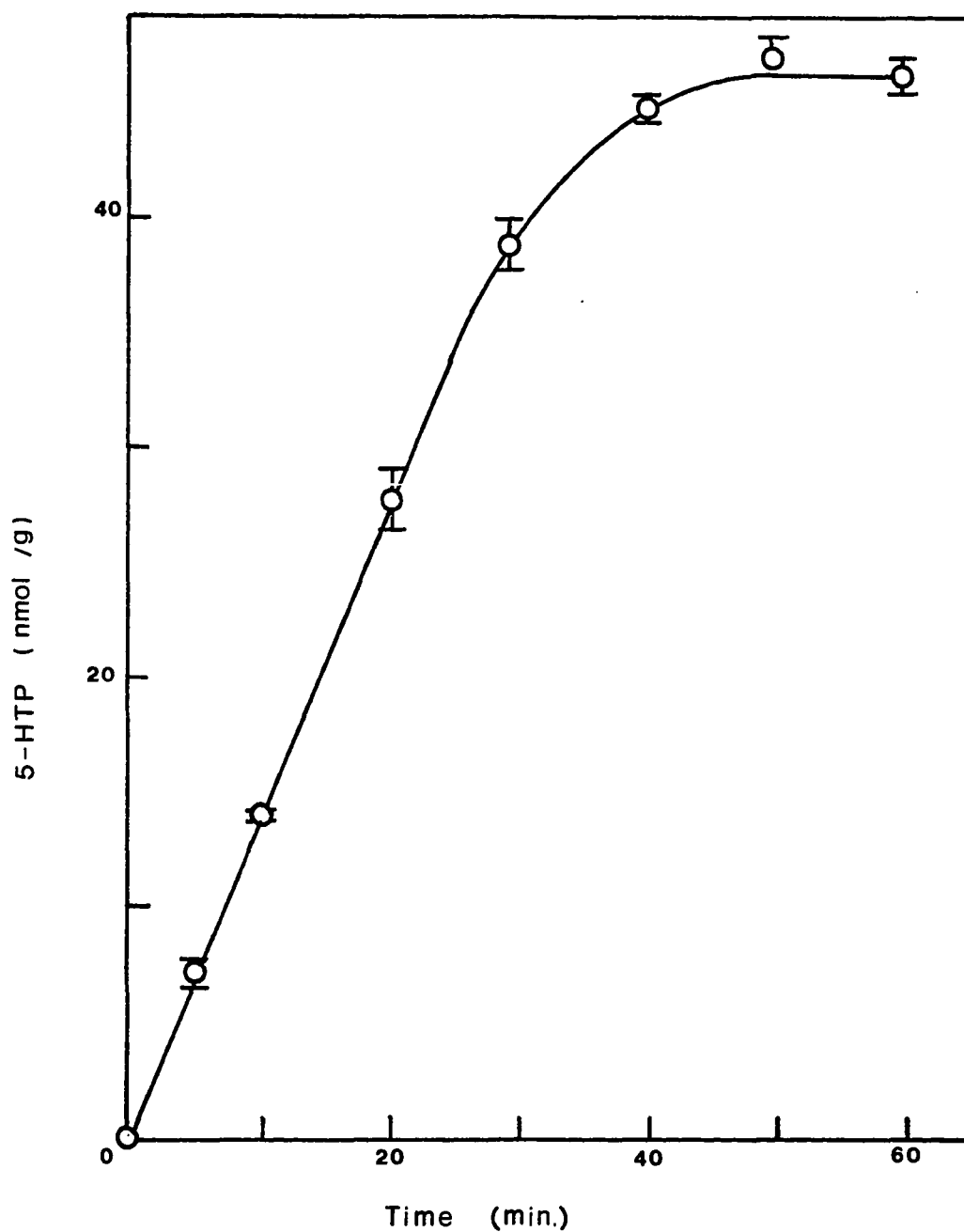
Figure 3.11 The Amount of 5-HTP Formed as a Function of the Incubation Time.
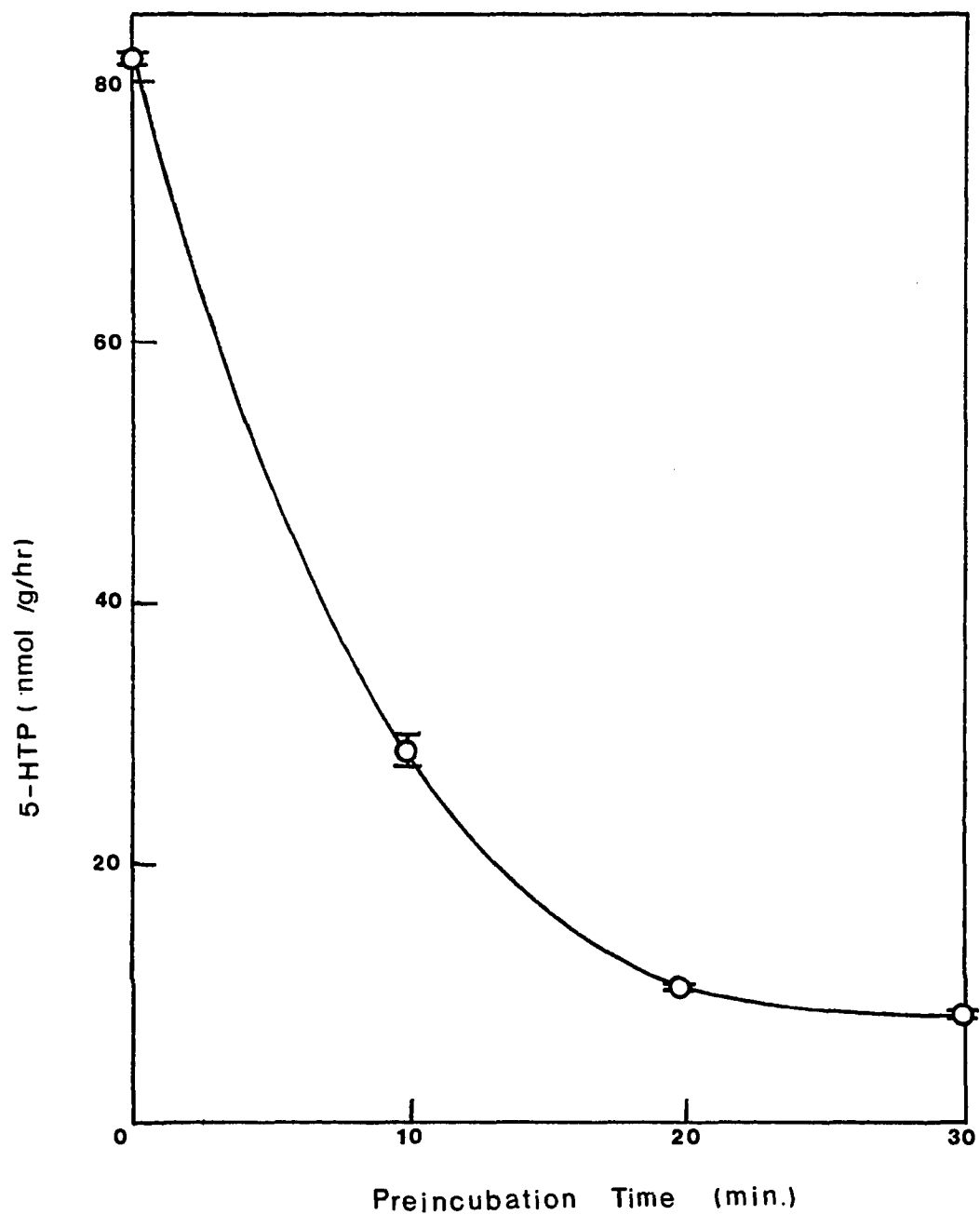
Figure 3.12 The Effect of the Preincubation Time on TPH
Activity.
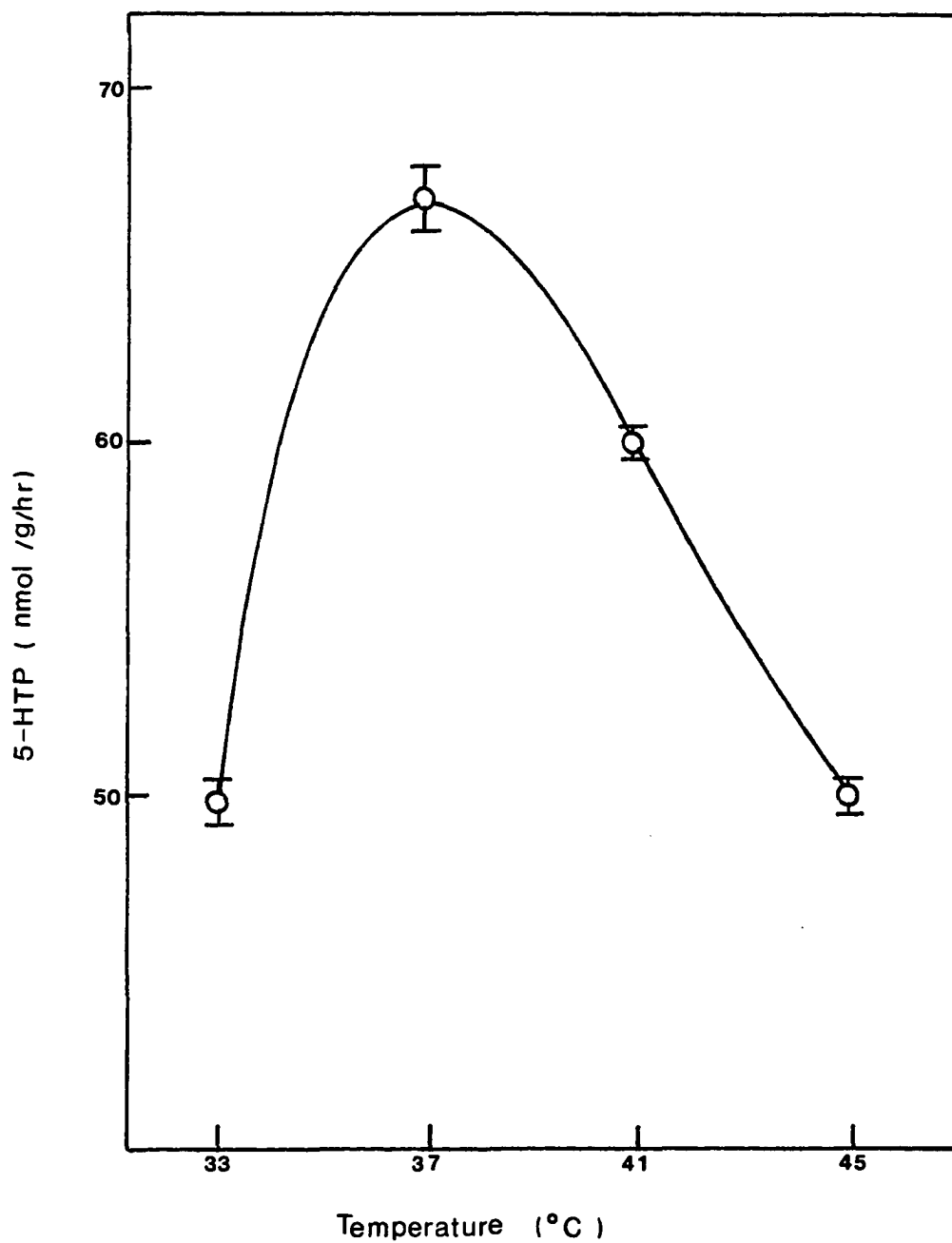
