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SASA, Suleiman Issa, 1937-
ANALYSIS OF BIOGENIC AMINES AND THEIR METABOLITES IN BRAIN TISSUE BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION.

The University of Oklahoma,
Ph.D., 1977
Chemistry, analytical

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THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

ANALYSIS OF BIOGENIC AMINES AND THEIR METABOLITES IN BRAIN TISSUE BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

By

SULEIMAN ISSA SASA

Norman, Oklahoma

1977

ANALYSIS OF BIOGENIC AMINES AND THEIR METABOLITES IN BRAIN TISSUE BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

APPROVED BY

DISSERTATION COMMITTEE

ACKNOWLEDGEMENTS

I wish to express my gratitude and appreciation to Dr. C. LeRoy Blank for guidance and support during the course of this research. It is impossible to adequately thank him for his patience, understanding, and enthusiasm.

Special thanks go to Dr. Glenn Dryhurst and Dr. Richard Taylor who always provided, together with Dr. C. LeRoy Blank, the real friendly and harmonious atmosphere in our analytical chemistry division.

Special thanks go to Dr. Eddie Smith who, as a graduate student advisor, always showed a real concern and interest in helping graduate students. Special thanks also go to Dr. Arnulf Hagen, whose help with the writing of the dissertation was certainly more than was required, and to Dr. Pushkar Kaul whose valuable instructions and notes will always be remembered.

Special thanks go to Dr. William Stavinoha, Dr. Arvin Modak and their group at the Health Sciences Center in San Antonio, Texas for providing brain parts and microwave facilities.

Special thanks go to Mr. Pat Cohenour for the

synthesis of the neurotoxins employed in this research. Special thanks also go to my fellow graduate students: Messrs. Robert Pike, David Wassil, Mike Bulawa, Jim Owens, Mike Cleary and Henry Marsh for their contributions of labor, ideas, and advice.

I wish to thank my loving wife, Nuha, and Mrs. Nancy Heinicke for their great help in preparing and typing this manuscript. Their help will always be remembered and appreciated.

Financial assistance in the form of a teaching assistantship from the Chemistry Department at the University of Oklahoma is acknowledged.

This work was supported in part by a Cottrell grant from Research Corporation and in part by Grant No. MH26866-01, awarded by the National Institutes of Mental Health, DHEW. This support is gratefully acknowledged.

I am most indebted to my parents who sacrificed for my education, and to my loving wife, Nuha, without whose patience and understanding, graduate work would have been impossible.

Finally, I heartily thank "Almighty Allah" from whom I always received assistance and protection.

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CHAPTER I

GENERAL INTRODUCTION

Over the recent decades, information has accumulated which suggests important roles for both the serotonergic and adrenergic nervous systems in the brain.[1,2] These systems utilize the indoleamine, 5-hydroxyptamine (5-HT, serotonin)*, and the catecholamines, norepinephrine (NE) and dopamine (DA), as neurotransmitters.

Serotonin was first isolated from beef serum in 1948, when it was demonstrated to exhibit vasoconstrictive activity.[3] Shortly thereafter, its structure was identified to be that of 5-hydroxytryptamine.[4] The material has since been shown to be widely distributed in nature. For example, many types of fruits such as pineapples and bananas contain extremely large amounts of 5-HT. Also, many animal tissues contain significant amounts of serotonin.[5] It is notably present in brain and innervated tissues, in blood platelets, and in intestinal mucosa.

---

*All abbreviations used in the text are included in Appendix I for reference by the reader.

While serotonin does occur naturally in brain it does not enter the brain readily from the blood stream because of its rejection by the blood-brain barrier. Therefore, the brain must synthesize its own serotonin (Figure 1-1). The first step in this synthesis involves the hydroxylation of the amino acid tryptophan to form 5-hydroxytryptophan (5-HTP) by the enzyme tryptophan hydroxylase (TPH). 5-HTP is then decarboxylated by 5-hydroxytryptophan decarboxylase to yield serotonin. Following synthesis, the major route of degradation for serotonin involves deamination[6] by the enzyme monoamine oxidase (MAO) to form 5-hydroxyindole-acetaldehyde. The aldehyde can be further oxidized to form 5-hydroxyindoleacetic acid (5-HIAA, the predominate product) or reduced to form 5-hydroxytryptophol. Enzymes have also been described in liver and in brain which can catabolize 5-HT without deamination through formation of the 5-O-sulfate ester.[6] This can then be transported out of the brain, possibly by the same acid excretion system handling 5-HIAA.

The catecholamines, norepinephrine and dopamine, are of increasing importance in many areas of neuroscience. Norepinephrine was first discovered in sympathetically innervated organs and sympathetic nerves by von Euler[7] in 1946. It was also observed in urine and the adrenal medulla by Holts[8] in 1947. Dopamine was found in animals at about the same time.[9,10,11]

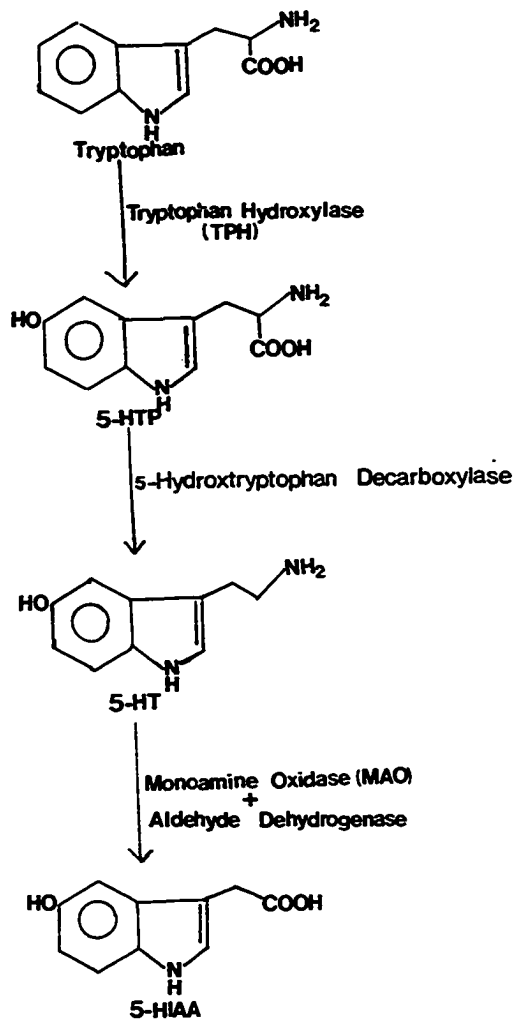

FIGURE 1-1. Major metabolic pathways for the synthesis and metabolism of serotonin.

These catecholamines are formed in brain, chromaffin cells, sympathetic nerves, and sympathetic ganglia from their amino acid precursor tyrosine by a sequence of enzymatic steps first postulated by Blaschko[12] in 1939. This sequence was finally confirmed by Nagatsu and coworkers[13] in 1964, with the demonstration that the last enzyme to be discovered (tyrosine hydroxylase) converted tyrosine to DOPA. The amino acid precursor, tyrosine, is normally present in the circulation in a concentration of about 5 to 8 x 10^{-5} M. It is taken up from the blood stream and concentrated within the brain and other sympathetically innvervated tissue by an active transport mechanism. Once inside the periperal neuron tyrosine undergoes the series of chemical transformations outlined (Figure 1-2), resulting in the ultimate formation of norepinephrine and dopamine.

Under normal conditions the biogenic amines are continuously being produced, released, and metabolized. Abnormal production and metabolism of these compounds may produce pathophysiological effects.[14] For example, there is an increasing body of evidence indicating abnormal indoleamine metabolism in the affective disorders.[15] A decrease in 5-HT and 5-HIAA concentrations has been reported in the brains of depressive suicides.[16] Loss of brain serotonin has been associated with overeating and increased body weight.[17] Lowering of 5-HT levels in both man and animals tends to cause overresponsiveness to both internal

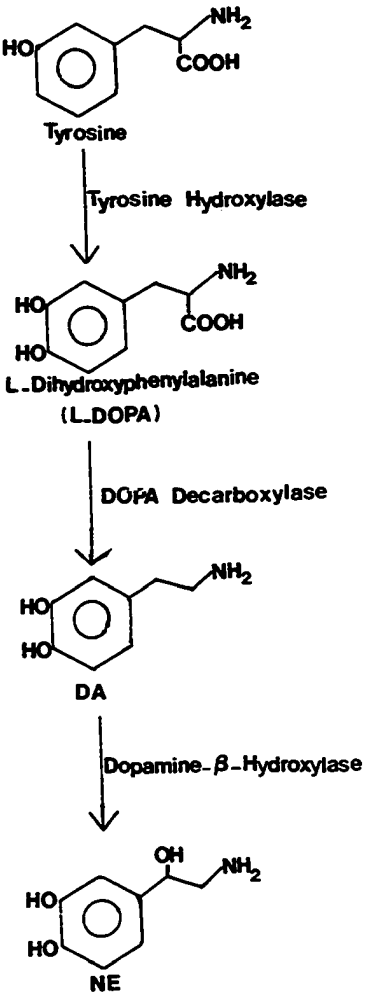

FIGURE 1-2. Biosynthesis of the catecholamines, norepinephrine (NE) and dopamine (DA).

and external stimulae. These can involve disturbances of sleep, increased susceptibility to convulsions, heightened sexuality, greater irritability, and aggressiveness.[18] Elevations of serotonin in rats also showed marked behavioral changes.[19] The involvement of catecholamines in affective disorders has been well established in the treatment of Parkinsons patients with L-DOPA to increase deficient brain dopamine levels.[20] Recently, damage to the norepinephrine system of the brain has been postulated to have important implications in schizophrenia.[21]

A great deal of recent research on biogenic amines attempts to separate the effects of each of the monoamines, NE, DA and 5-HT, in behavior and affective disorders. This is usually done by comparing the monamine level in the brains of control animals to those pretreated with a drug or precursor, while observing the behavior of both groups. However, accumulating evidence strongly indicates that the catecholamines and indoleamine systems interact with each other as well as with other systems involved in neurotransmission, e.g., the cholinergic system.[22,23,24] Also, it has been shown that the regional brain concentrations of biogenic amines are selectively affected by certain drugs,[25] and these regional concentrations, thus, may be much more relevant in behavioral studies than are whole brain values.

Analytical Methodology Background

Based on the above observations, an assay that can simultaneously determine both adrenergic and serotonergic neurotransmitters was deemed highly desireable. This assay should be selective, sensitive enough to analyse individual mouse brain parts, and inexpensive so that it can be used routinely.

The techniques frequently employed for the determination of biogenic amines in tissue samples[26-40] can all be broken down into three basic steps: (1) homogenization followed by precipitation of macromolecules and subsequent removal by centrifugation, (2) isolation and purification of the biogenic amines, and (3) following physical separations of the biogenic amines or the division of the sample into two or more distinct samples, the separate quantitation of each species.

The initial precipitation step is usually performed by treatment with an acid. Isolation and purification of the amines has been accomplished by either liquid chromatographic or extraction methods. The chromatography has primarily utilized methodology similar to the cation exchange approach described by Taylor and Laverty.[27] Extractions have typically involved a pH adjustment, extraction into a non-aqueous solvent, and a back extraction into an acidic aqueous solution. Such an extraction procedure is described by Shore and Olin[38] for the determination of

catecholamines. Catecholamines, of course, can also be isolated by the highly selective alumina adsorption, as described by Anton and Sayre.[41]

Finally, the detection and quantitation of the amines has been performed by fluorescence, gas chromatography (with or without mass spectrometry, GC or GCMS), and radiochemical techniques. The most commonly employed analyses have utilized fluorescence as a convenient mode of detection and quantitation. Although the fluorometric determinations of 5-HT may be performed directly in 3 M HCl, many workers seem to prefer derivatization with o-phthaladehyde, as used by Maickel et al.,[30] or with ninhydrin, as introduced by Vanable,[31] to increase the sensitivity. The catecholamines, on the other hand, are chemically converted to indole species by an oxidative rearrangement known as the trihydroxyindole method,[32] prior to fluorometric determination. This oxidative technique produces much more selective and intense fluorescence than the unchanged molecules and is still the method of choice. As mentioned, however, the determination of NE, DA and 5-HT by fluorometry usually involves chemical transformation to other compounds as well as the use of spectroscopically pure reagents and various other precautions to avoid possible interferences. The detection limit of the technique is only in the nanomole range. These restrictions frequently require sample pooling for the analysis of individual brain parts to obtain the

necessary sensitivity. Additionally, the selectivity of fluorescence in the quantitation of these samples leaves a great deal to be desired.

Gas liquid chromatography (GLC) with electron capture detection for the analysis of tissue samples containing NE, DA, and 5-HT has been suggested by Arnold and Ford[34] and others.[35] Yet, the sensitivity limit obtainable by this method was only ca. 50 ng/g of wet tissue for sample weights of 500 mg. The analysis time was also reported to be five hours for six samples. The combined technique of GCMS has been shown by Koslow et al.[36] to be applicable to such determinations. While offering excellent selectivity and detection limits in the subpicomole range, GCMS is, undeniably, a very expensive technique to employ on a routine basis.

A rather recent entry into the area of biogenic amines determinations is the radiochemical techniques.[39,40] In these assays labeled S-adenosyl-L-methionine (methyl-^{14}C or methyl-^{3}H) and an appropriate methyltransferase enzyme e.g., catechol-O-methyltransferase (COMT) for the catecholamines, are incubated with the sample to convert the amine to a labeled methylated metabolite. The metabolite is then isolated by extraction into an organic solvent such as chloroform or toluene and assayed for ^{14}C or ^{3}H by scintillation counting. These enzymatic isotopic assays are highly specific, and sensitive to the picogram level. They have

been proven to be precise and reproducible. However, they do require careful handling of reagents and cofactors as well as experience in working with enzyme preparations since they involve the use of purified enzymes which are not presently available commercially.

As one can readily observe, the highly sensitive assays for biogenic amines require the use of either the expensive GCMS or radiochemicals and scintillation counting equipment. Moreover, in all the techniques available, some form of chemical transformation is typically required which may add to the analysis error and lengthen the time required.

The technique of high performance liquid chromatography (HPLC) has provided rapid qualitative and quantitative analysis of mixtures. A major limitation in the wider application of this technique, however, has been the lack of sensitivity of the detection systems available.[42] The most sensitive of these systems has previously been found to be ineffective for measuring nanogram and sub-nanogram quantities of catecholamines and indoleamines.

The development of the thin-layer electrochemical detector (EC) by Kissinger and coworkers[43] has provided the sensitivity and selectivity needed for the detection of many important biological and pharmacological compounds. This system has the capability of detecting as little as 3 femtomole (fmole = 10^{-15} mole) of some molecules.[44]

The specificity of the detector is due to the fact that it can only "see" the compounds which are electrochemically active (capable of absorbing or releasing electrons) at the particular potential chosen. This selectivity is of great value in the analysis of complex biological tissues and fluids since most of biological compounds are not electrochemically active, a property which eliminates them from being possible interferences. Additionally, some of the interferences due to electrochemically active compounds can be eliminated by the particular choice of electrode potential. Moreover, the electrochemical detection has been shown[45] to exhibit a linear dynamic range of ca. 10^5 to 10^6.

High performance liquid chromatography combined with electrochemical detection (LCEC) is now routinely used for the analysis of catecholamines.[37] However, this methodology[37] cannot be directly applied to the analysis of serotonin; the catecholamines in this technique are isolated by their highly specific adsorption onto alumina, a procedure which will eliminate serotonin due to its lack of similar adsorption. Simultaneous determination of serotonin and the catecholamines has been accomplished by extraction at a low pH value[38] and by a few, essential modifications in the previous LCEC system. Details of development of the methodology will be discussed later.

In short, the present dissertation is primarily

a report of the development of two new, major biogenic amine assays: (1) the simultaneous determination of DA and 5-HT, and (2) the simultaneous determination of NE, DA, and 5-HT. The sensitivity of these methods permits direct application to individual small parts of mouse brain. Chemical conversion prior to measurement of the biogenic amines has been eliminated and, in comparison to the previous techniques, substantially less initial monetary investment is required. Additionally, this methodology, utilizing LCEC, provides sensitivity and selectivity comparable to that of the exquisite GCMS, Finally, to demonstrate the broad applicability of these assays, a variety of practical neurochemical investigations of interest were undertaken.

CHAPTER II

EXPERIMENTAL

1. Reagents:

Generally, all chemicals were used without any further purification. Both reagent grade n-butanol from Mallinckrodt and n-butanol purified according to Shore and Olin[38] (by successive washings with 1 M HCl, 1 M NaOH, and deionized water) were examined for use in the extraction. Likewise, Baker analyzed n-heptane and heptane purified according to Shore and Olin[38] were investigated. Ethylenediaminetetraacetic acid dihydrate (EDTA) was obtained as the disodium salt from Fisher Scientific. Sodium chloride was reagent grade from Mallinckrodt. The biogenic amines, NE as norepinephrine hydrochloride, DA as dopamine hydrochloride, and 5-HT as serotonin creatinine sulfate monohydrate, were purchased in the highest possible purity from Aldrich and Regis Chemical Cos., respectively. All concentrations of these compounds are expressed as the free base. The internal standard, 3,4-dihydroxybenzylamine·HBr (DHBA, 98% pure) was obtained from Aldrich. The alternate internal standard, α-methyldopamine·HBr, (α-MeDA)

was prepared in our laboratory according to Borgman et al.[46] All other compounds tested for possible interference in the procedure, were obtained in the highest possible purity from Regis Chemical Co. Ascorbic acid was obtained from Aldrich. The components of the mobile phase, i.e., sodium acetate, acetic acid, sodium hydroxide, and citric acid, were all reagent grade from Mallinckrodt. All water used was deionized.