Figure 3.13   The Effect of the Incubation Temperature on
TPH Activity.
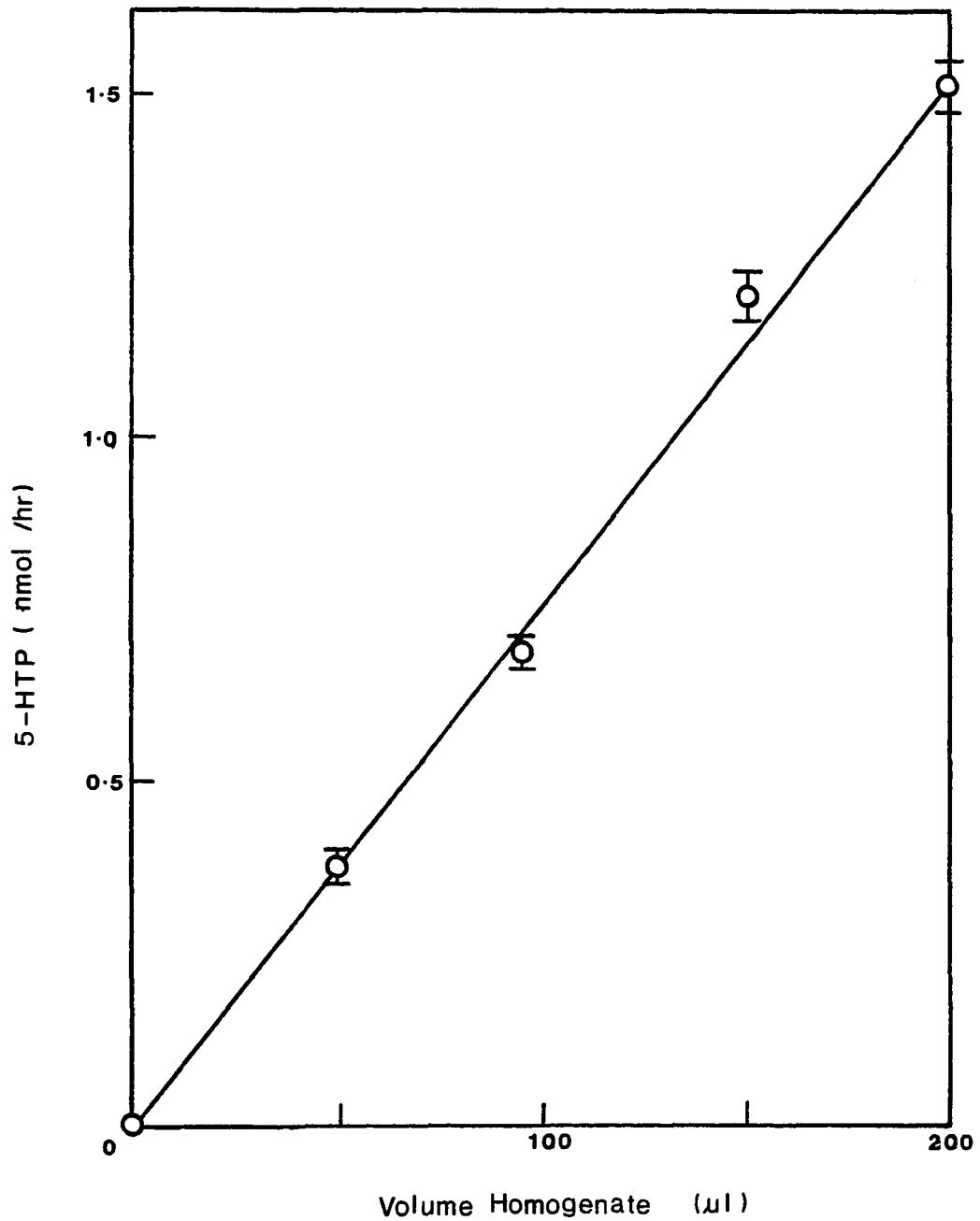
Figure 3.14   The Amount of 5-HTP Formed as Function of the
Volume of Brain Homogenate Added to the
Incubation Mixture.

mixture is adequate for routine utilization.

13. Summary. The parameters optimized, ranges investigated, and the resulting optimal conditions are presented in Table 3.3.

G. Final Procedure. The in vitro tryptophan hydroxylase procedure presently believed to yield optimal activity and signal/noise ratios is obtained by using the optimized values of Table 3.3 in the initially presented procedure. Employing these values, a typical assay produced a result of 81.0±0.6 nmol/g/hr for whole mouse brain. Since there are no directly comparable results found in the literature, we decided to examine the similarity of the present results to those obtained for rat brain stem. In rat brain stem, Gal and Patterson (5) reported 4.0-4.6 nmol/mg protein/hr, Meek et al. (4) reported 4.7±0.5 nmol/mg protein/hr, and Kizer et al. reported 0.88±0.09 nmol/mg protein/hr. Assuming a brain protein content of 10%, our value would correspond to 0.81±0.01 nmol/mg protein/hr. While not being directly comparable, our results can, nonetheless, be seen to be quite reasonable. Higher values from rat brain stem are to be expected, since this region contains a much larger fraction of serotonin-containing neurons and thus, presumably, a much larger fraction of TPH than the whole brain.

The detection limit of the finally derived procedure

TABLE 3.3

Summary.   Optimization of TPH Incubation Parameters*

Parameter	Initial Conditions	Range Investigated	Optimal Value
Buffer type	Acetate	Acetate, Tris	Acetate
Fe^{+2}	0.1 m$\underline{M}$	0.1-1.0 m$\underline{M}$	None
Ca^{+2}	none	0.1-10 m$\underline{M}$	10 m$\underline{M}$
Buffer pH	7.6	7.2-8.4	8.0
2-Mercapto- ethanol	0.10 $\underline{M}$	0-0.60 $\underline{M}$	0.020 $\underline{M}$
NSD-1015	0.40 m$\underline{M}$	0-1.0 m$\underline{M}$	0.010 m$\underline{M}$
6-MPH_4	0.20 m$\underline{M}$	0.10-1.0 m$\underline{M}$	0.50 m$\underline{M}$
Catalase	0.84 unit/μl	0-5.25 unit/μl	5.25 unit/μl
L-Tryptophan	0.10 m$\underline{M}$	0.010-0.60 m$\underline{M}$	0.40 m$\underline{M}$
Incubation Time	30 min	5-60 min	25 min
Preincubation	0 min	0-30 min	0 min
Temperature	37°C	34-45°C	37°C

*Fixed parameters:

Shaking rate - slow, 3.0 cycle/sec.

Incubation tube orientation - vertical, uncapped.

appears to be slightly better than those previously re-
ported. Employing a minimal signal/noise ratio of two,
we can currently detect about 0.2 ng, or 1 pmol, of 5-HTP
produced. This corresponds to the determination of TPH
activity in as little as 6 µg of rat brain stem assuming
a 55% recovery in the isolation step. This is compared
to the 5-HTP detection limit of 20 ng, or 100 pmol, reported
for the fluorometric assay of Gal and Peterson (5), and
the detection limit of 0.5 ng, or 2.5 pmol, reported for
the radioenzymatic procedure of Kizer et al. (11).

The LC/EC procedure also exhibits a greater selecti-
vity than these alternative methods. Endogenous and exo-
genous indolic compounds may interfere in either the fluoro-
metric or radiochemical procedures. But, at the present
time, none of these species is known or anticipated to
interfere with the quantitation of 5-HTP in the LC/EC
procedure.

III. <u>References</u>

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CHAPTER 4

A RAPID AND SENSITIVE PROCEDURE FOR THE DETER-

MINATION OF SEROTONIN IN MOUSE BRAIN

TISSUE

I. Introduction

Our laboratory recently became involved in an in-

vestigation of serotonin levels in various regions of

mouse brain. The number of samples anticipated quickly

caused us to consider the possibility of a more rapid pro-

cedure than the butanol extraction process combined with

LC/EC quantitation (11). The sensitivity and selectivity

needed, as well as other practical considerations, still

indicated the necessity for LC/EC. Thus, we focused our

attention on shortening or eliminating the butanol isolation

step. The maximum savings in time, of course, would be

obtained by a total elimination of this step, and this is

where we directed our initial efforts.

The liquid chromatography employed for these studies

was of the rather low resolution, large particle size (30-

40 μ) variety. This selection was based upon two major

criteria. The alternative high resolution, small particle

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size column (5 μ) would be expected to offer a considerable
increase in efficiency. But, such high resolution is not
necessarily required when one is only concerned with a
single compound. And, we anticipated that the smaller
particle size would lead to more frequent clogging problems
when injecting supernates from brain homogenates directly
into the chromatograph.

The remainder of this chapter discusses the develop-
ment and application of a procedure for serotonin which
employs only homogenization, centrifugation, and quantitation
by LC/EC. The isolation step is eliminated from the usual
procedure.

II. Materials and Methods

A. Materials

1. Apparatus.

a. LC/EC. The liquid chromatograph with
electrochemical detection has been discussed in general
in Chapter 1. The present system is composed of the
following specific components:

> Pump - Milton Roy Minipump, Model 13906-1.
>
> Column packing - Vydac CX, pellicular
>> (30-40 μm) cation exchange resin from
>>
>> The Separation Group, Hesperia, CA.
>
> Zipax SCX - pellicular (30-44 μm) cation
>> exchange resin from Dupont Instruments,
>>
>> Wilmington, DE.

Columns - composed of glass: 250x3 mm,

150x3 mm, and 500x3 mm obtained from

Altex, Inc., Santa Barbara, CA.

Detector - Home-made as described in Chapter

1.

Chromatographic Conditions -

Mobile Phase: Citrate/acetate buffer,

pH 5.1, containing 0.025 $\underline{M}$ citric acid,

0.050 $\underline{M}$ sodium acetate, 0.060 $\underline{M}$ NaOH,

0.020 $\underline{M}$ acetic acid, and 0.20 $\underline{M}$ NaCl.

Flow Rate: 1.0 ml/min.

Detector Potential: 0.65 V $\underline{vs}$. Ag/AgCl.

Column: 500x3 mm Vydac CX or

250x3 mm Vydac CX + 150x3 mm

Zipax SCX

b. Tissue Homogenizer. The samples were homogenized with an ultrasonic cell disruptor, Model W 200P, obtained from Ultrasonics, Inc. This was equipped with a long probe tip designed for small volume work.

c. Centrifuge. A Sorvall Model RC2-B, equipped with an SM-24 rotor, was used for all centrifugal separations.

d. Centrifuge Tubes. 1.5 ml polypropylene tube with V-shaped bottoms were used to house samples during homogenization and centrifugation.