The stock standard solution, containing ca. 40 µg/ml NE, 90 µg/ml DA and 65 µg/ml 5-HT, was prepared by dissolving the appropriate amount of their salts, accurately weighed, in 100 ml of 0.01 M HCl. The HCl was previously deaerated for 15 minutes with O_2-free N_2.* This solution was stable for up to one month if stored in the refrigerator at 4°C. However, we never utilized a solution stored for more than two weeks. The working standard solution was always prepared on the day of use by diluting a 1.00 ml aliquot of the stock standard solution to 100 ml with deaerated 0.01 M HCl. Thus, the working standard contained ca. 400 ng/ml NE, 900 ng/ml DA, and 650 ng/ml 5-HT. This is comparable to the whole brain concentrations (expressed as ng/g tissue).

The internal standard, a solution of ca. 10^{-5} M DHBA, was prepared by dissolving the appropriate amount of

---

*Described in Section 2 of this chapter.

the hydrobromide salt of DHBA in deaerated 0.01 M HCl. The concentration of the internal standard need not be accurately known provided that the same volume of the same solution is added to both the standards and the samples. This solution was also stable up to one month when stored at 4°C. Lower concentrations of DHBA were used in the determination of mouse brain parts and are clearly noted in that section of the text.

Ascorbic acid solution was always prepared immediately before use by dissolving 11 mg in 1 ml of 0.01 M HCl. Lower concentrations of this solution were utilized with the standard solutions employed in the determinations of brain parts. These concentrations will be clearly noted in the final assay for brain parts.

The mobile phase finally selected for the LCEC, a pH 5.1 acetate/citrate buffer, was prepared by dissolving 8.2 g anhydrous sodium acetate, 2.1 ml glacial acetic acid, 4.8 g sodium hydroxide, and 10.5 g of citric acid monohydrate in 1 liter of water. Before use, it was filtered through a 0.45 micron Millipore filter to prolong pump life and to avoid column blockage by any particulate matter.

2. LCEC Apparatus:

The liquid chromatograph was constructed entirely from separate components. A simplified drawing of the basic setup is shown in Figure 2-1. Since tank nitrogen is not

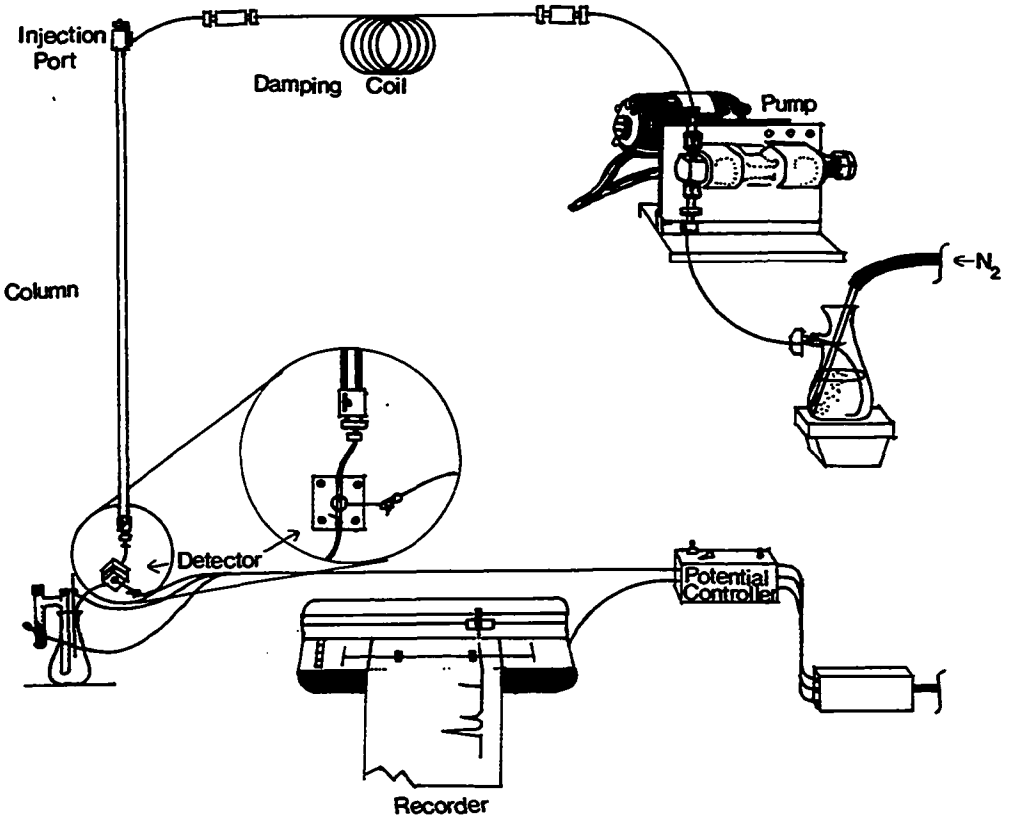

FIGURE 2-1. Apparatus for liquid chromatography with electrochemical detection.

sufficiently free of oxygen, it must be "scrubbed" by passing it through two gas washing bottles in series containing 5% chromous perchlorate in 10% perchloric acid.[47] The pumping system utilized was a Milton Roy reciprocating minipump (max. flow-rate, 160 ml/hr, max. pressure, 5000 p.s.i.). This pump was connected to a coil (ca. 25 m) of 0.8 mm diameter Teflon tubing to partially dampen pulsations.

The injection ports and standard connectors were obtained from Altex Scientific, Inc. (Berkeley, California). The glass columns, also available from the same supplier, were 2 mm diameter by 500 mm length, 3 mm diameter by 500 mm length, and 3 mm diameter by 250 mm length. These columns were packed with a pellicular strong cation exchange resin: typically, either Zipax SCX from DuPont (Wilmington, Delaware) or Vydac SCX from the Separations Group (Hesperia, California) were employed. Specifically, the columns finally shown to be most useful for the DA/5-HT assay were a 3 mm diameter by 250 mm length unit connected through a minimum dead volume fitting to a second unit, 3 mm diameter by 500 mm length, both columns being packed with Zipax SCX. For the NE/DA/5-HT assay, the columns used were: a 3 mm diameter by 250 mm length unit packed with Zipax SCX in tandum with 2 mm diameter by 500 mm length unit packed with Vydac SCX. Chromatographic injections were made with micro syringes (Hamilton CR 700-50) fitted with 20 gauge platinum needles. The needles are available

from the same supplier and are not particularly expensive.

The detector (Figure 2-2) was simply two blocks of lucite, each 44 mm in length, 35 mm in width, and 12 mm in thickness (these dimensions are not critical). The two blocks were separated by a thin (0.1 mm) Teflon spacer cut from commercial sheet supplied by Small Parts, Inc. (Miami, Florida). This spacer, shown separately in Figure 2-2-C, has a slit 2 mm wide and 25 mm long, cut in it. One of the blocks (the left side of Figure 2-2-A) has two holes, cut at the approximately indicated angles, to provide an inlet (In) and outlet (Out) flow stream from the column through the Teflon slit region. These holes were drilled slightly oversized and standard 0.8 mm Teflon tubing was inserted in each. The tubing was sliced flush at the inner face of the block and the excess was trimmed smooth with a razor blade. The upper end of the inlet tube was connected to a standard diameter Altex male tubing connector which was recessed and secured with epoxy cement to the top of the block.

The opposite block (right side of Figure 2-2-A) contained a recessed well of ca. 5 mm diameter and 9 mm in depth. Again, the well dimensions are not critical. It should, however, be slightly greater in diameter than the spacer slit width and positioned slightly downstream of the point where the inlet tube emerges from the opposite block. Entering the side near the bottom of the well is

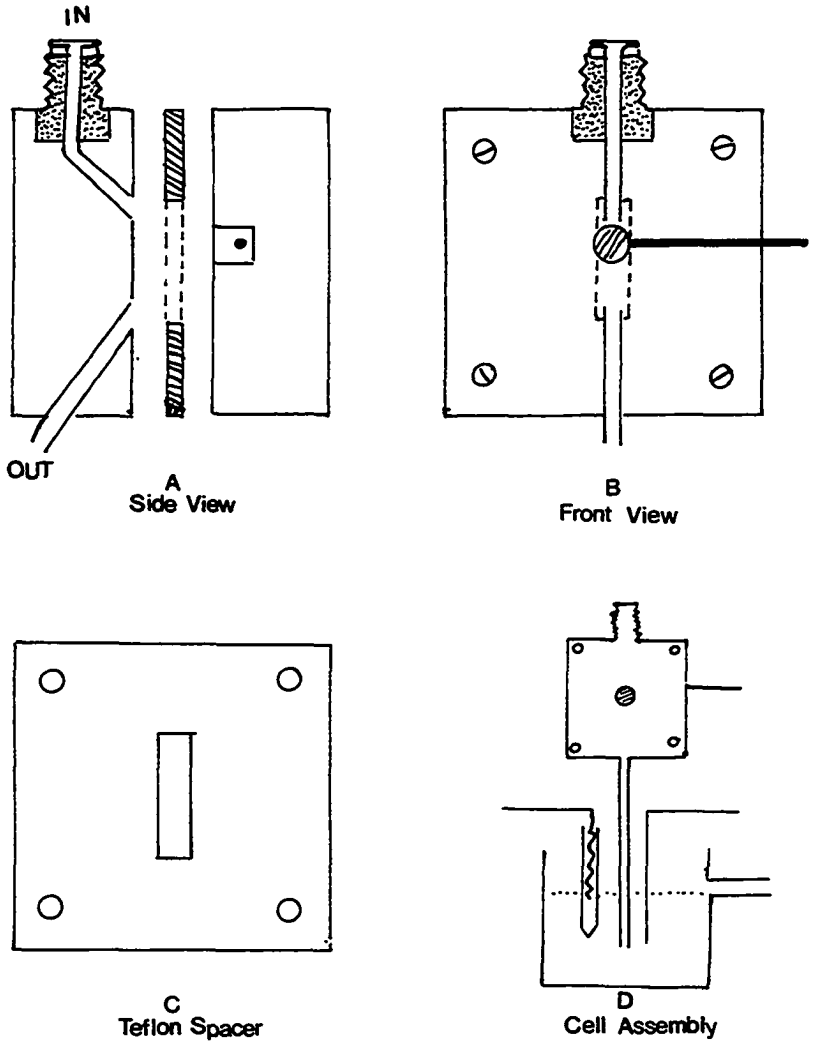

FIGURE 2-2. Carbon-paste electrochemical detector.

a drill hole into which a platinum wire contact was sealed (the platinum wire is shown as a solid black dot emerging perpendicular to the page in Figure 2-2-A). The well was packed flush to the rim with carbon paste (will be described later in this section) which forms the working electrode surface. The lucite block with the carbon paste well was then rubbed to a smooth surface on computer cards.[48]

Figure 2-2-D shows the connection from the outlet tube of the carbon paste detector to the overflow unit, a 125 ml suction flask fitted on the top with a rubber septum. The reference electrode (saturated calomel electrode, SCE) and the auxiliary electrode (a platinum wire) were inserted in holes pierced through the septum to dip into the overflow solution providing the three electrode cell. The side arm of the section flask was then used as the natural overflow outlet for the chromatographic system.

The carbon paste for the working electrode was made by thoroughly mixing 3 g of Ultra Pure Graphite (UPG) powder (Ultra Carbon Corp., Bay City, Michigan) with 2 ml of Nujol. Thorough mixing requires considerable time, but a single batch suffices for many electrodes.

The electronics used to control the potential of the working electrode and monitor the current have been previously described.[47] The potential controller, incidentally, is now commercially available from the Bioanalytical Systems Inc. (West Lafayette, Indiana). The

output, directly proportional to the current flowing in the carbon paste electrode, was monitored as a function of time by directing it to an Omniscribe (Houston Instruments, Austin, Texas) single pen recorder.

3. Animals:

Mice used in the various investigations were adult males of the ARS-HA/ICR strain (Sprague-Dawley, Madison, Wisconsin) weighing 20-30 grams at the time of sacrifice. These were maintained in 12 hr. dark/12 hr. light cycle (lights on from 0700 to 1900 hr.) and allowed access to food and water ad libitum. No mice were employed in the experiments until, at least, one week after their arrival so that they could become accustomed to these conditions. Also, since diurnal fluctuation in the biogenic amine levels was reasonably expected,[49] the animals were only sacrificed at a fixed time each day (10:00 - 10:30 a.m.).

The mice were sacrificed by cervical dislocation. The brains were removed as rapidly as possible, frozen in liquid N_2, and stored on dry ice. Each brain was weighed to the nearest mg and transferred to 30 ml screw-cap vial. The vials were kept on dry ice during the addition of reagents. When brain parts were to be analyzed, the whole brain was placed on an ice cold glass plate for dissection immediately after removal. The seven parts (cerebellum, medulla-pons, midbrain, diencephalon, hippocampus, striatum,

and cortex) were then removed by a procedure described in detail by Wassil.[50] The author is indebted to Mr. David Wassil (Chemistry Department, Oklahoma University) and Ms. Joyce Valkovitch (Pharmacology, Health Sciences Center, San Antonio, Texas) for providing all the brain parts included for this work.

4. Centrifuge:

The centrifuge employed was a refrigerated centrifuge (Sorvall RC-2B) with an SM-24 rotor. The Teflon centrifuge tubes, each 15 mm diameter by 100 mm length and having a round bottom, employed were from DuPont Instruments (Wilmington, Delaware).

5. Shaker:

An Eberbach shaker, operated at 280 cycles/minute and immersed in an air bath at 15°C, was employed for the extractions. Vials were always placed on their sides with the long axis parallel to the direction of shaking.

6. Microwave Oven:

Sacrificing animals by microwave exposure was kindly performed by Dr. William Stavinoha's group at the Health Sciences Center in San Antonio, Texas. For properly focusing the beam of radiation, the animal had to be placed in a modified 50 ml plastic syringe. For enzymatic deactivation (and killing), the head was exposed to 6 kw of microwave irradiation at 2450 MHz for 250 msec unless clearly noted otherwise.

7. Tissue Homogenizers:

All brain tissue samples, unless otherwise noted, were homogenized with an ultrasonic homogenizer (W200P, Ultrasonics, Inc.) equipped with a long probe tip for small volume work. A ground glass homogenizer (Kontes-Martin) was also briefly used in an investigation of the possible ill effects of ultrasonic treatment on 5-HT levels.

8. Calculations:

Whenever an assay was performed, working standard samples were always treated exactly like the tissue samples. The calculations were then based on the peak height ratios of NE, DA, and 5-HT, respectively, to DHBA in standard samples vs. the same ratios in brain samples. A typical calculation (for DA) is given as:

$$\text{if } R = \frac{\text{peak height, DA}}{\text{peak height, DHBA}}$$

$$\text{then DA(ng/g)} = \frac{R \text{ (sample)} \times \text{ng DA (standard)}}{R \text{ (standard)} \times \text{tissue wt (g)}}$$

Results of a typical analysis are tabulated below. The sample data are taken from the peak heights of a whole mouse brain determination. The actual standard utilized contained 274 ng of DA. The exact calculation for DA content is:

	Peak Heights (mm)		Peak Heights Ratios
	DA	DHBA	DA/DHBA
Standard	99	106	0.934
Brain Sample (wt. 0.462 g)	132	102	1.294

$$DA(ng/g) = \frac{(1.294)(273)}{(0.934)(0.462)} = 819 \text{ ng/g}$$

This is similar to the calculation of Refshauge et al.[37] in the analysis of catecholamines.

An important point to be noted here concerns the addition of an internal standard to both brain samples and working standards. This eliminates the need to reproduce the injection volumes, since only peak height ratios are used in the calculations. Also, the working standards are subjected to exactly the same extraction procedure as tissue samples. Thus, the final results do not need to be corrected for percent recovery except when this percent recovery is different for the working standards and tissue samples (i.e., for 5-HT as will be shown in Chapter 3).

For calculations of percent recovery, the peak heights of the individual amines were first established by injection of a sample of the mixture to be extracted. Then the percent recovery was determined by proceeding through the steps of the extraction procedure. The heights before and after extraction, obtained with the same

injection volume, were then compared. The results were corrected for dilution as follows:

$$\text{percent recovery} = \frac{\text{peak height after extraction}}{\text{peak height before extraction}} \times \frac{\text{final volume}}{\text{initial volume}} \times 100$$

All uncertainties for multiple determinations were expressed as the standard error of the mean (SEM), since this is the usual parameter applied to such determinations. Statistical levels of significance were calculated using the student's t-distribution.[51]

CHAPTER III

DEVELOPMENT OF BIOGENIC AMINES LCEC ANALYSES

A. Initial Chromatographic Studies

The major goal of this research was to develop a more sensitive, selective, and widely applicable technique for the analysis of the biogenic amines (NE, DA, and 5-HT). This assay should be performed relatively rapidly to allow the maximal amount of sample throughput. Therefore, an intensive search for a rapid and efficient chromatographic separation of these compounds was undertaken. Preliminary investigations utilized a synthetic whole brain mixture of the biogenic amines, ca. 10^{-6} M each, and ascorbic acid, ca. 10^{-3} M, dissolved in deaerated 0.01 M HCl. Ascorbic acid was added to mimic brain ascorbic acid levels and retard oxidation of the amines.

Since these biogenic amines are known to have pK_b values of ca. 5[1] they may be considered to be fully protonated at pH values $\leq$ 7. But, these species are also known to be highly sensitive to oxygen, very unstable in aqueous alkaline solution, and relatively stable at acidic pH

values.[1] Therefore, a deaerated, acidic eluting solvent appeared chromatographically most reasonable. Also, for these same reasons, the choice of a cation exchange resin for preliminary studies was a quite logical choice as the chromatographic packing material.