2. Animals. See Chapter 2.

3. Reagents. See Chapters 2 and 3.

4. Solutions.

    a. Stock standard solution - 8.63 mg of serotonin

        creatinine sulphate was dissolved in 50

        ml of deaerated 0.10 $\underline{M}$ HCl.

    b. Working standard solution - On the day of

        use, 1.00 ml of the stock standard solu-

        tion was diluted to 100 ml with deaerated

        0.10 $\underline{M}$ HCl to give a solution containing

        750 ng 5-HT/ml (expressed as the free

        base).

    c. Internal standard stock solution - 12.2 mg

        of N_{ω}-methyl-5-hydroxytryptamine oxalate

        (NM-5-HT) was dissolved in 50 ml of

        deaerated 0.10 $\underline{M}$ HCl to give a solution

        having a concentration of $\underline{ca}$. 9×10^{-4} $\underline{M}$.

B. <u>Routine Procedure</u>. The method described in this
section is directly applicable to the routine determination
of serotonin in whole mouse brain. The development of this
method is discussed in the immediately following sections.

    After sacrificing the animals, the brains are removed
as quickly as possible, placed in small polypropylene centri-
fuge tubes, frozen in liquid nitrogen and stored on dry
ice. At the time of analysis, the small centrifuge tubes
containing the brains are weighed. To each tube, 750 µl
of a homogenizing solution is added. The homogenizing

solution is prepared by diluting 200 µl of the stock NM-5-HT
solution to 50 ml with deaerated 1.0 $\underline{M}$ HCl. Homogenization
of the tissue is accomplished with the ultrasonic cell dis-
ruptor, set for 20 sec at a 50% pulse rate and a medium
(No. 6) output power rating. The homogenate is then cen-
trifuged at 27,750xg and 4°C for 30 minutes. Finally, about
5 µl of the supernate is injected into the LC/EC for
quantitation.

For standards, 450 µl of a working standard solution
is used to replace the brain in the above procedure. After
thorough mixing this solution is injected directly into
the LC/EC without being subjected to the ultrasonic
treatment.

Each of seven individual mouse brain parts may also
be analyzed by a procedure similar to that outlined for
the whole brain. Some of the parameters used for the whole
brain, however, require modification to make them more
appropriate to the brain parts. A summary of the reagents
applicable to whole mouse brain and brain parts is pre-
sented in Table 4.1.

C. <u>Data Treatment</u>. The 5-HT level in a tissue sample
is established by comparing the peak height ratio of 5-HT
and the internal standard for the sample to the average
ratio for the standards:

$$5\text{-HT (ng/g)} = \frac{R_{sample}}{R_{std}} \frac{ng\ 5\text{-HT in std}}{wt.\ tissue\ (g)}$$

TABLE 4.1

Reagents Used in the Determination of Serotonin in Mouse Whole Brain and Brain Parts

Brain Parts	Standard			Sample		
	Working** Std (μl)	Internal* Std (μl)	1.0 M HCl (μl)	Typical Tissue wt (mg)	Internal* std (μl)	1.0 M HCl (μl)
Diencephelon	50	200	0	44	200	0
Hippocampus	50	150	50	33	150	50
Cerebellum	50	100	100	57	100	100
Medulla-pons	50	200	0	43	200	0
Midbrain	50	200	0	36	200	0
Striatum	50	150	50	23	150	50
Cortex	200	150	150	198	150	150
Whole brain	450	750	0	450	750	0

*Internal standard is a 1:250 dilution of the stock NM-5-HT solution in deaerated 1.0 M HCl.

**Working standard is a 1:100 dilution of the 5-HT stock standard solution in deaerated 0.10 M HCl.

where $R_{sample} = (\dfrac{\text{Peak height of 5-HT for sample}}{\text{Peak height of NM-5-HT for sample}})$

$R_{std} = (\dfrac{\text{Peak height of 5-HT for std}}{\text{Peak height of NM-5-HT for std}})_{ave}$

All results for multiple determinations are expressed as the mean ± the standard error of the mean (SEM) and represent at least five separate measurements. Statistical values were calculated using Student's t-distribution (1).

III. Development of Procedure

A. Chromatographic Conditions. In implementing a direct injection scheme for the analysis of 5-HT, a number of potentially interfering compounds must be considered. From previous work (11), it was known that the compound of interest, 5-HT, would be highly retained by a styrene-divinylbenzene based cation exchange resin such as Vydac SC. We, thus, proceeded to investigate the use of such a column. After trying a variety of conditions, it was found that a 500x3 mm column with a mobile phase consisting of a citrate/acetate buffer and a high concentration of NaCl would provide the desired separation of 5-HT and the internal standard, NM-5-HT. The high ionic strength of the mobile phase shifts essentially every potentially interfering cationic species to the solvent front. Potential interferences of the amino acid, neutral, and acidic variety are not retained due to the nature of the packing material and the pH of the mobile phase. A typical chromatogram for a whole mouse brain 5-HT assay is shown in Figure 4.1.
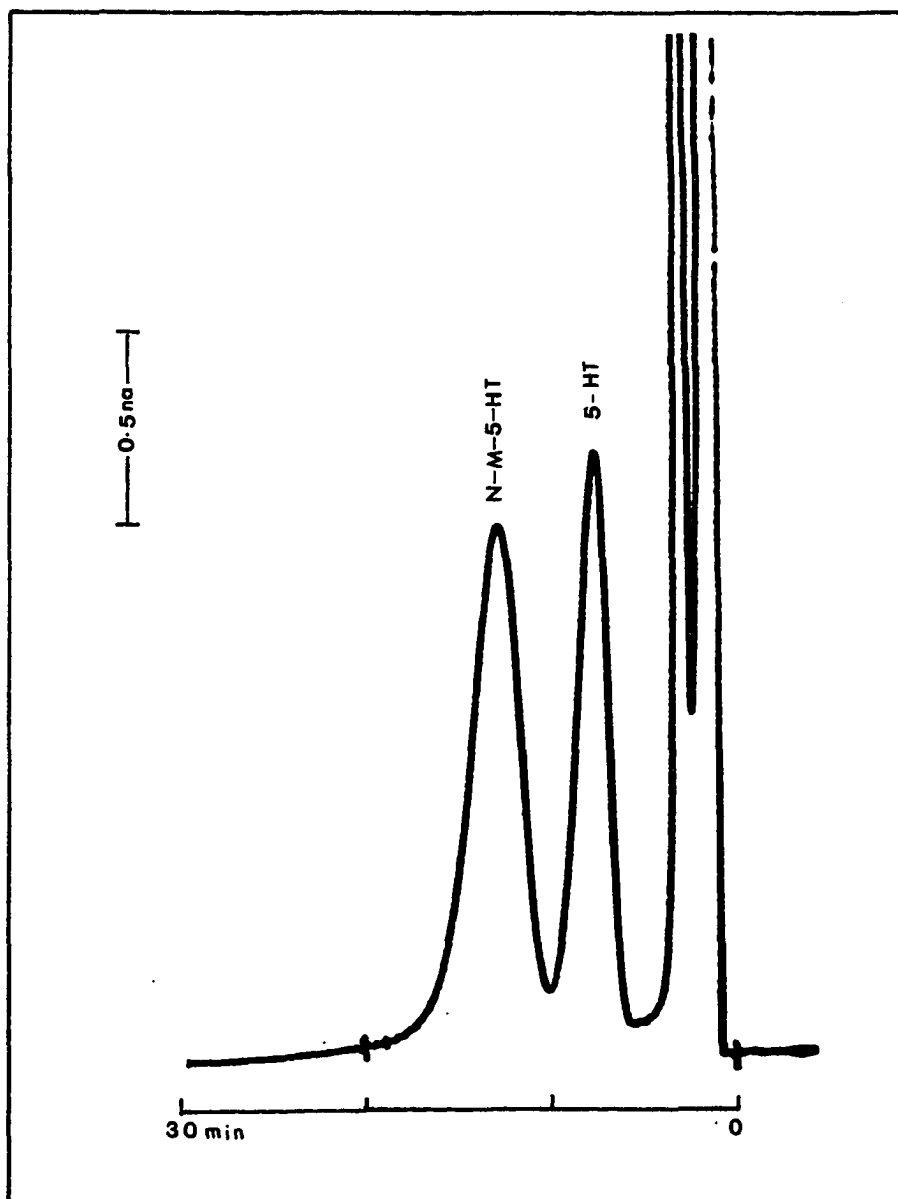
Figure 4.1   A Typical Chromatogram Obtained From a Whole
Mouse Brain 5-HT Assay.

However, Ponzio and Jonsson (2) had previously used a 2.1x200 mm Vydac CX column with a citrate/acetate mobile phase for the determination of 5-HT and reported that the dopamine metabolite, 3-methoxytyramine, interfered with 5-HT. This was particularly of concern for brain regions with a dense dopaminergic innervation such as the neostriatum. Using the present chromatographic conditions, as seen in Figure 4.2, however, no such interference was observed. The 3-methoxytyramine (3-MT) is clearly separated from 5-HT.

It should be mentioned that, after this work was completed, the use of a combination of column materials was found to provide an even better separation than that presented above. Thus, a tandem arrangement of Zipax SCX, 150x3 mm, and Vydac SC, 250x3 mm, is now being routinely employed in our laboratory. The Zipax provides a better resolution for 5-HT and NM-5-HT, while the Vydac provides the necessarily strong retention of both.

B. **Attempted Whole Brain Analysis.** During the initial phase of this study, a 0.025 $\underline{M}$ HCl solution was employed as the homogenizing medium. To our surprise, this resulted in a measured 5-HT level of 300 ng/g for the whole mouse brain. As seen in Table 4.2, the average reported value for 5-HT is more like 500-800 ng/g. This direct injection approach, involving no clean-up or isolation step procedures, would, thus, have been expected to provide a value
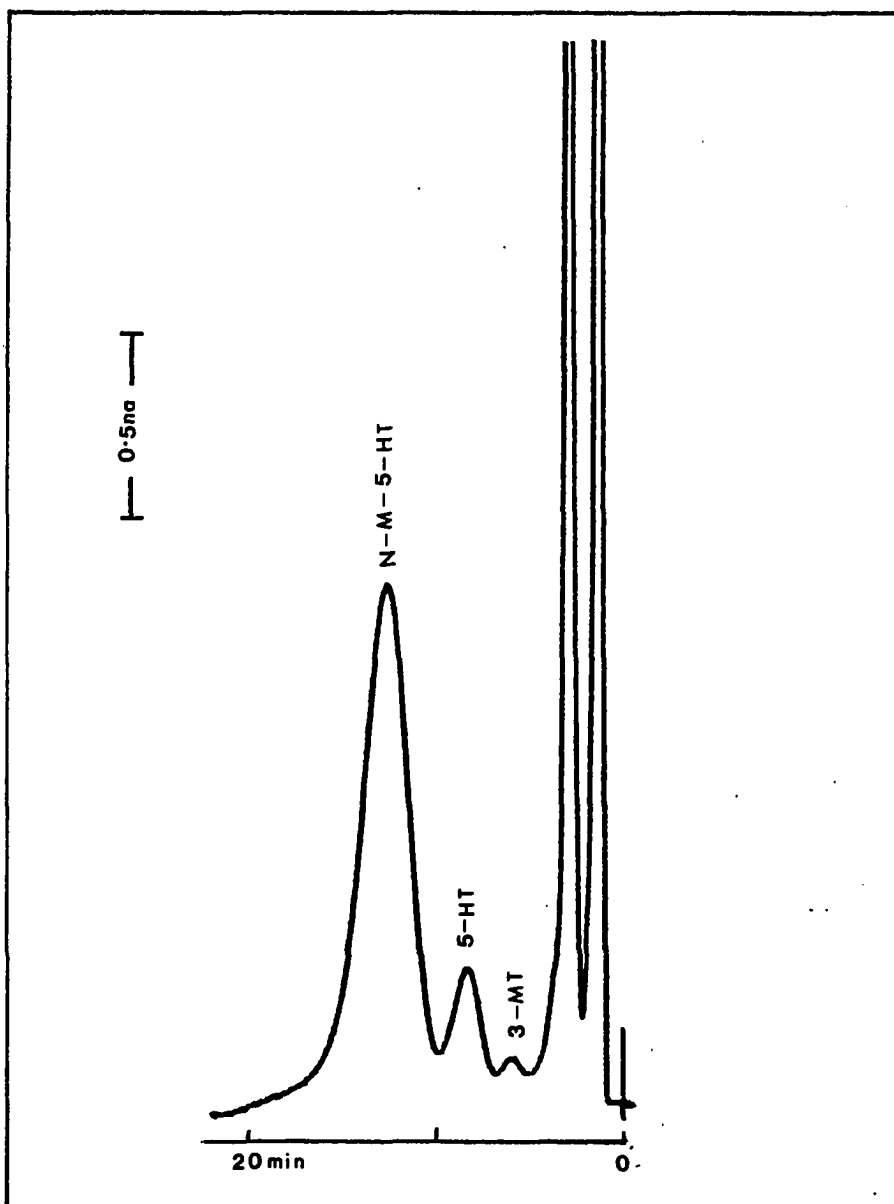
Figure 4.2 A Typical Chromatogram Obtained During the
Determination of 5-HT in Mouse Brain Striatum.

TABLE 4.2

Whole Mouse Brain 5-HT Levels Reported by Various Workers

Investigator	Technique	Homogenizing Medium	5-HT (ng/g)
Carlsson et al. (16)	Fluorescence	0.40 $\underline{M}$ $HClO_4$	520
Leroy (15)	"	0.10 $\underline{M}$ HCl	520±16
Weintraub et al. (10)	GC/MS	-	670±40
Sasa & Blank (11)	LC/EC	0.025 $\underline{M}$ HCl	805±12
Fleming et al. (5)	Fluorescence	acetone	550±10
Wiegand and Perry (6)	"	0.010 $\underline{M}$ HCl	820±12
Welch and Welch (7)	"	0.010 $\underline{M}$ HCl	600-800
Smith (8)	"	0.50 $\underline{M}$ HCl	660±13
Agrawal et al. (9)	"	-	410±16
Weintraub et al. (10)	"	0.010 $\underline{M}$ HCl in butanol	550±20

which was equal to or greater than the previous results. This led us to investigate the only chemical parameters, the acid type and concentration, involved in the homogenization.

C. Investigation of Homogenizing Medium. Three different types of acid are typically used for protein precipitation in tissue homogenization: HCl, $HClO_4$ and Cl_3CCOOH. Thus, we determined the 5-HT level of mouse brain using various concentrations of these three acids. As seen in Table 4.3, indiscriminate selection of either acid type or concentration could lead to quite diverse results. The 5-HT levels measured ranged from 123 to 728 ng/g.