Our first choice for a column packing was Zipax SCX.* This decision was primarily based upon two facts: (1) this material had already provided a baseline separation in the assay for catecholamines (NE and DA) developed by Refshauge[47] employing 0.1 M perchloric acid as a mobile phase, (2) Zipax SCX is unique among strong cation exchangers in having a fluoropolymer backbone (all others have some type of silica or polystyrene-divinylbenzene backbone).

Our first attempt at obtaining the desired chromatographic separations thus utilized various column lengths (500 to 1000 mm), various flow rates (to ca. 2 ml/min), and various concentrations of the eluting solvent, $HClO_4$ (0.05 to 0.20 M). The flow rate maximum, it should be noted, was effectively controlled by the pressure; the glass/Teflon system can only be reasonably operated for extended periods of time at a pressure of $\leq$ 800 p.s.i.

Two major difficulties arose under these conditions. First, ascorbic acid, being predominately uncharged at these pH values, was not retained by the column. Thus,

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*A fluoropolymer based packing with $-SO_3^-$ as a functional group and a particle size of 30-40 μm.[52]

at flow rates reasonable for rapid analysis, it always comprised a rather large "solvent front"* which significantly overlapped with the only slightly retarded NE. The second problem was that the anomalous iron peak[47] observed in this acidic medium (as Fe^{++}) was almost completely isographic with serotonin. Neither of these problems could be adequately resolved by the adjustments of chromatographic parameters mentioned above. Additionally, the same problems were again encountered when the solvent used in the analysis of tyrosine hydroxylase activity by Pike[53] was employed. This solvent was composed of 0.03 M $HClO_4$ containing 0.025 M $KClO_4$.

Being still confident that Zipax could achieve the desired separation, we moved a step further in trying to remove the Fe^{++} by adding to the mobile phase some reagent that could complex this interference. In the complexed form, it would either be no longer electrochemically active due to a shift in oxidation potential or, at least, it would no longer exhibit the same retention time due to a change in its charge and/or structure. Therefore, we first added o-phenanthroline and then EDTA (each to a concentration of 0.002 M) to the 0.1 M $HClO_4$ eluting solvent.

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*This "solvent front" was shown later by the study of interferences to be due not only to ascorbic acid but also to the acidic and neutral metabolites of both catecholamines and indoleamines as well as other unidentified tissue components.

But the anomalous Fe^{++} peak was still present and isographic with serotonin. Looking for possible chloride complexation, we also tried the following eluting solvents: 0.1 M KCl in 0.01 M HCl and 0.1 M NaCl in 0.01 M HCl. Again, both proved to be unsuccessful in achieving the desired separation or elimination of Fe^{++}. Employing solutions of 0.1 to 0.2 M oxalic acid, which is known to complex Fe^{++} in the oxalate form in moderate to high pH values, did not result in any better results. Citric acid solutions (.05 to 0.2 M) ended in the same lack of success.

At this point, it became quite obvious that the use of Zipax SCX with an acidic eluting solvent would continually present us with the Fe^{++} problem. A quite obvious choice, therefore, was to examine eluting solvents of a higher pH value. To our surprise, and a happy surprise it was, the Fe^{++} peak simply disappeared from the chromatograms when the eluting solvent had a pH $\geq$ 3. That this effect was not due to the choice of buffer materials was shown by reproducing the result for each of acetate, phosphate, citrate/phosphate, and acetate/citrate buffer combinations (all with pH $\geq$ 3). For further investigations we selected the pH 5.1 citrate/acetate buffer[54] previously mentioned in the experimental section. This section was based upon three criteria: (1) it had previously[54] been shown to provide a reasonably rapid separation of the biogenic amines and some of their metabolites on a Bondapak/

Corasil strong cation exchange (Waters Associates, Milford, Massachusetts) packing; (2) it eliminated the Fe^{++} problem; and (3) preliminary investigations showed that, in combination with Zipax SCX, this buffer provided almost ideal resolution of the individual biogenic amines.

Optimal Chromatographic Conditions for DA/5-HT Analysis

Having selected an adequate column packing/eluting solvent combination, we proceeded to optimize the various chromatographic conditions for biogenic amine analyses. The following parameters were found to provide both a baseline separation of DHBA,* DA, and 5-HT and the shortest possible time between injections: (1) a Zipax SCX strong cation exchange resin, 3 mm diameter by 750 mm length, (2) the pH 5.1 citrate/acetate buffer as a mobile phase, (3) a flow rate of ca. 1.3 ml/min, which yielded a pressure of ca. 600 p.s.i., (4) a working electrode potential of +0.60 volts vs. SCE. Utilizing these conditions, individual injections could be made every 10 min; and, this analysis time was effectively halved by employing the dual parallel apparatus of Blank.[45] NE was not sufficiently separated from the solvent front under the optimal conditions to allow its simultaneous quantitation. Much slower flow rates or greatly weakened (diluted ca. 1:10) eluting solvent did yield an NE/solvent front separation. But these conditions would make the time between injections approach

*DHBA was initially selected as the internal standard due to its chemical similarity to the biogenic amines and its excellent chromatographic properties.

1 hr., a clearly unacceptable throughput. However, the importance of DA and 5-HT alone warranted the further development of an analysis for these two species.

Investigation of Chromatographic Interferences in the DHBA/DA/5-HT Separation

Before establishing an appropriate isolation procedure for the nervous tissue analysis of DA and 5-HT, it was decided that possible chromatographic interferences should be examined. There are, in fact, a relatively small number of components which would feasibly interfere with the projected assay. Such species (1) must be carried through the isolation procedure to be developed, (2) must have a percent recovery and endogenous concentration sufficient to yield a final molar concentration at least within a factor of 100 of the determined compounds, (3) must be retained by the cation exchange resin, and (4) must be electrochemically active on the carbon paste electrode at +0.60 V vs. SCE in this medium. The most likely candidates are, thus, the basic metabolites in the catecholamine and indoleamine biosynthetic pathways. We also investigated neutral metabolites, acidic metabolites, and ascorbic acid. The results of these investigations are presented in Table 3-1.

Ascorbic acid fulfills all the requirements for interference except one. It is not retained by the column

Table 3-1

Potential Interferences Examined

Compound	Retention time t_R (min)	Peak width (min)
Ascorbic acid	2.5	1.0
3,4-Dihydroxyphenethyl alcohol	2.5	1.0
3,4-Dihydroxyphenylglycol	2.5	1.0
L-3,4-Dihydroxyphenylalanine (DOPA)	2.0	0.7
L-3-O-Methyl DOPA	2.5	1.0
3,4-Dihydroxyphenylacetic acid	2.5	1.0
3,4-Dihydroxymandelic acid	2.5	1.0
3,4-Dihydroxyphenylalanine	2.0	0.7
5-Hydroxytryptophan	2.2	1.1
Norepinephrine	2.6	1.0
Normetanephrine	4.0	2.0
Dihydroxybenzylamine	4.0	2.0
Epinephrine	5.2	2.3
Dopamine	6.2	2.4
5-Hydroxytryptamine	9.3	3.8
Tryptamine*	24.5	21.3
Epinine(N-methyldopamine)	18.5	7.0
α-Methyldopamine	18.2	7.0
5-Methoxytryptamine*	28.0	31.7
Metanephrine	23.0	8.0
3-Methoxytyramine	46.0	17.0
4-Methoxy-3-hydroxyphenethylamine	68.0	20.0

*Chromatograms of these compounds were only obtainable at a voltage of +0.94 V vs. SCE.

and, thus, forms a major portion of the solvent front (t_R=2.5 min). Aromatic amino acids, including 3,4-dihydroxyphenylalanine and 5-hydroxytryptophan, are all in the zwitterion form at the pH of the eluting solvent. Thus, they also elute on the solvent front. Tyrosine and tryptophan, incidentally, are not observed because of their inability to be oxidized at this potential. Both 3,4-dihydroxyphenethyl alcohol and 3,4-dihydroxyphenyl glycol (neutral metabolites) elute under the solvent front. 3,4-Dihydroxyphenylacetic acid and 3,4-dihydroxymandelic acid, acidic catecholamine metabolites, also appear under the solvent front.

Biogenic amines related to DA and 5-HT comprise another group examined. Norepinephrine (NE) is slightly separated from the solvent front. But because of the large tissue concentrations of ascorbic acid, NE is effectively buried and, unfortunately, not determinable. Epinine (N-methyldopamine) is completely separated (t_R=18.5 min) from the 5-HT peak (t_R=9.3 min) and the procedure could be utilized directly to determine this species in appropriate samples. Tryptamine is not detectable at the potential chosen; but chromatograms run at +0.94 V vs. SCE display a very broad peak for tryptamine at t_R=24.5 min. Epinephrine (E) elutes (t_R=5.2) almost directly between DHBA (t_R=4.0 min) and DA (t_R=6.2 min), but is rather poorly resolved (R=0.77 and 0.67, respectively). However, E is normally present in brain tissue in only very small amounts. Even if present

in equimolar concentrations with DA, E would contribute only ca. 6% to the measured peak height for the former. Thus, as long as E cannot be visually detected in the chromatography of samples, it will not present any major problems. Quantitative separation of DA and E can, if necessary, be accomplished by either diluting the citrate/acetate solution solvent or utilizing the column conditions reported by Refshauge.[47]

Methoxylated metabolites form the other major group examined. 3-Methoxy-4-hydroxyphenylalanine (3-O-methyl-DOPA) elutes with the solvent front as do the other amino acids. 5-Methoxytryptamine is not electrochemically active at the potential chosen; however, it does exhibit a broad, poorly resolved peak (t_R=28.0) at a potential of +0.94 V vs. SCE. Metabolites which are observed under the given conditions include normetanephrine (t_R=4.0 min), 3-methoxy-tyramine (3-MT, t_R=46 min), 4-methoxy-3-hydroxy-phenethylamine (t_R=68 min), and metanephrine (t_R=23 min). Of these, only normetanephrine, which is isographic with DHBA, is of concern. The other species are clearly separated from the components of interest; also, their endogenous concentrations are generally so low that they are not observed in routine determinations. Normetanephrine (NMN), as the other methoxylated catecholamines, is only partially oxidized (ca. 15% relative to maximum obtainable) at the potential chosen. Additionally, NMN is present

in brain tissue at relatively low concentrations (65±4 ng/g, personal observation). Mouse brain determinations in which DHBA was omitted showed no discernible NMN peak at the sensitivity employed (less than 1% of the usual DHBA peak). Thus, NMN should not normally cause any significant interference.

Since both NMN and E overlap the chosen internal standard, other species were investigated for possible use in samples where either of these may pose problems. α-Methyldopamine (α-MeDA, t_R=18.5 min) was found to be quite adequate for this purpose. But, one should remember that this will increase the time required for individual determinations. Also, α-methyldopamine is isographic with epinine.

In short, however, there are no major known interferences in the procedure.

Development of Optimal Chromatographic Separation for NE/DA and 5-HT Analysis

As shown in the interferences study discussed immediately above, NE was "buried" under the "solvent" peak. A separation of NE, DA, and 5-HT was still desired to expand the capabilities of LCEC to include all three components simultaneously. Therefore, chromatographic conditions capable of attaining the adequate resolution of these species in a reasonable period of time were sought.

Preliminary investigations utilized Partisil-10 SCX* and the same pH 5.1 citrate/acetate buffer diluted to 40% of its original concentration by deionized water. These investigations showed all three of the biogenic amines to be strongly retarded and thus, adequately separated from the "solvent" peak. But, the individual amines were poorly resolved. Also, the use of the metal column, in which this packing was provided, in direct conjunction with the electrochemical detector contributed a substantial amount of electronic noise to the base line.

Therefore, it was decided to use the Partisil-10 SCX metal column in tandem with a glass column of 2 mm diameter by 250 mm length packed with Zipax SCX. This combination, with the use of the diluted (40%) pH 5.1 citrate/acetate buffer as an eluting solvent achieved the separation desired and the noise was considerably reduced. One shortcoming appeared to be the relatively low analysis time involved (ca. 32 min per injection). Yet, the separation was worth considering for direct injection of tissue homogenates.

The direct brain injection analysis included homo-

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*This is a cation exchange resin of 10 μm particle size. It contains a silica gel backbone with $-SO_3^-$ functional groups.[52] The column utilized was a metal column of 4.1 mm diameter by 250 mm length supplied by Altex Scientific Corporation, Berkeley, California. Unfortunately, this material cannot adequately be packed in a glass column due to the very small particle size.

genization of the brain tissue in 0.1 M $HClO_4$, centrifugation, and simple injection of a part of the supernatant into the chromatographic system. Such an assay would be very fast since it does not require any chemical manipulation, extraction, and/or adsorption. The selectivity and the sensitivity of the LCEC system makes such an assay appear at the outset to be quite feasible. Thus, we decided to investigate this procedure for the possible routine determination of the biogenic amines.

Unfortunately, the system utilized for direct analysis provided, in routine application, a far more serious problem than just finding the initial separation conditions. After only a minimal number of analyses, the cation exchange resin (particularly the Zipax SCX) was observed to significantly deteriorate as shown in Figures 3-1 and 3-2. Since ten (or less) injections was deemed an unacceptable column life, this approach was abandoned.

Simultaneous to this investigation we decided to try the same Partisil-10 SCX/Zipax SCX combination at a higher pressure* (ca. 2500 p.s.i.) coupled with a weaker ionic strength of the same citrate/acetate buffer (diluted 1:3 with deionized water). This attempt, although it provided the desired separation in a reasonable time as

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*For this investigation the pump utilized was a high pressure pump model 6000 from Waters Associates, Inc., Milford, Massachusetts. The pump was coupled to Model U6K injector from the same source.

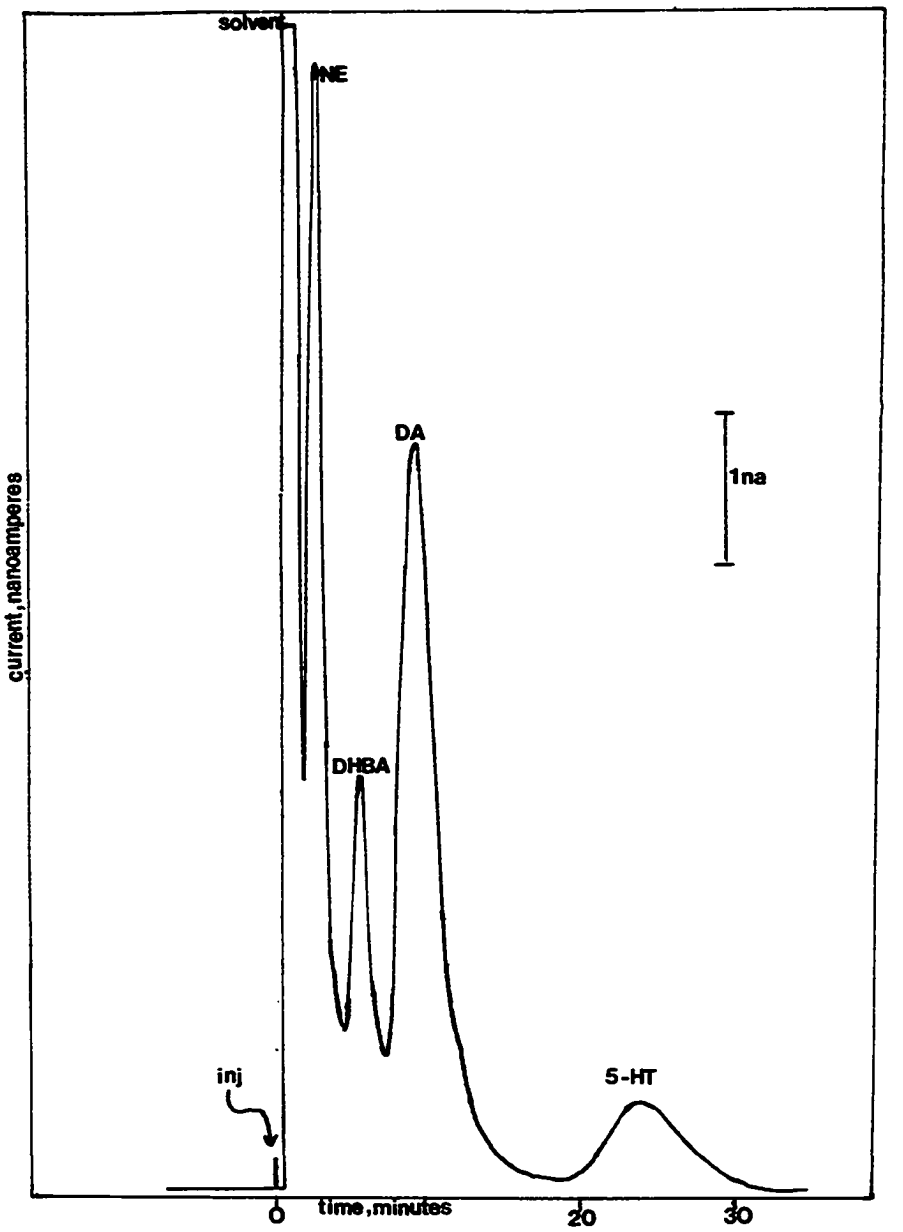

FIGURE 3-1. A typical whole mouse brain chromatogram, direct brain injection (50 µl of homogenate supernatant). Eluting solvent; citrate/acetate buffer diluted 2:3 with deionized water, electrode potential +0.60 V vs. SCE.