Since deproteinization by acid treatment is so commonly employed by investigators, we further examined the cause of these variable results. Thus, we investigated the percent recovery of 5-HT from different homogenizing mediums. For each acid, three groups of samples were prepared according to Table 4.4. After thoroughly mixing the samples from all groups, those from groups 1 and 2 were centrifuged. An accurately measured volume of supernatant from each sample in all groups was then injected into the LC/EC, and the absolute peak heights of 5-HT were measured. To calculate the percent recovery of 5-HT, the difference in the peak heights for samples from groups 1 and 2 were compared to the peak heights obtained for group 3. The resulting

TABLE 4.3

The Effect of Acid Concentration in the Homogenizing Medium

on Whole Mouse Brain 5-HT Levels*

Acid Concentration in Homogenizing Medium, $\underline{M}$	5-HT Found (ng/g)		
	HCl	CCl_3COOH	$HClO_4$
1.1	712±12	613±9	683±18
0.57	728±19	588±26	689±26
0.11	667±27	689±33	705±33
0.057	575±11	523±7	634±11
0.014	479±7	146±6	261±15
0.0057	354±12	123±5	151±16

*Each brain was homogenized in 750 µl of the listed acid concentration and composition containing an appropriate amount of NM-5-HT.

percent recovery of 5-HT for the three acids investigated is presented in Figure 4.3. Comparable, although not exactly equivalent, results were obtained for NM-5-HT. Based on this data, an HCl homogenizing medium of 0.50 to 1.0 $\underline{M}$ was selected to be optimal for routine applications.

Sasa had previously reported (12) that a direct injection of the supernate in the determination of dopamine and serotonin produced a deterioration in the observed
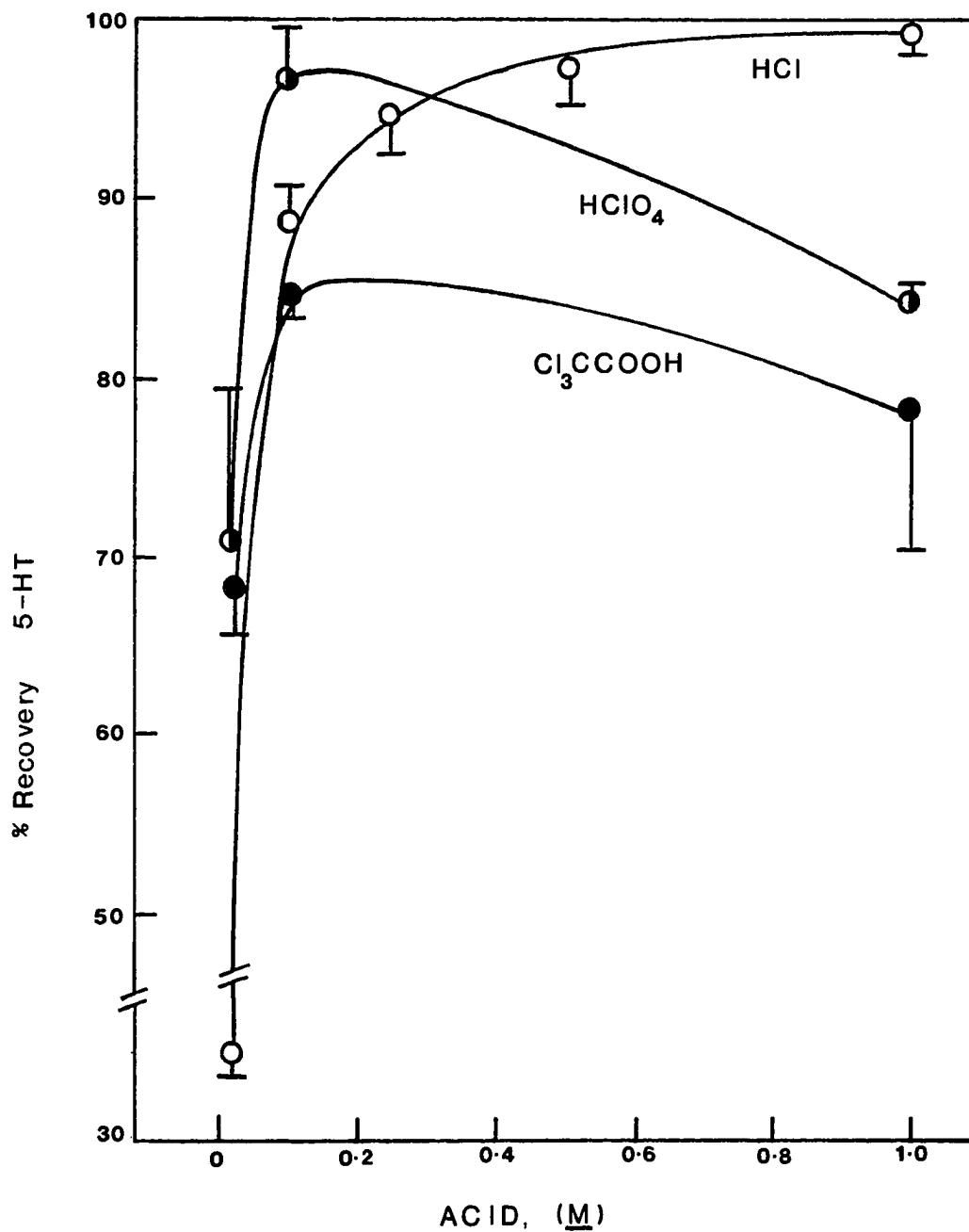
Figure 4.3   The Effect of Different Concentrations of
Various Homogenizing Acids on the Recovery
of 5-HT.

TABLE 4.4

Composition of Samples Used to Determine the Percent

Recovery of 5-HT and NM-5-HT from

Different Homogenizing Mediums

Reagent	Sample Group		
	1	2	3
Brain homogenate*	500 μl	500 μl	
NM-5-HT**	100 μl	100 μl	100 μl
5-HT***		100 μl	100 μl
x M HA****	100 μl		500 μl

*Several brains were pooled together in a ratio of 1 brain

per 1.5 ml homogenizing acid solution, where the homogenizing

acid investigated is HA and its concentration is x $\underline{M}$.

Homogenization was effected with ultrasonic disruption (out-

put setting No. 6, 20 sec/brain).

**NM-5-HT was prepared as a 1:50 dilution of its stock

solution in deaerated homogenizing acid (HA) of concentration

x $\underline{M}$.

***5-HT was prepared as a 1:100 dilution of the stock

standard solution in deaerated homogenizing acid (HA) of

concentration x $\underline{M}$.

****HA is the acid investigated (HCl, $HClO_4$, or CCl_3COOH).

The concentration examined is x $\underline{M}$.

chromatographic resolution after only ten injections. Our initial studies, using the same acid concentration (0.025 $\underline{M}$ HCl) for deproteinization had also resulted in rather rapid worsening of the column performance. Further investigations, however, revealed that this performance was also largely related to the acid strength of the homogenizing medium. As can be seen from Figure 4.4, a 1.0 $\underline{M}$ HCl homogenizing medium not only yields the highest recovery of 5-HT, but also provides a much better column performance. Using the 1.0 $\underline{M}$ HCl, hundreds of samples can be injected without the column showing signs of significant deterioration.