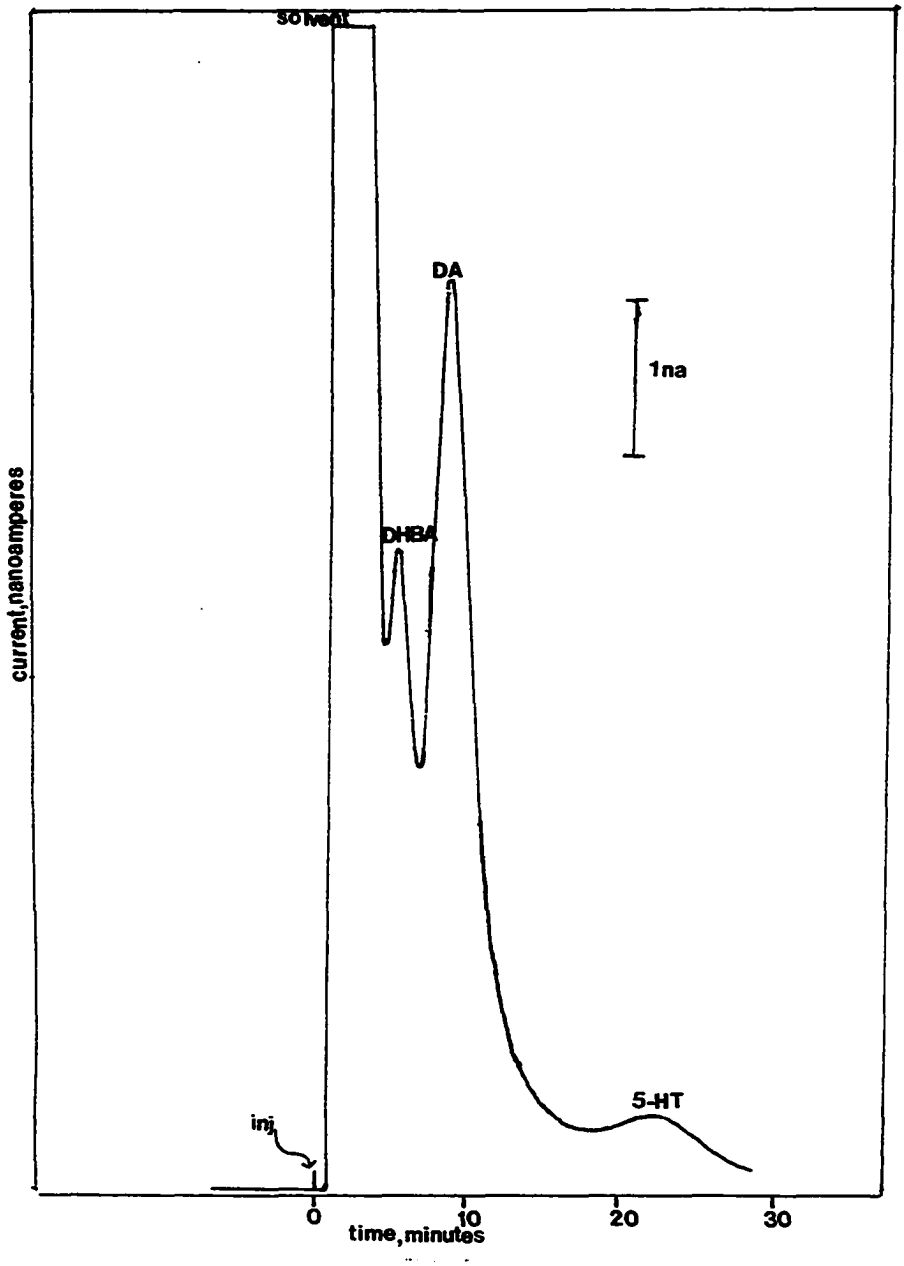

FIGURE 3-2. A whole mouse brain chromatogram after less than 10 direct injections (each was 50 µl of homogenate supernatant). Same conditions as Figure 3-1.

shown by the chromatogram of Figure 3-3, exhibited even more difficulties. With only the synthetic brain mixture of ascorbic acid, NE, DA, and 5-HT, it was obvious that the quantitation of NE would be impossible due to the anomalous solvent front behavior. Additionally, the base line exhibited an unpredictable and inexplicable amount of electrical noise which significantly decreased the sensitivity of the detector. Therefore, this high pressure approach was temporarily abandoned.

At this stage, it was obvious that the best resolution of the individual components was provided by the fluorocarbon based Zipax SCX resin. Yet, this same material was inadequate in providing sufficient separation of NE from the solvent front. The silica based strong cation exchangers (as represented by Partisil-10 SCX), on the other hand, elicited just the opposite behavior: adequate NE/solvent front separation but inadequate resolution of individual amines. And, the metal columns in which Partisil-10 SCX is obtained, led to unacceptable levels of background noise. Thus, we began to investigate other packing materials to replace the Partisil-10 SCX in the above Zipax/Partisil combination. All the materials examined were selected to have a particle size (30-40 μm or greater) sufficient to allow hand packing into glass columns, thus eliminating the metal noise problem.

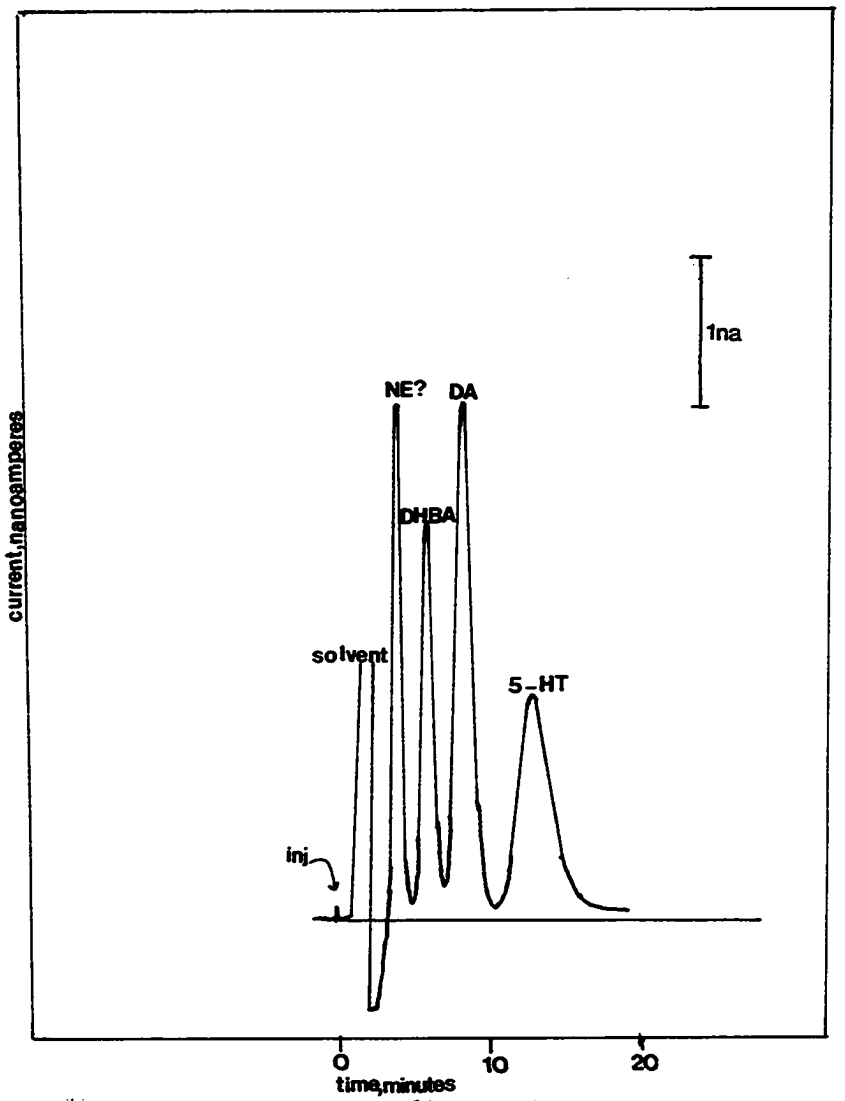

FIGURE 3-3. A typical LCEC chromatogram employing the high pressure system; 2500 p.s.i., flow rate ca. 2.5 ml/min, 50 µl injection. Eluting solvent was citrate/acetate buffer diluted 1:3 with deionized water.

High Capacity Pellionex SCX* proved to be inadequate for our purposes. The resolution of solvent front from NE as well as that for each of the individual amines was extremely poor. Eluting solvents tried included: the pH 5.1 acetate/citrate buffer, a pH 5.0 McIlvaine buffer,** and a 0.2 M NaH_2PO_4 solution of pH 4.2. Each of these attempts clearly showed that, in addition to the poor resolution noted, extremely long analysis times were required. Thus, Pellionex was discarded as a possible answer to our problem.

Another polystyrene-divinylbenzene copolymer packing tried was Vydac SCX (see Chapter 2). This packing, in combination with the pH 5.1 citrate/acetate buffer provided considerable success in the separation of the solvent front and NE peaks, yet it achieved a very poor resolution for the NE, DHBA, DA, and 5-HT peaks. Therefore, a combination of the two packings (Zipax and Vydac) was deemed worthy of investigation.

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*This is a cation exchange resin of 37-53 μm particle size. Its structure is a polystyrene-divinylbenzene copolymer backbone with $-SO_3^-$ functional groups.[52] It was supplied by Reeve Angel and Co., Inc., New Jersey.

**Prepared by dissolving 73.6 g $Na_2HPO_4 \cdot 12H_2O$, 20.4 g citric acid monohydrate, and 36.4 g NaCl in 1 liter of deionized water.

Optimal Chromatographic Conditions for NE/DA/5-HT Analysis

By employing various column lengths, flow rates, and ionic strengths of the mobile phase, we were able to optimize chromatographic conditions for NE/DA/5-HT analysis. These optimal parameters were as follows. The columns consisted of a 3 mm diameter by 250 mm length Zipax SCX unit in combination with a 2 mm diameter by 500 mm length Vydac SCX unit. The elution solvent was the pH 5.1 citrate/acetate buffer (see Chapter 2) diluted to 60% of its previous ionic strength with deionized water. The flow rate was reduced to ca. 0.6 ml/min resulting in a pressure of ca. 600 p.s.i. The potential was kept at +0.60 V vs. SCE.

Later application of these chromatographic conditions to the analysis of brain tissue clearly exhibited the applicability of these parameters to the quantitation of NE, DA, and 5-HT.

Unfortunately, under these conditions, 5-HT is relatively more strongly retarded by the Vydac SCX than the catecholamines NE, DHBA, and DA. Therefore, the total analysis time is ca. 22 min per sample. Yet, this time can be effectively halved by employing the dual parallel apparatus previously mentioned.[45] Operating with the dual system, thus, provides analysis of all three biogenic amines within a relatively reasonable period of time.

Investigation of Chromatographic Interferences in NE/DHBA/DA/5-HT Separation

Since the chromatographic conditions utilized for this separation were different from the DA/5-HT conditions, it was essential to reinvestigate the possible interferences. Table 3-2 lists the compounds investigated according to their elution order.

On the basis of conditions required for a compound to interfere (see previous investigation of DA/5-HT interferences in this chapter) the only compounds of concern are: normetanephrine (NMN, t_R=6.6 min), which elutes between DHBA (t_R=5.5 min) and DA (t_R=8.0 min); metanephrine (M, t_R=16 min); 3-methoxytyramine (3-MT, t_R=27.0 min); and epinine (t_R=15.0 min). However, NMN and M both are again only partially oxidized (ca. 15% of the maximum possible) at the potential chosen. Additionally, NMN and M are present in brain tissue at relatively low concentrations (NMN=65±4 ng/g, personal observation, and M is not detectable[55]). 3-MT elutes directly after 5-HT, yet its peak height in brain tissue samples is normally less than 5% of those of the biogenic amines and, thus, causes no major interference. 3-MT can be quantitated, although the analysis time will be extended to 30 min. A typical whole mouse brain chromatogram with 3-MT is provided later. In short, however, there are no significant interferences under these optimal chromatographic conditions.

Table 3-2

Potential Interferences Examined[a]

Compound	Retention time t_R (min)	Peak width (min)
3,4-Dihydroxyphenethyl alcohol	2.4	1.2
3,4-Dihydroxyphenyl glycol	2.3	1.1
3,4-Dihydroxyphenylacetic acid	2.3	1.2
3,4-Dihydroxymandelic acid	2.3	1.2
3,4-Dihydroxyphenylalanine (DOPA)	2.3	1.2
L-3-O-Methyl DOPA	2.3	1.1
5-hydroxytryptophan	2.3	1.2
Norepinephrine	3.5	1.0
Dihydroxybenzylamine	5.5	2.0
Normetanephrine	6.6	2.3
Dopamine	8.0	3.0
5-Hydroxytryptamine	20.0	5.0
Tryptamine[b]	31.0	30.0
Epinine (N-methyldopamine)	15.0	5.0
Metanephrine	16.0	6.0
5-Methoxytryptamine[b]	21.0	33.0
3-Methoxytyramine	27.0	6.0
4-Methoxy-3-hydroxyphenethylamine	41.0	13.0

[a]All compounds (except two[b]) investigated with the column conditions described in the text and used for routine analysis.

[b]Chromatograms of these two compounds were only obtainable at a voltage of +0.94 V vs. SCE.

B. Isolation Procedure

In the previous investigation of brain tissue analysis based on direct injection into the LCEC it was noted that the Zipax SCX column packing deteriorated after only a few injections. This deterioration is assumed to be due to some endogenous tissue components which can "poison" the chromatographic column packing. Therefore, a procedure for the isolation of these biogenic amines from these endogenous components was necessary.

The isolation of catecholamines NE and DA (but not the indoleamine 5-HT) by adsorption onto alumina at pH=8.6 is known to be highly selective.[41] Yet, it was thought that 5-HT might also be isolated by Al_2O_3 adsorption at pH < 8.6. Thus, alumina adsorption followed by acid elution was attempted as a method to extract all three of the biogenic amines NE, DA and 5-HT from synthetic aqueous solutions of pH values ranging from 2 to 8. Chromatographic analysis of each of these solutions after adsorption showed that 5-HT could not be adsorbed onto alumina from aqueous solutions.

Another possible attack of the 5-HT isolation and purification focused upon extraction into a non-aqueous phase, drying the solvent, and subsequent adsorption onto alumina. Although this would conceivably involve more time per analysis, it would also be projected to provide more selectivity. Elution from the nonaqueous Al_2O_3

would be followed by LCEC analysis. Similar isolation of 5-HT by alumina column separation had already been accomplished using methanol as a solvent.[56] Feasibility of this adsorption was also partially supported by the existent data obtained for phenolic compounds (5-HT is a phenolic species) by Higgins and Richards.[57] Using their data for phenol, and assuming a nonaqueous volume of 25 ml containing ca. 1×10^{-7} M solute (comparable to the contents of a single rat brain), the calculated recovery would be approximately 100% utilizing heptane as the solvent and 50% utilizing ethyl acetate as the solvent per gram of Al_2O_3. Therefore, this method appeared worthy of investigation.

A 10^{-6} M serotonin solution was prepared. Two mls of this solution were added to 40 mls of ethyl acetate in a separatory funnel and shaken for 20 min. After shaking, the aqueous layer (lower layer) was removed and the ethyl acetate layer was dried by shaking with ca. 0.5 g of anhydrous magnesium sulfate for ca. 10 min. The ethyl acetate layer was filtered, and 10 mls of this filtrate were shaken with 0.1 g of acid washed alumina,[41] for 30 min. The alumina was then dried at room temperature in a desiccator under vacuum for 45 to 60 min. The dry alumina was shaken for 2 to 5 min with 200 µl 0.1 M $HClO_4$ to elute any 5-HT possibly adsorbed. Volumes of 10 to 50 µl of this eluent were injected into the LCEC, but no 5-HT was

detected indicating no adsorption on alumina. This experiment was repeated with nitrobenzene and chloroform as solvents, but the results again indicated no adsorption of 5-HT on Al_2O_3.

Isolation of NE, DA, and 5-HT by chromatographic separation using strong cation exchange resins such as Dowex-50, Amberlite CG-120, or Duolite C-25[1] was principally rejected since that would just be two successive treatments with such resins when used in conjunction with the LCEC.

Finally, it was realized that a very highly selective method of isolation was simply not obtainable. Therefore, an investigation of the less selective Shore and Olin[38] butanol extraction procedure was undertaken. This procedure can be briefly described as [1] aqueous sample to n-butanol extraction (volume ratios, ca. 1:10 aqueous to butanol), [2] removal of the butanol layer and addition of heptane to butanol (volume ratios ca. 1:1.7, butanol to heptane) followed by [3] back extraction into an acidic aqueous solution. Naturally, this would be followed by injection of the final aqueous solution into the LCEC apparatus.

Although the Shore and Olin[38] extraction procedure was projected to be less selective than, e.g., the Al_2O_3 procedures would have been, many factors led us to believe it would still form a quite selective analytical procedure when combined with the LCEC. These

factors include: (1) the selectivity of the electrochemical detector at the potential chosen (described in Chapter 1), (2) the utilization of cation exchange resin with a mobile phase of pH 5.1 would cause all the neutral and acidic metabolites extracted from brain tissue to elute before any of these amines (see Chromatographic Interferences, this chapter). These factors, added to the ease and rapidity of this isolation procedure, made it a method definitely worthy of investigation.

However, the Shore and Olin extraction procedure was previously utilized only in combination with fluorescence as a detection technique. Since we intended to employ it with LCEC, an intensive examination of the parameters involved was undertaken.

C. Optimization of the Extraction Procedure for DA/5-HT Analysis

The chromatographic separation of DHBA, DA, and 5-HT proved to be faster (10 min) than that of NE, DHBA, DA, and 5-HT (22 min). Therefore, the extraction conditions for DHBA, DA, and 5-HT was initially investigated in some detail. The reinvestigation of only the appropriate parameters for the extended NE/DHBA/DA/5-HT extraction were then undertaken.

In the original method of extraction,[38] the authors did not specify the time required for the extraction into butanol in the case of standard solutions. However, they

concluded that the standard solutions should be shaken for a period less than that required for the brain tissue to minimize losses due to oxidation. Therefore, the following study of the time of initial extraction was carried out.

A solution containing 500 µl of the working standard (containing DHBA, DA, and 5-HT, see Chapter 2), 1.75 ml of the acetate/citrate buffer, and 200 µl of 10^{-5} M DHBA was prepared. 50 µl samples of this solution were injected into the LCEC to establish the peak heights prior to extraction. Then 1.20 ml of the mixture (pH 5.1) was transferred to a 30 ml screw-cap vial containing 12.0 ml of purified butanol and 1 g NaCl. Separate samples were shaken for 5, 20, 40, or 60 minutes. A 10.0 ml pipet of the butanol layer was transferred to a second vial containing 17.0 ml purified heptane and 500 µl of 0.01 M HCl. After shaking 5 minutes and allowing 10 minutes for the layers to separate, a 50 µl aliquot of the HCl layer was injected into LCEC. The percent recovery was established by measuring the absolute peak heights, comparing these to the same for the non-extracted samples, and correcting for dilution as previously described. The results are given in Table 3-3. The optimal time appears at 20 minutes. This value was chosen for all subsequent investigations of standard solutions.

Brain tissue is known to contain large amounts of easily oxidized molecules like ascorbic acid. These mole-

Table 3-3

Percent Recovery of Amines as a Function of Initial Shaking Time--Standard Solutions[a]

Shaking time, min	Recovery, % DA	5-HT	DHBA
5	47.8±0.9	47.2±1.0	42.0±0.7
20	48.3±1.9	48.8±1.9	42.8±0.8
40	43.7±0.2	46.9±0.3	40.9±0.2
60	40.6±1.5	43.1±3.9	42.8±2.2

[a]Purified butanol and heptane used. All results expressed as mean of at least six separate determinations ± SEM.

cules act as antioxidants providing protection to the biogenic amines. Therefore, in an attempt to minimize losses due to oxidation, the addition of ascorbic acid and EDTA was studied. 10 µl of ascorbic acid (11 mg/ml) and 100 µl of 0.1 M EDTA were added to the previous solution prior to extraction. The results are presented in Table 3-4.

Comparing this traditional ascorbate/EDTA treatment to the results of Table 3-3 points to a better recovery for the addition case. In a separate experiment, 50 µl of 1 M $NaHSO_3$ replaced the ascorbate as a possible antioxidant. However, the $NaHSO_3$ caused a significant loss in 5-HT recovery, as previously reported with the similar metabisulfite by Welch and Welch.[26] The improved recoveries in the case of EDTA and ascorbic acid addition,

Table 3-4

Percent Recovery of Amines When Extracted in Presence of EDTA and Ascorbic Acid[a]

DA	5-HT	DHBA
48.3±0.3	52.9±0.8	50.5±0.8

[a]Purified butanol and heptane used. Initial shaking time 20 min. All results expressed as mean of six determinations ± SEM.

however, do not necessarily mean that these species act solely as antioxidants. The results may also indicate an effect of these additives on the distribution coefficients of the amines in the extraction process. Nonetheless, the addition was included in all subsequent investigations because of the improved recoveries.

In fluorometric analyses of biogenic amines, repeatedly washed butanol and heptane[38] or the expensive, spectroscopically pure reagents, must be utilized.[26] This extreme purity is required to eliminate interferences. LCEC, on the other hand, should not be sensitive to such components. Thus, we examined the effect of using simple reagent grade butanol and heptane for extraction instead of their tediously purified counterparts. The results are given in Table 3-5.

Although the purified reagents do display a significantly better recovery for each of the components ($P < 0.02$, $n=5$) the relatively small difference (6 to 8% increase)

Table 3-5

Percent Recovery of Amines for Purified and Unpurified Butanol and Heptane Extraction Solvents[a]

Solvent	DA	5-HT	DHBA
Non-purified	48.1±0.3	46.6±0.3	41.4±0.3
Purified	52.8±0.8	50.5±0.8	44.8±0.2

[a]Purified butanol and heptane prepared by successive washing with 1 M HCl, 1 M NaOH, and deionized water according to Shore and Olin.[38] Unpurified solvents were reagent grade butanol and Baker Analyzed heptane. All results expressed as mean ± SEM for five separate determinations. $P < 0.02$ for each compound.

was not deemed enough to justify the time-consuming purifications. Thus, reagent grade butanol and heptane were utilized for all the following investigations.

Another important factor that determines the sensitivity of the technique used is the volume injected in LCEC. Optimization of this volume maximizes the detector response. It is desirable that the largest possible volume be injected. But, if the volume becomes too large, band spreading will destroy the essential separation of components. Therefore, we investigated the resolution of adjacent peaks for various injected volumes of a mixture composed of 500 µl of the working standard, 100 µl of 10^{-5} M DHBA, and 10 µl of ascorbic acid (solvent front). The results are presented in Table 3-6.

The results display adequate (> 1) resolution for

Table 3-6

Resolution Between Adjacent Chromatographic Peaks as a Function of Injected Volume

Injection volume, µl	Resolution		
	Solvent front/ DHBA	DHBA/DA	DA/5-HT
10	1.5	1.5	1.3
25	1.5	1.4	1.2
50	1.3	1.3	1.2
75	1.1	0.87	1.1

all pairs of peak except the DHBA/DA combination at 75 µl. Figure 3-4 shows the separation obtained for 50 and 75 µl. This lack of resolution at larger volumes complicates the calculations of results, so 50 µl was chosen as the optimal injection volume. However, it is also desirable to have this 50 µl contain the largest possible concentration of biogenic amines to maximize the LCEC response. A theoretical examination of the (1) aqueous to butanol and (2) butanol/ heptane to aqueous extractions shows optimal results will be provided if the smallest possible volumes of the initial and final aqueous solutions are used.

For the initial aqueous solution a minimum volume consists of 100 µl of 0.1 M EDTA, 100 µl of 10^{-5} M DHBA, 750 µl of acetate/citrate buffer and a 500 µl working standard aliquot. Selecting smaller volumes produces

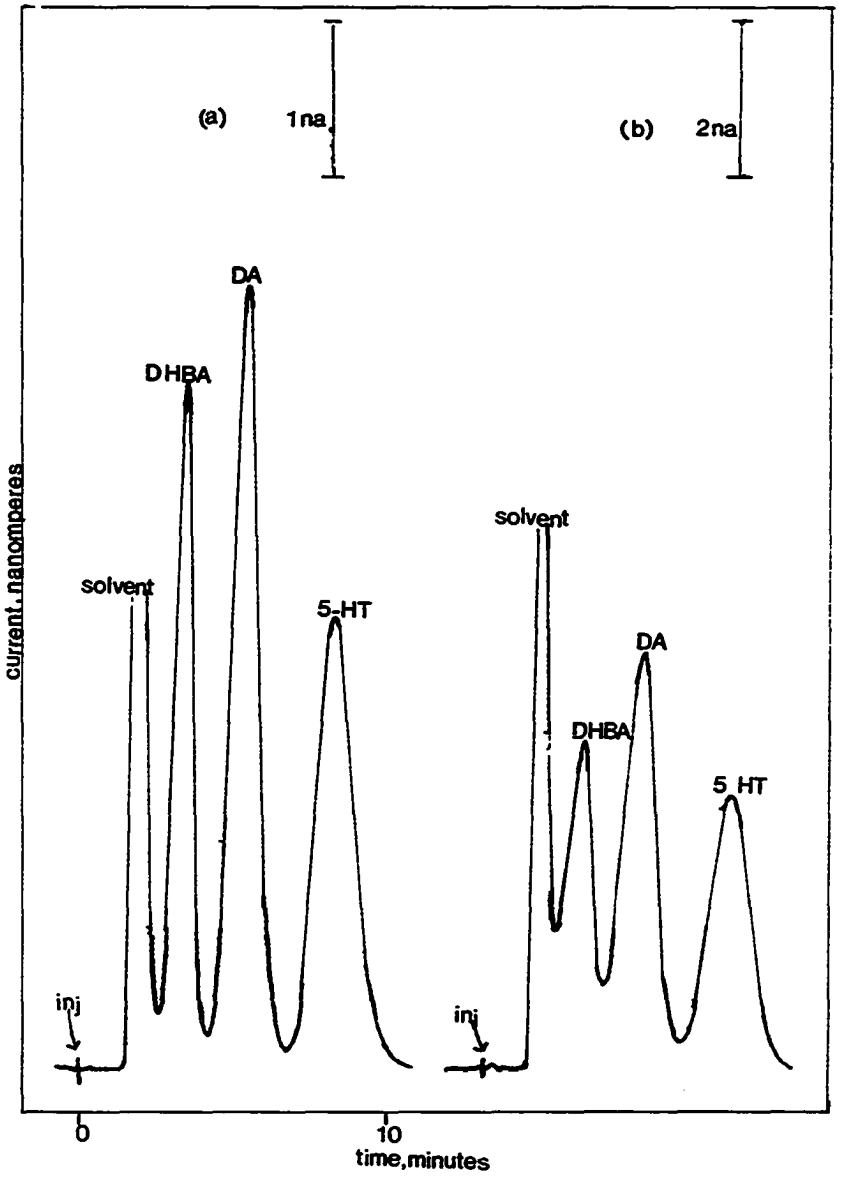

FIGURE 3-4. Separations obtained with (a) a 50 µl injection and (b) a 75 µl injection of the same DHBA/DA/5-HT mixture described in text.

samples which are too viscous to homogenize and manipulate for the brain tissues (see Chapter 4). The 0.01 M HCl solution in the final extraction was reduced from 500 to 200 µl. Smaller volumes are physically difficult to handle. It should be noted that this decrease in final volume causes a definite decrease in the absolute percent recovery; but the amount of biogenic amine (nmol or ng) contained in the 50 µl LCEC injection is increased.

Using the modifications noted immediately above, an investigation of the effect of the pH of the initial aqueous solution of standards (measured just prior to extraction) was undertaken. The results are given in Table 3-7.

As can be seen from Table 3-7, the percent recovery for each of the amines is fairly constant over the pH range of 1.1 to 5.2. This result agrees well with the report of Shore and Olin.[38] It also shows that if the standards or samples should become a bit too acidic prior to their initial extraction, the final result would not be very adversely affected.

All the previously described investigations were performed in the absence of tissue. Thus, some examinations were deemed necessary in presence of brain tissue. Two parameters we felt would be of importance were the initial and final times of extraction for brain samples. These had been studied by Shore and Olin[38] and showed a maximal

Table 3-7

Percent Recovery as a Function of Initial Aqueous pH Value[a]

pH	Recovery, %		
	DA	5-HT	DHBA
1.1	29.9±1.1	21.4±1.1	23.0±0.6
2.7	35.5±0.3	25.2±0.4	26.2±0.7
3.2	33.2±0.5	24.1±0.6	23.3±0.4
5.2	32.9±0.6	25.4±0.7	24.2±0.3

[a]All values are reported as mean ± SEM for at least six separate determinations.

recovery when the shaking time for the initial extraction into butanol was extended to 1 hour and the time for the final extraction was 5 min. We repeated this experiment at shaking times of 5, 20, 40 and 60 min. Indeed, our results also displayed significantly higher recoveries for brain tissue at 60 min. The shaking for the second extraction (butanol/heptane to 0.01 M HCl) was also examined at 5, 20, 40 and 60 min; but this later time did not significantly effect the results. So, 60 min initial shaking and 5 min final shaking times were used for all tissue samples.

There is no a priori reason to believe that extraction of the biogenic amines is the same in the presence of a considerable amount of tissue as in its absence. Therefore, a study of recoveries in the presence and absence of tissue was carried out as follows. A brain homogenate

was prepared by sonicating ten mouse brains in a mixture of 1.00 ml of 0.1 M EDTA, 7.50 ml of 0.025 M HCl, and 1.00 ml of deionized water (to replace the usual DHBA). A 1.00 ml pipet of the homogenate was transferred to each of ten separate vials. To five of these homogenate samples (Group A) was added a 50 µl aliquot of a solution containing 2.8×10^{-6} M DHBA, 4.4×10^{-6} M DA, and 3.0×10^{-6} M 5-HT. The second group of five homogenate samples (Group B) remained untreated. Five additional vials (Group C) were then prepared each containing 100 µl of 0.1 M EDTA, 10 µl of 11 mg/ml ascorbic acid, 900 µl of the citrate/acetate buffer, and 50 µl of the same solution of DHBA, DA and 5-HT.

After salt saturation, all fifteen vials were subjected to the extraction procedure outlined above. The DHBA, DA, and 5-HT mixture, appropriately diluted, was injected to establish peak heights prior to extraction. Samples containing only the amines (Group C) were used to determine percent recovery in the absence of tissue. Percent recovery in the presence of tissue was determined by subtracting the samples containing only brain (Group B) from those containing both brain and standard addition (Group A) and comparing to the data obtained from the unextracted samples.

The results are presented in Table 3-8. DHBA and DA produced results completely comparable in both cases. However, 5-HT yielded significantly lower recovery in the

presence of brain tissue than in its absence (19.5% compared to 25.2%, $P < 0.001$, n=8). Thus, when using the procedure outlined for tissue samples, the final 5-HT results must be multiplied by 1.29 (=25.2/19.5) to obtain the proper results.

Table 3-8

Percent Recovery in Presence and Absence of Brain Tissue[a]

	Recovery, %		
	DA	5-HT	DHBA
In presence of brain tissue	31.0±0.7	19.5±0.7	25.2±0.7
In absence of brain tissue	31.3±0.8	25.2±0.6	24.3±0.6

[a]All results in presence of brain tissue represent eight separate determinations. All results in absence of tissue represent thirteen separate determinations. Results expressed as mean ± SEM. Recoveries significantly different only for 5-HT ($P < 0.001$).

The reason for this difference in percent recovery of 5-HT between standards and tissue samples is not clearly understood. This anomaly could be due to two possibilities, however, which may either be operating separately or together.

The first of these possibilities is that the brain homogenates contain, in addition to ascorbate, other substances which protect the amines during the solvent extraction procedure. Thus, the brain samples would more

effectively retard the oxidation of catecholamines and indoleamines in the tissue samples as compared to the standards. This explanation is based on the assumption that there are two competing processes involved in maximizing the recovery. The first process encompasses a slow equilibrium between the aqueous and nonaqueous phase (in the aqueous to butanol extraction) and is maximized by increasing the time of extraction. The second involves the oxidation of the extracted compounds and is maximized by decreasing the time of extraction.

The second explanation could be that 5-HT in the brain tissue samples is in fact bound in some way. Thus, we may be encountering a very slow equilibrium, kinetically controlled, which first allows the release of serotonin from the binding (be it protein[58] or vesicular in nature) into the aqueous medium, coupled with the possible slow equilibrium between the aqueous and nonaqueous phases during extraction. This would explain the long time required for maximum recovery. Again, oxidation would be the limiting factor at very long times, even though it might be a relatively minimal process in the short run.

Trying to protect the biogenic amines from oxidation during extraction, we decided that shaking at 15°C might minimize losses. Thus, we examined the percent recovery of the three amines DHBA, DA, and 5-HT with shaking at 15°C and room temperature (21-25°C). Samples were run both in

the presence and absence of brain tissue. In all cases, the lower shaking temperature produced significantly better recoveries. Individual results were relatively some 11-22% better for those samples isolated at 15°C than those isolated at room temperature. However, shaking at temperatures <15°C did not improve the recovery.

D. Final Assay Procedure for DA and 5-HT Determination

Based on the individual optimizations of both the extraction procedure and the LCEC response, we arrived at the optimal procedure for the determination of whole mouse brains having a typical weight of 450-500 mg. This procedure is reported here in its entirety for easy reference.

The mice were sacrificed by cervical dislocation. The brains were removed as rapidly as possible, frozen in liquid N_2, and stored on dry ice. Each brain was weighed to the nearest mg and transferred to 30 ml screw-cap vial. The vials were kept on dry ice during the addition of all reagents. The following solutions were added to each vial: 100 µl of 0.1 M EDTA, 100 µl of 10 M DHBA, and 750 µl of 0.025 M HCl. While the concentration of DHBA used need not be accurately known, the same solution should be used throughout a given analysis for both brains and standards.

The cold (but not frozen) mixture was subjected to ultrasonic homogenization for ca. 20 sec. resulting in a homogenate pH of ca. 5.1. Salt saturation was accomplished

by adding 1 g of NaCl. A 12.0 ml aliquot of n-butanol was added to the vial and the samples were shaken for 60 min. A 10.0 ml aliquot of the butanol layer was then removed and placed in a second 30 ml screw-cap vial containing 17.0 ml heptane and 200 µl of 0.01 M HCl. The second bottle was shaken for 5 min and the aqueous layer was allowed 10 min to separate. The aqueous layer was then transferred by a small disposable pipet to a 3 ml vial. After removal of the remaining traces of the nonaqueous layer, a 50 µl sample of the HCl solution (or a volume compatible with the sensitivy of the EC system used) was injected into the LCEC apparatus using the optimal chromatographic conditions described previously. If the injection was not performed directly after extraction, the final HCl solution was stored on dry ice until a few minutes before injection. A typical chromatogram of whole mouse brain is presented in Figure 3-5.