D. <u>Sonication Effects</u>. The only other homogenizing parameter is represented by the fact that the samples are sonicated to elicit homogenization. This was of some concern since Alliger (14) had reported that extensive ultrasonic treatment could produce destruction of the indole nucleus of tryptophan. To investigate the possible effect of ultrasonic treatment on 5-HT and NM-5-HT, we decided to first examine treated and untreated standard samples. These samples were prepared as described in the Materials and Methods section of this chapter and divided into two groups. One group was ultrasonically treated (power setting 6, 40 sec, 50% duty cycle) while the other was not. The results are shown in Table 4.5. As can be seen, sonication significantly affects both the peak heights and the peak
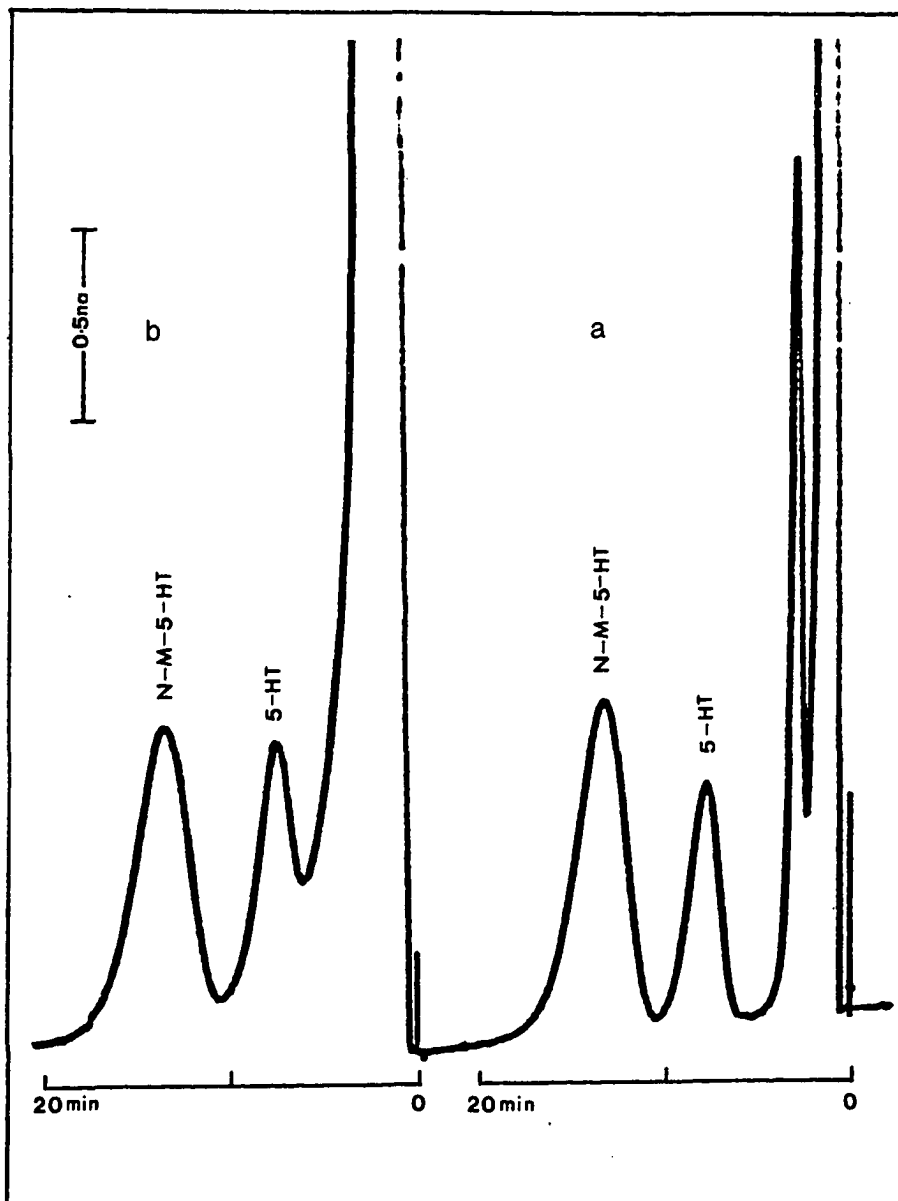
Figure 4.4 Chromatograms from Whole Mouse Brain 5-HT
Assays. a. Brain homogenized in 1,0 $\underline{M}$ HCl;
b. Brain homogenized in 0.10 $\underline{M}$ HCl.

TABLE 4.5

The Effect of Ultrasonic Treatment on 5-HT and NM-5-HT

Ultrasonic Treatment	Peak Height, mm 5-HT	NM-5-HT	Ratio 5-HT/NM-5-HT
No	99.6±0.9	74.8±0.65	1.33±0.01
Yes	50.4±1.9	32.8±1.7	1.54±0.06
P-value	P <0.001	P <0.001	P <0.001

height ratio for 5-HT and NM-5-HT.

Having observed these rather disturbing effects of sonication upon standards, we proceeded to examine the same for tissue samples. The individual samples were prepared as described in the Materials and Methods section of this chapter. Different groups of samples were subjected to different times of sonication. The results are shown in Table 4.6. From this data, the peak height for 5-HT is seen to exhibit a maximum at 21 sec. Therefore, a sonication time of 21 seconds was used for all subsequent investigations.

Once the optimal sonication time for tissue samples was obtained, we proceeded to examine if sonication at this value might differently effect the percent recovery of 5-HT and NM-5-HT in the samples and standards. For this study, four groups of samples are prepared according to

TABLE 4.6

The Effect of Sonication Time on Peak Heights and Peak

Height Ratio for 5-HT and NM-5-HT

in Mouse Brain Tissue

Peak Height or	Sonication Time (sec)**				
Peak Height Ratio*	8	21	42	64	86
H_{5-HT}/Sample wt	177±7	199±9	188±10	167±7	156±15
$H_{NM-5-HT}$	53±2	51±1	47±3	45±3	40±5
H_{5-HT}/($H_{NM-5-HT}$·Sample Wt)	3.33	3.90	4.00	3.71	3.90

*Peak heights (H) given in mm.

**Medium (No. 6) power setting; 50% duty cycle.

TABLE 4.7

Composition of Samples Used to Investigate the Percent

Recovery of 5-HT and NM-5-HT in the Absence

and Presence of Brain Tissue After Sonication

Reagents	Group			
	1	2	3	4
Brain Homogenate,* µl	500	500		
NM-5-HT, µl	100	100	100	100
5-HT, µl	100		100	100
0.10 M HCl, µl		100	500	500
Sonication Time, sec	21	21	21	0

*The brain homogenate was prepared by sonicating 5 brains in 5.00 ml of 1.0 M HCl for 20 sec/brain.

Table 4.7. To obtain the percent recovery of 5-HT in the presence of brain, the difference between peak heights for samples from group 1 and group 2 are compared to those of group 4. For the percent recovery of NM-5-HT in the presence of tissue, the peak heights for samples from either group 1 or group 2 are compared with those of group 4. To obtain the percent recovery of either 5-HT or NM-5-HT in the absence of brain, peak heights for samples from group 3 are compared to those from group 4. The results are presented in Table 4.8.

The ratio in the absence of brain tissue (1.02) is not much different from 1.00. But, the individual components involved obviously show considerable losses due to sonication. Thus, sonication of standards was deleted from the procedure. The tissue samples, however, must be sonicated to effect homogenization. Thus, the final results for 5-HT assays must be divided by 0.929 (or multiplied by 1/0.929 = 1.08) to obtain accurate values. This adjustment of results is necessitated by the ratio of the percent recoveries for 5-HT and NM-5-HT.

E. _Linearity_. The 5-HT concentration-electrode response relationship has been tested before (2,3,4) to demonstrate linearity over a wide range of concentration. In all these studies, however, the samples used were either standard solutions or solutions obtained after the purification

TABLE 4.8

Percent Recovery of 5-HT and NM-5-HT in the

Presence and Absence of Brain Tissue

after Ultrasonic Treatment

| | % Recovery | | Ratio (5-HT/NM-5-HT) |
	5-HT	NM-5-HT	
With Brain Tissue	85.1±5.8%	91.6±1.6%	0.929
Without Brain Tissue	78.9±2.7%	77.2±3.0%	1.02

by solvent extraction or ion exchange adsorption/desorption.
To ascertain that samples obtained from simple deproteinization
contained no interfering materials, an investigation of
linearity was undertaken. The samples used in this investiga-
tion were prepared as described for group 1 in Table 4.7
with a variable concentration of added 5-HT. As can be seen
from Figure 4.5, an excellent linearity is obtained over
a wide range of added 5-HT values.