Working standards (0.500 ml each) were treated exactly as the brain samples and were always injected into the LCEC on the same day of analysis. The only differences for standards were: (1) the initial solution, prior to homogenization, utilized 750 µl of the acetate/citrate buffer instead of the 0.025 M HCl to make the pH comparable to that of a brain sample at this stage; (2) 10 µl of the ascorbic acid was added to the homogenization mixture to mimic brain ascorbic acid levels and to help prevent

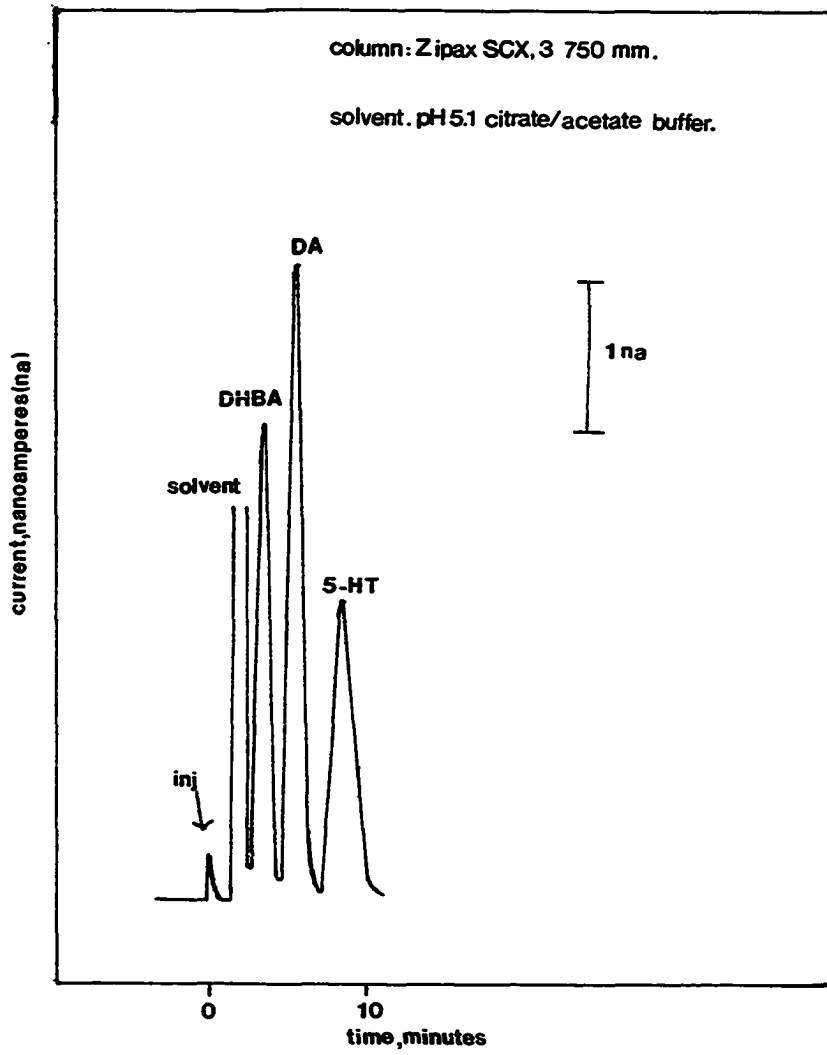

FIGURE 3-5. A typical whole mouse brain determination; 50 µl of final HCl solution containing ca. 14 ng/DHBA, 27 ng DA, and 18 ng 5-HT; flow rate ca. 1.3 ml/min; electrode potential +0.60 V vs. SCE.

oxidation of the amines; and, (3) the shaking time for the initial extraction into butanol was shortened to 20 min. Calculations of all results were performed as described in Chapter 2.

E. Optimization of the Extraction Procedure for NE, DA and 5-HT Analysis

The optimal extraction procedure as described in Section D of this chapter for DA/5-HT was determined to be partially unsatisfactory to achieve sufficient resolution of NE from the solvent front. This was mainly due to the incomplete separation of butanol and butanol/heptane layers from the aqueous layers. Such an incomplete separation was found to cause some physical carryover of the original homogenate into the butanol or butanol/heptane layer and, thus, into the final aqueous layer. This caused the occurrence of a very large solvent front which overlapped with NE. Therefore, solutions had to be centrifuged for 10 min at 7,900xg after the aqueous to butanol extraction and for 1 min at 121xg after the butanol/heptane to aqueous extraction.

Since addition of these centrifugation steps constituted a new procedure and since the percent recovery for NE was not established in the DA/5-HT analysis, a reinvestigation of percent recovery was deemed necessary for each component in the present method. The procedure

used to establish percent recovery is comparable to that employed in Section C of this chapter with some minor changes. However, it will be described in detail here for clarity.

A brain homogenate was prepared by sonicating ten mouse brains in a mixture of 1.00 ml of 0.1 M EDTA, 7.50 ml of 0.025 M HCl, and 1.00 ml of 0.01 M HCl (to replace the usual DHBA). A 1.00 ml pipet of the homogenate was transferred to each of ten separate 30 ml centrifuge tubes. To five of these was added a 50 µl pipet of a solution containing 2.1×10^{-6} M NE, 2.4×10^{-6} M DHBA, 3.7×10^{-6} M DA, and 3.2×10^{-6} M 5-HT. These five tubes will be referred to as group A. To another five, known as group B, a 50 µl pipet of 0.1 M HCl was added to equalize the volumes. Additional centrifuge tubes, constituting group C, were prepared with each containing 100 µl of 0.1 M EDTA, 10 µl of 11 mg/ml ascorbic acid solution, 900 µl of the citrate/acetate buffer, and 50 µl of the same standard solution of NE, DHBA, DA, and 5-HT.

Some of the NE, DHBA, DA, and 5-HT samples (group C) were injected to establish peak heights prior to extraction. Each mixture, whether or not it contained brain tissue, was subjected to ultrasonic homogenization for ca. 20 sec, resulting in a "homogenate" pH of ca. 5.1. Salt saturation was accomplished by adding 1 g of NaCl.

After salt saturation, a 10.5 ml aliquot of butanol

was added to each tube. The tubes containing brain tissue samples were shaken for 60 min, whereas those tubes containing standard solutions were only shaken for 20 min. The samples were then centrifuged at 7,900xg for 10 min at 4°C to completely separate the butanol layer from the aqueous layer.

After centrifugation, a 9.6 ml pipet of the butanol layer was removed and placed in a second 30 ml vial containing 17.0 ml heptane and 200 µl of 0.01 M HCl. The second tube was shaken for 5 min and centrifuged for 1 min at 121xg and 4°C. After removal of the nonaqueous layer, a 20 µl sample, or a volume compatible with the sensitivity of detector, was injected into the LCEC apparatus.

Samples which were carried through the extraction and contained only amines (group C) were used to determine percent recovery in the absence of tissue by comparison to the unextracted samples. Percent recovery in the presence of tissue was determined by subtracting the peak heights of the samples containing only brains (group B) from those containing both brain and standard addition (group A) and compared to the unextracted results.

The results are presented in Table 3-9. NE, DHBA, and DA produced results completely comparable in both the presence and absence of tissue. However, 5-HT again yielded significantly lower recovery in the presence of brain tissue than in its absence (22.8% as compared to

Table 3-9

Percent Recovery in Presence and Absence of Brain Tissue[a]

	Recovery, %			
	NE	DHBA	DA	5-HT
In presence of brain tissue	36.1±0.6	36.0±0.9	42.6±1.1	22.8±0.2
In absence of brain tissue	36.8±0.4	35.8±0.9	41.4±1.0	29.4±0.3

[a]All results represent eight separate determinations. Results expressed as mean < SEM. Recoveries significantly different only for 5-HT ($P < 0.001$).

29.4%, $P < 0.001$, $n=8$). Thus, we still need to multiply the final 5-HT results by 1.29 to obtain the proper results for this analysis.

It should be noted that in this particular investigation, we utilized a 10.5 ml aliquot of butanol in the initial extraction and transferred 9.6 ml of this solvent to the second vial. This initial volume (10.5 ml) was chosen to make the butanol to aqueous ratio 10:1. However, we found that in performing the routine analyses it was easier to utilize 12.0 ml of the butanol, of which 10.0 ml was then transferred to the second vial.

There was some concern about the stability of the biogenic amines in the final extract (0.01 M HCl), especially after observing that simply replacing Mallinckrodt n-butanol by the Baker Analyzed product caused the peaks to be non-gaussian in character and the amines to be highly

unstable. Therefore, a study of the stability of this extract was deemed necessary in the presence and absence of brain tissue after storage at room temperature and on dry ice. This study was carried out as follows. In separate centrifuge tubes, three brain homogenates were prepared by sonicating each of three mouse brains in 100 µl of 0.1 M EDTA, 750 µl of 0.025 M HCl and 50 µl of the same standard solution as used in the percent recovery study. Each homogenate was salt saturated by adding 1 g of NaCl. In the same time, 500 µl samples of the standard was treated in the same manner as the individual brains.

After salt saturation, the vials were subjected to the extraction procedure outlined above. Immediately after extraction, 20 µl of the final extract was injected. A second 20 µl injection was then made, after storage either at room temperature or on dry ice, at 1 to 24 hours following the first. Results for the room temperature samples after 1 hr are given in Tables 3-10 and 3-11.

Although there is some decrease in peak ratios after leaving the extract at room temperature, this decrease is only significant for NE in the absence of brain tissue. On the other hand, the similar study performed with storage on dry ice showed no decrease in all peak ratios after 24 hours. Therefore, in every assay performed in our laboratory, the final extracts were always kept on

Table 3-10

Study of the Stability of the Final Extract at Room Temperature (ca. 23°C) in Presence of Brain Tissue[a]

	Peak Ratios		
	NE/DHBA	DA/DHBA	5-HT/DHBA
Immediately after extraction	2.4±0.2	2.3±0.2	0.84±0.02
1 hr. after extraction	2.3±0.1	2.0±0.1	0.82±0.01
P-value	N.S.	N.S.	N.S.

[a]All values reported as mean ± SEM for six separate determinations.

Table 3-11

Study of the Stability of the Final Extract at Room Temperature (ca. 23°C) in Absence of Brain Tissue[a]

	Peak ratios		
	NE/DHBA	DA/DHBA	5-HT/DHBA
Immediately after extraction	1.54±0.02	1.38±0.02	0.51±0.02
1 hr. after extraction	1.44±0.02	1.32±0.03	0.50±0.02
P-value	P <0.01	N.S.	N.S.

[a]All values are reported as mean ± SEM for six separate determinations.

dry ice until 3 min before injection. Additionally, storage of the final extracts on dry ice never exceeded 24 hours.

F. Final Assay Procedure for the NE, DA, and 5-HT Determination

This assay was performed exactly in the same way as the DA/5-HT assay described previously in Section D of this chapter. The only differences are (1) the analysis should be accomplished in Teflon centrifuge tubes (see Chapter 2), (2) the solutions should be centrifuged after the aqueous to butanol extraction at 7,900xg and 4°C, (3) the solutions should be centrifuged at 121xg and 4°C after the heptane/butanol to aqueous extraction, and (4) the working standards utilized should contain NE, DA, and 5-HT in concentrations comparable to the brain (see Chapter 2).

Injections should be made into the LCEC system described for this assay in Section A of this chapter. A typical whole mouse brain chromatogram for the NE/DA/5-HT analysis is shown in Figure 3-6.

G. Modification of the NE/DA/5-HT Assay for the Analysis of Biogenic Amines in Brain Parts

Appropriate modifications were required to compensate for the differing amine concentrations of individual brain parts. However, in applying these modifications: (1) the ratio of the aqueous to the butanol phases in the

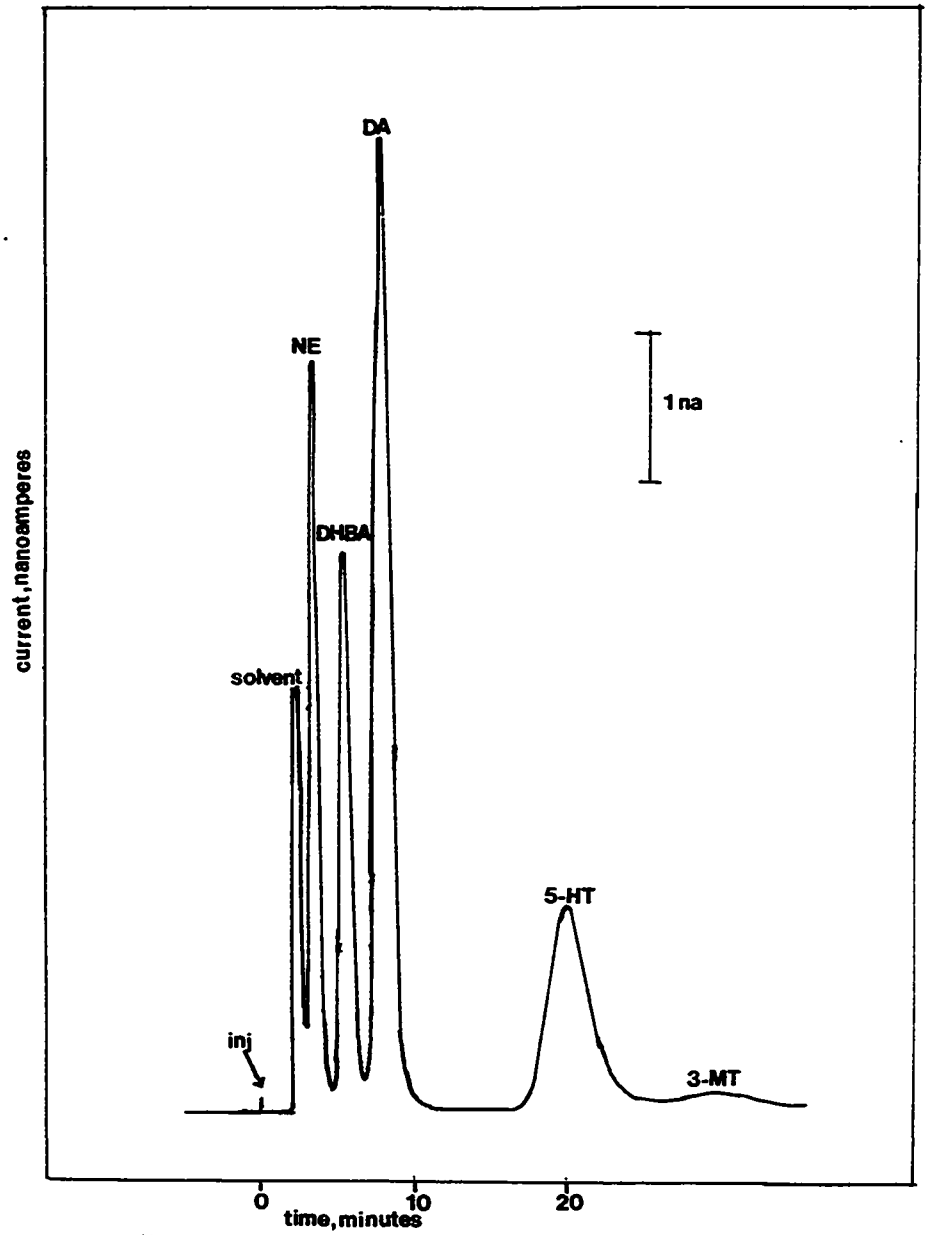

FIGURE 3-6. A typical whole mouse brain chromatogram, 15 µl injection.

initial extraction was always maintained at ca. 1:10; (2) the ratio of butanol to heptane was always maintained at ca. 1:1.75; (3) the volume of 0.01 M HCl solution in the final extraction was always 200 µl; (4) the volume of standards (in µl), run at the same time as the samples, was comparable to the sample weight (in mg); (5) the times of the first and second extractions were maintained at 60 and 5 min, respectively, for the samples, and 20 and 5 min for the standards; and (6) the concentration of DHBA, the internal standard, was appropriately reduced so that its peak height became comparable to those of the biogenic amines in the part analyzed. It should also be noted that the volumes of both the working standard solution and the DHBA solution used were chosen to be large enough (50 µl or more) such that reproducibility was easily obtainable.

Considering the remarks immediately noted above, the following assay procedure was deemed optimal for the determination of cerebellum, hippocampus, medulla-pons, midbrain and diencephalon. These brain parts had an average wt. of 25-50 mg.

The weighed brain part was transferred to a Teflon centrifuge tube. The following solutions were added to the tube: 10 µl of 0.1 M EDTA, 50 µl of 10^{-6} M DHBA, and 100 µl of 0.025 M HCl. The mixture was subjected to ultrasonic homogenization for ca. 10 sec. The homogenate was

salt saturated by adding 0.05 g NaCl. A 2.2 ml aliquot of butanol was added to the tube and the samples were shaken for 60 min. The tube was then centrifuged at 7,900xg at 4°C. A 1.5 ml portion of the butanol layer was then removed and placed in a second Teflon centrifuge tube containing 2.5 ml heptane and 200 µl of 0.01 M HCl. The second tube was shaken for 5 min and then centrifuged at 121xg and 4°C. The aqueous layer was transferred by a disposable pipet to a 2 ml vial. After removing the remaining traces of the butanol/heptane layer, a volume of the HCl solution compatible with the detector sensitivity was injected into the LCEC apparatus. The solution contents and volumes for these brain parts and their standards, as well as the two succeeding parts, are summarized in Tables 3-12 to 3-14.

Working standards for these brain parts (50 µl) were treated exactly as the brain tissue samples. The only differences were: (1) the initial solution prior to homogenization utilized 100 µl of the acetate/citrate buffer instead of the 0.025 M HCl; (2) 5 µl of the ascorbic acid solution (2 mg/ml) was added to the homogenization mixture; and (3) the shaking time for extraction into butanol was shortened to 20 min.

For the analysis of striatum (average wt. ca. 25 mg), 100 µl of 4×10^{-6} M DHBA was utilized to result in a peak height comparable to that of DA. This necessitated

Table 3-12

Solutions Added Prior to Homogenization for Brain Parts Analyses[a]

Brain part	0.1 M EDTA vol., µl	Conc.	DHBA M	vol., µl	volume (µl) 0.025 M HCl (samples) or buffer[b] (standards)[c]
Cerebellum	10	1	50		100
Hippocampus	10	1	50		100
Medulla-pons	10	1	50		100
Midbrain	10	1	50		100
Diencephalon	10	1	50		150
Striatum	10	4	100		350
Cortex	50	4	100		350

[a]The EDTA and DHBA solutions were acced to both the tissue samples and standards.

[b]Citrate/acetate buffer, pH=5.1.

[c]5 µl of ascorbic acid (2 mg/ml) was added to each standard except in the case of cortex where 5 µl of 11 mg/ml was added.

Table 3-13

Materials Used for Extraction in Brain Parts Analyses[a]

Brain part	Initial volume of butanol added, ml	Volume of butanol obtained after initial extraction ml	Volume of heptane added for final extraction, ml
Cerebellum	2.2	1.5	2.5
Hippocampus	2.2	1.5	2.5
Medulla-pons	2.2	1.5	2.5
Midbrain	2.2	1.5	2.5
Diencephalon	2.2	1.5	2.5
Striatum	3.2	2.5	4.2
Cortex	7.0	5.0	9.0

[a]In the second extraction, the butanol obtained after the first extraction was combined with the heptane and the amines were extracted into 200 µl of 0.01 M HCl. Numbers given refer to both the tissue samples and standards.

Table 3-14

Working Standards Used in Brain Parts Analyses of NE, DA, and 5-HT[a]

Brain part	Volume employed in analysis, µl	Concentrations NE(ng/ml)	DA(ng/ml)	5-HT(ng/ml)
Cerebellum	50	432	824	712
Hippocampus	50	432	824	712
Medulla-pons	50	432	824	712
Midbrain	50	432	824	712
Diencephalon	50	432	824	712
Striatum	50	335	1665	624
Cortex	200	432	824	712

[a]All standards were prepared in a deaerated solution of 0.01 M HCl.

adding 150 µl of 0.025 M HCl to the tissue sample or 150 µl of the citrate/acetate buffer to the standard solutions. Consequently, the volume of the butanol aliquot added was 3.2 ml, of which 2.5 ml was then pipetted into a second tube containing 4.2 ml heptane and 200 µl of 0.01 M HCl. Other procedural details for striatum remained essentially the same as those noted immediately above.

In the case of cortex (average wt ca. 200 mg), the volume of standards employed was 200 µl. Also, 100 µl of $4x10^{-6}$ M DHBA was utilized for both standards and samples. 350 µl of 0.025 M HCl was added to the tissue samples, and 350 µl of pH 5.1 acetate/citrate buffer to the standard solution. Therefore, the volume of butanol aliquot was chosen to be 7.00 ml of which 5.00 ml was pipetted into the second tube containing 200 µl of 0.01 M HCl and 9.00 ml of heptane.

The same assay procedures as those outlined above are directly applicable to the determinations of only DA and 5-HT. However, for the DA/5-HT determinations, one need not include the centrifugation steps after each extraction and the LCEC conditions used should obviously be the more rapid DA/5-HT conditions (not the longer NE/DA/5-HT ones used here).

CHAPTER IV

DETERMINATION OF DOPAMINE AND SEROTONIN IN MOUSE BRAIN TISSUE---APPLICATIONS

A. Whole Mouse Brain

The assay developed for the analysis of dopamine and serotonin, as described in Section D, Chapter 3, was applied to whole mouse brain analysis to demonstrate the general applicability of the procedure.

The result for a total of 40 samples is given in Table 4-1, along with the reported values of other investigators. As can be readily seen, the levels for both DA and 5-HT are well within the range of previously reported determinations. Yet, the present method yields both values simultaneously; no sample splitting, pooling, derivatization, or enzymatic reactions are required. The assay is quite simple and easy to perform.

Methods of Homogenization

During the development of this assay procedure, Alliger[64] reported that long exposure of biological samples to ultrasonic irradiation caused a destruction of the

Table 4-1

Whole Mouse Brain Dopamine and Serotonin Concentrations[a]

Investigators	Technique of Detection	DA,ng/g±SEM (n)	5-HT,ng/g±SEM (n)
Present Report	LCEC	973±16(40)	805±12(40)
Fleming et al.[59]	Fluorescence	770±6(13)	550±10(10)
Wiegand & Perry[60]	Fluorescence	870±46(12)	820±12(3)
Welch & Welch[26]	Fluorescence	700-900(1000)	600-800(100)
Smith[61]	Fluorescence	820±31(5)	660±13(5)
Agrawal et al.[62]	Fluorescence	1330±98(6)	410±16(6)
Weintraub et al.[63]	GCMS	800±30(5)	670±40(5)

[a]All results reported as mean ± SEM.

indole nucleus of tryptophan. Since 5-HT also contains an indole moiety, we compared ultrasonic homogenization to that performed with a ground glass apparatus. As presented in Table 4-2, there was no significant effect on the results.

Routine Application

With the present single column setup, it has been possible to run as many as 30 samples during a normal eight-hour day. This required that the tissue samples were already weighed at the start of the day. However, it also included sufficient time to clean all appropriate glassware while the chromatography was being run. When sacrificing and tissue removal were included, 20 samples

Table 4-2

Effect of Homogenization Method on Whole Mouse Brain Determination of Dopamine and Serotonin[a]

Method of homogenization	DA, ng/g	5-HT, ng/g
Ultrasonic	1023±60	837±44
Ground glass	985±30	810±23

[a]All values reported as mean ± SEM for five separate determinations. There is no significant difference between methods of homogenization for either DA or 5-HT.

could be easily accommodated in the same time period. The number of samples per day, however, may be increased significantly by utilizing the dual parallel LCEC apparatus[45] previously discussed.

Column Stability

In our laboratory, we utilized the same column of Zipax SCX for more than one year without any noticeable change in resolution. However, to extend the column life, we would recommend that, if the column is not to be used for a few weeks, it should be washed with deionized water and 0.01 M HCl. This will maintain the column at a pH of 2 and inhibit bacterial growth.

B. Mouse Brain Parts

The assay for the analysis of biogenic amines in mouse brain parts has been described in Section G, Chapter 3. This assay was applied to the analysis of brain parts

dissected from decapitated and from microwaved mice primarily to display the sensitivity of this method.

Rapid post mortem changes in the levels of a variety of important neurochemicals have been recently demonstrated. Unless the enzymes involved in the metabolism of these compounds are rapidly inactivated in situ, extraction and subsequent analysis of these compounds results in erroneous values. Rapid freezing techniques and the more rapid microwave irradiation have adequately demonstrated this for acetylcholine,[65,66] the cyclic nucleotides,[67] γ-aminobutyric acid,[68] and some high energy phosphate molecules.[69]

In the analysis of NE, DA, and 5-HT in whole mouse brains, the rapid enzymatic inactivation by microwave irradiation has previously been found to be unnecessary.[63] The present research was performed to see the effect of sacrificing by microwave exposure on the regional concentrations of these biogenic amines.

Sacrificing of the mice was accomplished by either (1) decapitation or (2) exposure to 6 kw of focused microwave irradiation at 2450 MHz for 250 msec. All animals were subjected to the following sequence: (1) sacrifice, (2) rapid brain removal and dissection into seven major parts,[50] (3) freezing and storage of the brain parts on dry ice until analysis, and (4) measurement of the biogenic amine levels in each part.

Results and Discussion

The results of the analyses for DA and 5-HT are presented in Table 4-3. Individual values represent the combination of two separate sets of data, each showing essentially the same results. As can be seen, the DA values in only the striatum are significantly larger for the microwaved animals than for the decapitated ones. 5-HT values are significantly higher for microwaved animals in five of the seven brain regions examined. These results were very disconcerting in view of the fact that whole brain biogenic amine values had previously[63] been shown to be unaffected by microwave vs. decapitation methods of sacrifice. Thus, we sought more extensive confirmation of this data.

An analysis of NE and DA, utilizing the alumina isolation procedure with LCEC,[50] completely verified the results for DA shown in Table 4-3 (i.e., microwaved and decapitated DA values only significantly different in the striatum). This NE/DA procedure also displayed no significant differences for NE values when microwaved and decapitated samples from all the parts were examined. Still remaining somewhat skeptical of the discovery of a DA and, possibly, a 5-HT "pool" which was so rapidly degraded after death, we fortified our results by two additional routes. First, we repeated the DA/5-HT analysis for striatum some five additional times, each verifying

Table 4-3

Dopamine and Serotonin Concentrations of Mouse Brain Parts[a]

Brain part	n[b]	Mean tissue weight (mg)	DA(ng/g) decapitated	DA(ng/g) microwaved	5-HT(ng/g)[c] decapitated	5-HT(ng/g)[c] microwaved
Cerebellum	9	52	19±2	23±2	125±4	293±15[d]
Hippocampus	11	26	65±8	94±22	520±19	747±28[d]
Medulla-pons	12	41	77±12	67±14	1023±46	1117±49
Midbrain	11	30	95±11	119±12	966±52	1231±94[f]
Diencephalon	10	41	199±21	225±20	889±90	1050±80
Striatum	10	22	4627±127	7790±210[d]	606±86	817±15[f]
Cortex	11	171	696±58	618±39	626±25	729±25[e]

[a]All values expressed as mean ± SEM. Decapitated and microwaved results are not significantly different (P >0.05) except where noted.

[b]Number of individual samples determined. In striatum, for example, there were 10 analyses each performed on the decapitated and microwaved animals.

[c]5-HT results corrected for percent recovery in presence of tissue by multiplying the final result obtained by 1.29.

[d]Significantly higher (P <0.001) results for microwaved animals.

[e]Significantly higher (P <0.01) results for microwaved animals.

[f]Significantly higher (P <0.05) results for microwaved animals.

the original. Secondly, we examined microwaved and decapitated mouse striatum by use of the GCMS method of Koslow et al.[36] Again, the original results for DA were confirmed.

In order to microwave animals, however, a quite rigid immobilization of the animals is required. For properly focusing the beam of radiation, the animal is typically placed in a modified 50 ml plastic syringe with the head exposed. The body and most of the head is then immobilized by "packing" the animal in this apparatus. The decapitated animals are typically not confined in this manner. But, this immobilization stress is certainly capable, by itself, of causing changes in the levels of biogenic amines. Thus, we examined the effect of immobilizing both the decapitated and microwaved animals prior to sacrifice. As shown in Table 4-4, the results are still significantly higher for microwaved animals ($P < 0.001$ for DA and 5-HT in striatum). Hence, immobilization stress is clearly not the source of the differences we observe.

At this stage, we were totally convinced that the microwave/decapitation differences were quite real and not simply due to analytical methodology or immobilization. But another possibility also existed, i.e., that the microwave irradiation could be somehow "releasing" some tightly bound form of DA or 5-HT. This tightly bound form could also be present in decapitated samples but was simply not

Table 4-4

Dopamine and Serotonin Concentrations in Mouse Striatum After Immobilization[a]

DA (ng/g)[b]		5-HT (ng/g)[b]	
decapitated	microwaved	decapitated	microwaved
8034±394	11134±262	673±29	927±35

[a]All values expressed as mean ± SEM, n=6. Both decapitated and microwaved animals were similarly immobilized just prior to sacrificing.

[b]P <0.001 for microwaved vs. decapitated samples in case of both DA and 5-HT.

measured due to its inaccessibility via the procedures chosen for analysis. Thus, we needed to examine a possible microwave artifact. The best method for testing this hypothetical artifact was determined to be through direct comparison of the striata of a normally microwaved group to a group that was first decapitated and then (5 minutes after decapitation) submitted to the same microwave treatment. In this test, the normally microwaved animals (DA= 9350±970 ng/g, n=9) were significantly greater (P <0.01) than those submitted to decapitation plus microwave treatment (DA=7750±130 ng/g, n=4). Additionally, a group of normally decapitated animals (DA=7720±610, n=12) were significantly different (P <0.01) from the normally microwaved group and not significantly different from the decapitated plus microwaved group. Thus, no microwave artifact can be operational in providing the results we

have obtained.

But what does the microwave treatment provide that is not present in decapitation? Surely, the most obvious characteristic of microwaving as a method of sacrificing animals is the phenomenal temperature rise it effects in the brain (from 37°C to 80-100°C in 200 msec). This temperature excursion virtually destroys all enzymatic activity through denaturation of the proteins. Knowledge concerning which enzyme is involved in the post mortem degradation of these amines could give us insight into the location of these highly labile "pools" that disappear between death itself (as measured in microwaved samples) and the time required for isolating the striatum (a time only relevant to the decapitated samples where the enzymes are still intact after death). As such, we decided to examine both DA levels and enzymatic activities for enzymes involved in DA metabolism as a function of time of microwave irradiation. Hopefully, the increase in DA seen between 0 msec (decapitated) and 200 msec of irradiation would closely match the loss of activity of one of the metabolic enzymes, thus allowing a correlation to be made. As presented in Table 4-5 and Figure 4-1, the DA appears to quite readily increase between 100 and 200 msec of microwave irradiation. The only enzyme examined to date, DOPA decarboxylase (DDC), does not show a good correlation with these DA results. In fact, some detectable amounts

Table 4-5

Concentrations of Dopamine in Striatum as a Function of Microwave Irradiation Time[a]

Microwave time (msec)	n	DA (ng/g)
0 (decapitated	12	7720±610
70	10	7760±640
100	11	7660±2020
150	11	8360±1190
200	9	9350±970

[a]All values expressed as mean ± SEM.

of DDC activity are already lost at only 70 msec of microwave treatment. We hope to obtain similar data for tyrosine hydroxylase, monoamine oxidase, and catechol-O-methyl transferase in the near future. However, until we do obtain such data, it is impossible to speculate upon the cellular localization of these "pools". Likewise, we hope to be able to more precisely pin down the location of the 5-HT pools via examination of the appropriate metabolic enzymes.

The present results do seem to display an apparently anomalous effect. When comparing microwaved to decapitated animals, NE shows no differences in all regions examined, DA is significantly different only in the striatum, and 5-HT is different in all but two of the seven areas. This same oddity, and its reproduction in subsequent experiments,

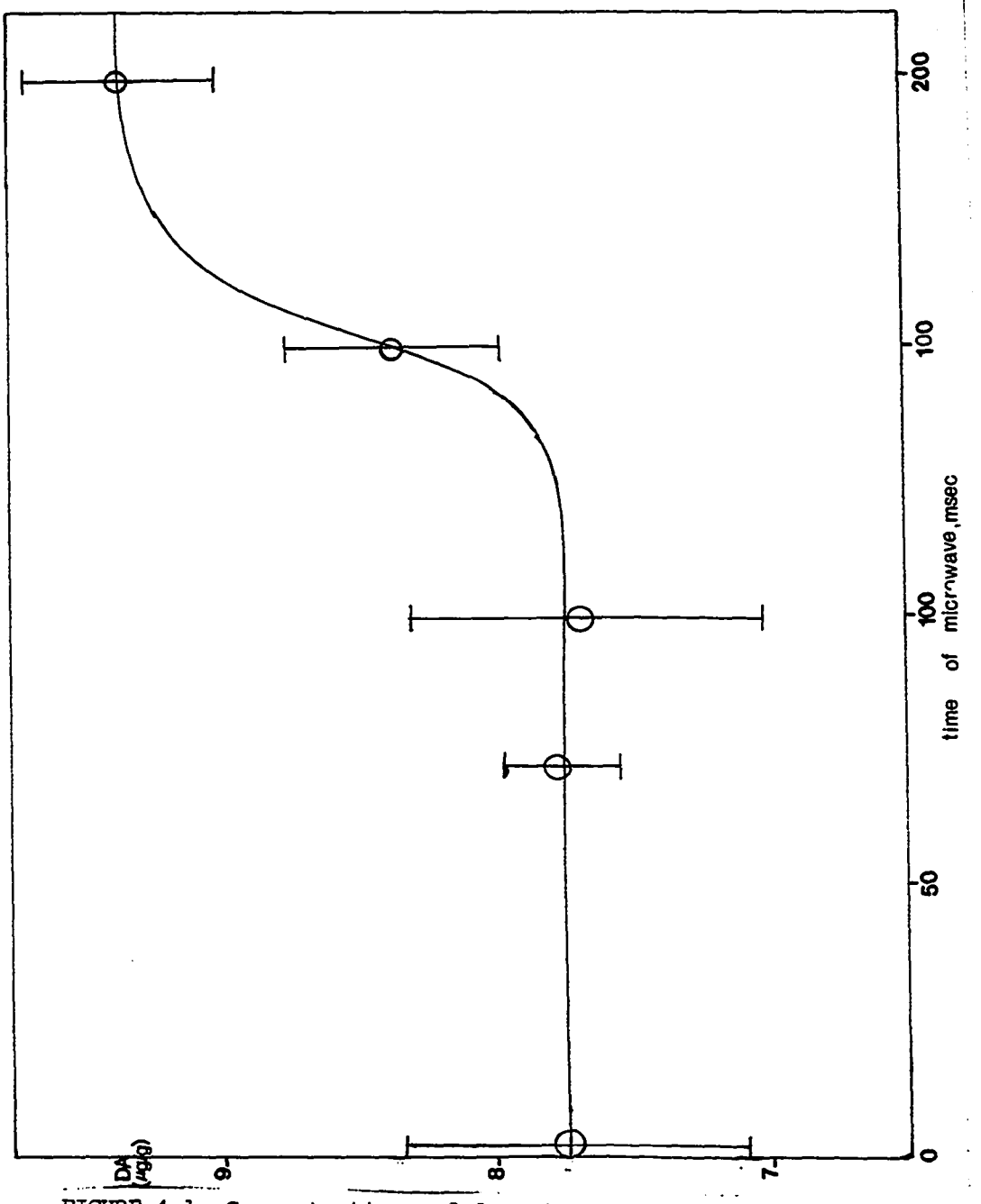

FIGURE 4-1. Concentrations of dopamine in striatum as a function of microwave irridiation time.

however, makes the differences, where they do appear, even more credible. Since these biogenic amines, by other techniques, typically yield half-lives on the order of hours, DA and 5-HT (in the regions showing significant differences) must also have "pools" or compartments with much shorter half-lives, i.e., minutes. Or, in these selected regions, these transmitters must be experiencing storage in multiple compartments.[70,71,72] While multiple compartmentation is a highly controversial issue, it has previously gained support for striatal DA by the long term post mortem studies in rat brain[73] and the 5-HT work of Grahame-Smith.[74] These multiple pools, it should be noted, may also exist in the regions showing no significant differences (microwave vs. decapitated) in the present study; but, they may be small enough that the instrumental and animal-to-animal variations simply camouflage their existence.