F. Final Procedure. Using the procedure described in
the Materials and Methods section, a typical assay yielded
a value for whole mouse brain 5-HT of 784±20 ng/g. This
is in good agreement with the previously reported values
listed in Table 4.2.

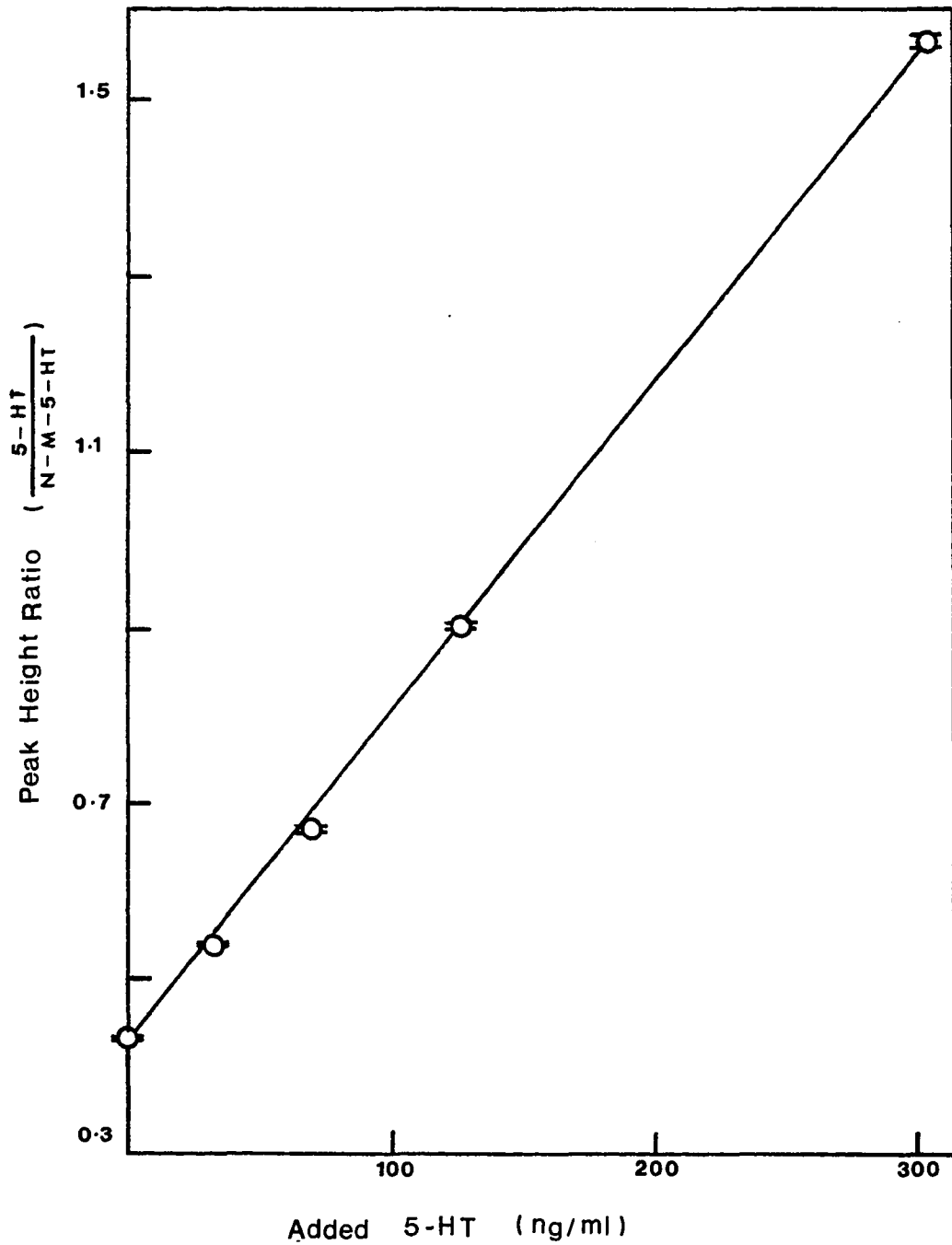With the presently employed procedure, the detector

Figure 4.5   The Peak Height Ratio of 5-HT to NM-5-HT
versus the Amount of 5-HT Added to the
Brain Homogenate.

is rather stable. This provides a very low noise level and enables the detection limit to be minimized. For example, an injected amount of 0.1 pmol of 5-HT clearly produced a peak height which was two times the noise level. This is comparable to the detection limit obtained by previously reported LC/EC procedures (2,3,11).

IV. Examination of Possible post mortem Alterations of

Serotonin in Mouse Brain

In an examination of the possible post mortem degradation of 5-HT in mouse brain, microwave irradiation (10) was employed to sacrifice the animals. This technique rapidly inactivates the metabolic enzyme monoamine oxidase and the biosynthetic enzymes tryptophan hydroxylase and 5-hydroxytryptophan decarboxylase. The 5-HT levels of microwave treated animals is then compared to those from decapitated animals to detect any rapid inactivation effects. Animals from a third group are microwaved 5 minutes after decapitation to eliminate the possibility of any microwave artifacts. One such investigation provided the results presented in Table 4.9. As expected, the t-test indicates there is no difference between the decapitated and decapitated/ microwaved samples (p >0.05) while the decapitated and microwaved samples are significantly different, (p <0.02). These results indicate that a rapid post mortem degradation of 5-HT does, indeed occur. These results further indicate

TABLE 4.9

5-HT Levels of Whole Mouse Brain After Sacrificing by

Microwave Irradiation and Decapitation

Method of Sacrifice	5-HT Level (ng/g)
Decapitated (D)	553±42
Decapitated/Microwaved (D/M)	543±39
Microwaved (M)	696±16

the lack of any instrument-related artifacts. But, at the present time, we have not been able to obtain results that are consistent in these microwave studies. This is most clearly seen in the results obtained for seven mouse brain parts (see Table 4.10 and 4.11).

The results listed here indicate that the microwaved samples generally have a higher 5-HT level than those from decapitated animals, suggesting a rapid post mortem degradation does occur. But the decapitated/microwaved samples, in most cases, have lower 5-HT levels than either the decapitated or microwaved samples. Instrumental artifacts should have produced a value for this combined result which would have been equivalent to the microwaved samples. No artifacts should produce results for the combined samples which are indiscernible from decapitated animals. The source of this discrepancy is not currently understood.

TABLE 4.10

5-HT Levels of Mouse Brain Parts After Different

Methods of Sacrificing

Brain Parts	5-HT Level (ng/g)		
	Decapitated (D)	Decapitated/ Microwaved (D/M)	Microwaved (M)
Diencephalon	838±72	331±43	1,222±41
Hippocampus	543±29	330±71	896±23
Striatum	598±45	436±88	710±32
Midbrain	809±54	297±48	1,356±39
Cortex	560±32	288±64	755±20
Cerebellum	72±9	68±14	338±13
Medulla-Pons	343±61	141±54	690±88

TABLE 4.11

T-Test for Results Listed in Table 4.10

Brain Parts	T-Test (P-values)*		
	D vs. D/M	D vs. M	D/M vs. M
Diencephalon	<0.001	0.002	<0.001
Hippocampus	0.03	<0.001	<0.001
Striatum	N.S.	N.S.	0.02
Midbrain	<0.001	<0.001	<0.001
Cortex	0.005	<0.001	<0.001
Cerebellum	N.S.	N.S.	<0.001
Medulla-Pons	0.04	0.02	<0.001

*N.S. indicates no significant difference.

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