Hopefully, as mentioned above, future studies in this area will allow us to find which particular metabolic enzymes are involved in the rapid post mortem degradation of biogenic amines seen after decapitation. Since the predominate locations of each of these metabolic enzymes has been well established, we should then be able to more precisely locate these rapid turnover "pools" and, therefore, be able to more accurately assess their function.

CHAPTER V

DETERMINATION OF NOREPINEPHRINE, DOPAMINE, AND SEROTONIN IN MOUSE BRAIN TISSUE---APPLICATIONS

A. Whole Mouse Brains

The assay developed for the analysis of norepinephrine, dopamine, and serotonin is described in Section F, Chapter 3. This assay was also applied to the analysis of whole mouse brains to demonstrate its general applicability.

The result of 48 separate whole brain determinations is given in Table 5-1. Some literature values obtained by other techniques are given for comparison in Table 5-2.

The mean values for the present method agree very well with the literature values. Yet, this method is the first to quantitate all three of these biogenic amines simultaneously in the same tissue sample without splitting, pooling, derivatization or enzymatic reactions.

B. Daily Rhythms in Norepinephrine, Dopamine, and Serotonin Concentration in Mouse Brain

The status of our present knowledge concerning

Table 5-1

Whole Mouse Brain Analysis-Liquid Chromatography Method

NE (ng/g) ±SEM (n)	DA (ng/g) ±SEM (n)	5-HT (ng/g±SEM (n)
437±7 (48)	927±11 (48)	783±9 (48)

Table 5-2

Whole Mouse Brain Analysis-Literature Values[a]

NE, ng/g±SEM (n)	DA, ng/g±SEM (n)	5-HT, ng/g±SEM (n)	Reference	Detection Technique[b]
350-450 (1000)	700-900 (1000)	600-800 (1000)	26	F
450±15 (11)	770±6 (13)	550±10 (10)	59	F
360±16 (10)	870±45 (12)	820±12 (3)	60	F
470±30 (5)	800±30 (5)	670±40 (5)	63	GCMS
350±30 (6)	850±20 (6)	550±20 (6)	63	F

[a]All results presented as mean ± SEM for the reported number of samples.

[b]F- Fluorescence
GCMS- Gas chromatography mass spectroscopy.

circadian rhythms of NE, DA, and 5-HT in mouse brains is very poor. A change in these levels as a function of the time of day is very important to many psychological experiments presently being performed by other workers. Halberg et al.,[49] in their study of 5-HT concentrations in mouse brains, are the only investigators to have reported results in this area. Therefore, we decided to investigate the diurnal variations in the concentrations of the biogenic amines in mouse brain.

Male Sprague-Dawley mice weighing 20 to 30 g were housed under constant environmental conditions for at least 7 days before the experiment, as previously described in Chapter 2. The lighting cycle consisted of lights on from 0700 to 1900 hr. and lights off from 1900 to 0700 hr. Groups of five mice were sacrificed by cervical dislocation every 4 hr. beginning at 0400 hr. Additionally, five mice were sacrificed 1 hr. before and after turning the lights on and 1 hr. before and after turning the lights off. The brains were removed as rapidly as possible, frozen in liquid N_2, stored on dry ice for 24 hr., and then assayed for NE, DA, and 5-HT. The results are shown in Table 5-3 and Figure 5-1.

From the results, it appears that all three biogenic amines exhibit a fairly reasonable diurnal variation with a single maximum and a single minimum at sometime during the 24 hr. period. Thus, NE displays its greatest value

Table 5-3

Norepinephrine, Dopamine, and Serotonin Concentration in Mouse Brain at Certain Times of Day[a]

Time of sacrifice	NE(ng/g)	DA(ng/g)	5-HT(ng/g)
0400	417±12	960±63	687±28
0600	394±10	883±52	620±25
0700	- - - Lights turned on - - -		
0800	357±12	736±24	555±11
1200	313±10	703±15	599±50
1600	348±9	694±24	568±20
1800	389±21	735±73	593±72
1900	- - - Lights turned off - - -		
2000	368±39	720±83	550±58
2400	463±11	799±35	614±27

[a]Each result represents the mean of 5 determinations ± SEM.

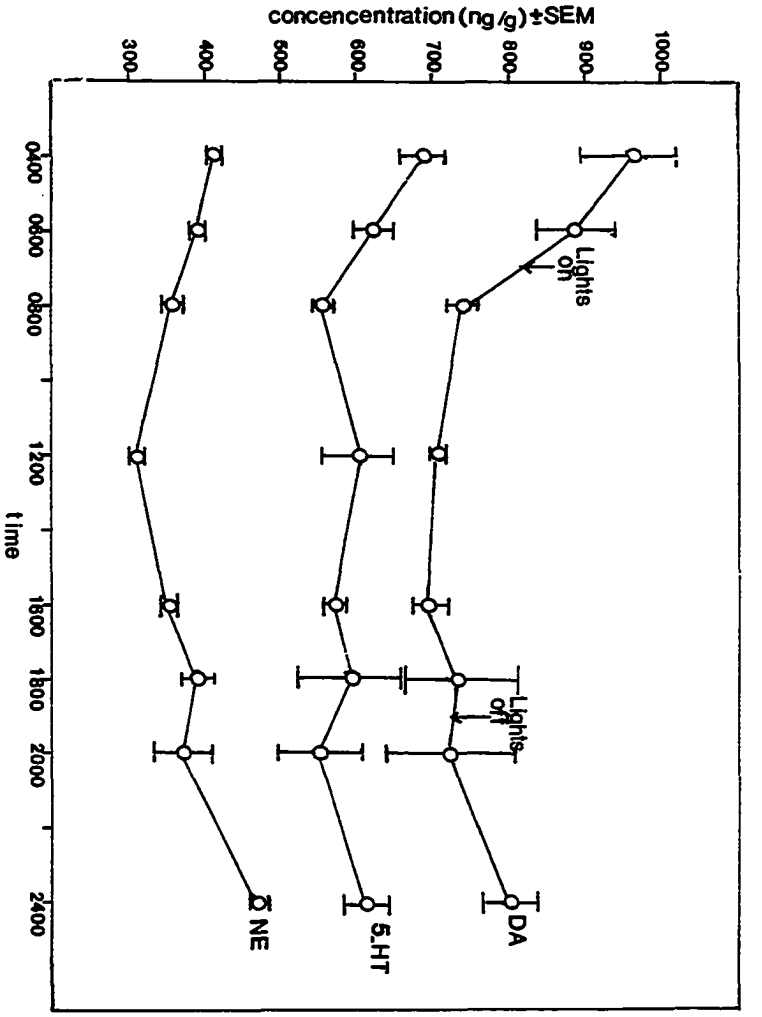

FIGURE 5-1. Daily rhythms in NE, DA, and 5-HT concentrations in mouse brain.

at 2400 hr. and its least value at 1200 hr. DA has a definitive maximum at 0400 hr. and an apparent minimum at 1600 hr. Serotonin, on the other hand exhibits a little less variation, with a maximum at 0400 hr. and a broad minimum between 0800 and 2000 hr.

The ratio of the maximal to minimal results for each amine was: NE, 1.48; DA, 1.38; and 5-HT, 1.25. And, the maximum value was significantly larger than the minimum value in all three cases ($P < 0.01$). This clearly shows that diurnal variations must be considered in any experimental results when the controls and treated animals are not sacrificed at the same time of day.

C. Comparison of the Effects of 6-Aminodopamine and α-Methyl-6-aminodopamine on the Central Nervous System

A major advance in knowledge concerning the functioning of both adrenergic and serotonergic systems has come from the use of neurochemically selective destroying agents. However, the selectivity of presently available agents is by no means absolute. Thus, 100% destruction of noradrenergic, dopaminergic, or serotonergic pathways has not been attained. Also, the destruction of any one of these systems is frequently accompanied by a concurrent disruption of another system.

6-Aminodopamine (6-ADA) is one of the compounds that has shown destruction of sympathetic neurons.[75,76] It was

first shown to deplete NE stores in mouse brain by Blank et al.[77] Its selectivity towards NE neurons was also shown to be somewhat higher than 6-hydroxydopamine (6-OHDA) by Oke et al.[78] However, the destructive effect of 6-ADA on NE neurons is still not complete.

Porter and coworkers,[79] have demonstrated that 6-aminodopamine, 6-hydroxydopamine, and their α-methyl derivatives are all capable of producing a long-lasting depletion of catecholamines from the hearts of mice as well as from certain peripheral tissues of dogs. Evidence has also been presented to suggest that α-methylation of 6-OHDA increases its potency towards NE neurons.[80] Therefore, we decided to study the effect of α-methyl-6-aminodopamine (α-Me-6-ADA) as compared to 6-ADA on NE, DA, and 5-HT in mouse brains utilizing the LCEC analysis.

6-ADA and α-Me-6-ADA were kindly prepared by Mr. Pat Cohenour via modifications of the method of Stone.[81] Solutions of both were prepared, immediately before injection, in thoroughly deaerated isotonic saline containing 1 mg/ml ascorbic acid to minimize air oxidation. Samples were introduced by direct intracranial injection into male ARS-HA/ICR mice weighing 20-30 g under light ether anesthesia. A microsyringe was used with an arresting stop which permitted a penetration depth of 3 mm. Mice were treated in groups of 24, including 8 controls and 16 experimentals. Injections, spaced apart at 24 hr. intervals,

contained 50 µg of the neurotoxin in a volume of 5 µl. Controls received equivalent injections of the isotonic saline with ascorbic acid. 24 hours after the last injection, the mice were killed by cervical dislocation. The brains were removed as rapidly as possible and assayed for NE, DA, and 5-HT as described before. The results are given in Table 5-4.

As shown in Table 5-4, α-methylation of 6-ADA significantly increased NE depletion ($P < 0.02$) while the effect on DA remained comparable to that of 6-ADA. This indicates higher potency of α-methylation in destroying NE neurons. But, this study also indicates that both 6-ADA and α-Me-6-ADA cause significant depletion ($P < 0.05$) of 5-HT. This result is potentially very disruptive to many existent behavior studies performed with 6-aminodopamine. Concurrent destruction of 5-HT in the use of 6-ADA or α-Me-6-ADA means that behavioral results obtained may also be experiencing an effect due to the loss of this transmitter; consideration of only the NE and DA depletions in such investigations, thus, may no longer be entirely adequate.

The present results also indicate that α-Me-6-ADA may be more toxic than 6-ADA, as shown by the number of survivals (5 out of 8 as compared to 7 out of 8 for 6-ADA). This data, while not being definitive at the present time, does point to the use of lower dosages in future investiga-

Table 5-4

Depletion of NE, DA, and 5-HT in Whole Mouse Brain by 6-ADA and α-Me-6-ADA[a]

Compound	Dosage		n[b]	NE		DA		5-HT	
	Schedule	Total		ng/g	% Control	ng/g	% Control	ng/g	% Control
α-Me-6-ADA	2x50 µg	100 µg	5	133±13	29	723±40	76	480±48	79
6-ADA	2x50 µg	100 µg	7	196±17	43	788±35	83	490±43	80

[a]All results expressed as mean ± SEM. Average results for controls (n=8): NE=460±18 ng/g, DA=948±38 ng/g, 5-HT=610±34 ng/g.

[b]Number of mice.

tions of α-Me-6-ADA to minimize these toxic effects.

D. Simultaneous Estimation of Norepinephrine, Dopamine, and Serotonin in Seven Discrete Areas of Mouse Brain

Obtaining data on discrete areas of brain rather than from the whole brain has already been shown to be of considerable importance.[82,83] The biogenic amine levels of the different brain areas have displayed a wide variation upon drug treatment.[25,84,85] While a particular amine may greatly decrease in one area after treatment, the same amine may show an increase or no change at all if examined in another area. In addition, since it is widely accepted that there is considerable functional interaction between the putative neurotransmitters, concurrent estimation of two or more neurotransmitter levels in the same sample of brain tissue should find increasing application. Therefore, we applied the assay described in Section G, Chapter 3 for the determination of NE, DA, and 5-HT to the smaller mouse brain parts to demonstrate its sensitivity and usefulness.

The results obtained comprise the first report of data for NE, DA, and 5-HT in these discrete areas of mouse brain. The method is intended to be widely applicable to drug screening and behavioral studies. It eliminates the necessity for pooling, which provides information about groups rather than individuals. Additionally, it avoids

the larger costs and longer analysis time associated with pooling.

Results and Discussion

The results of these brain parts analyses are given in Table 5-5. Although we could not find any references to NE, DA, and 5-HT in these regions of mouse brain, the results do agree fairly well with those reported for rat brain parts.[29,30,86-88]

The method was also used to determine 3-MT in striatum (456±74 ng/g, n=6). Figure 5-2 shows a typical chromatogram for such a striatum determination. The sensitivity of the method, however, is probably best shown in Figure 5-3 which represents a typical chromatogram for cerebellum. This is a small part (57 mg) with an extremely low concentration of DA (28 ng/g). Yet all the biogenic amines can still be measured and quantitated.

In conclusion, the procedure described for the simultaneous determination of NE, DA, and 5-HT (and even 3-MT, if desired) in mouse brain tissue by LCEC is rapid, selective, inexpensive, and comparable or better in sensitivity to the more expensive GCMS and radiochemical techniques. It has been used routinely for the analysis of ca. 15 individual brain part samples per day.

Table 5-5

Concentrations of NE, DA, and 5-HT in Regions of Mouse Brain[a]

Brain region	Average weight (mg)	NE(ng/g)	DA(ng/g)	5-HT(ng/g)
Cerebellum	57	296±14	28±4	103±8
Medulla-pons	43	535±40	59±7	812±54
Midbrain	36	616±39	126±9	1015±49
Diencephalon	44	625±41	200±12	974±76
Hippocampus	33	254±46	84±28	453±93
Striatum	23	153±13	4777±380	579±25
Cortex	198	256±34	673±67	630±47

[a]All results represent mean values for 11 determinations ± SEM.

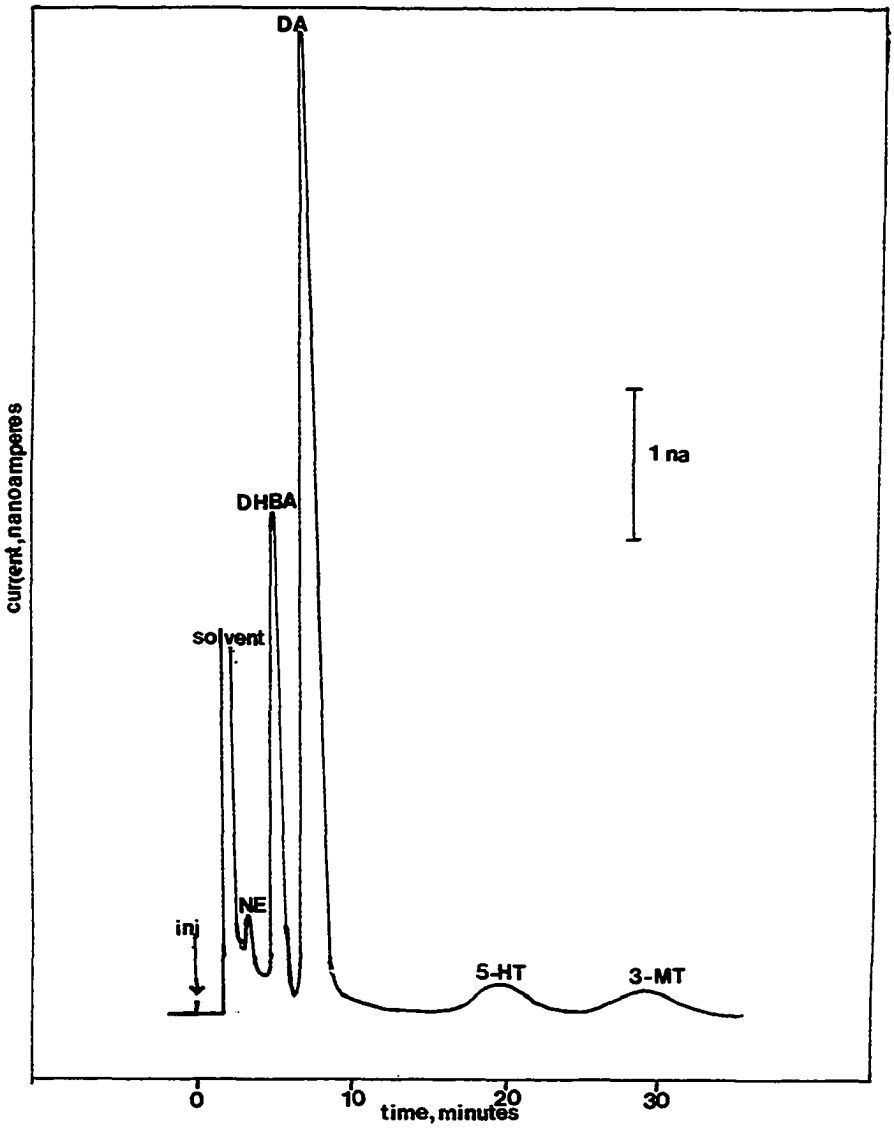

FIGURE 5-2. A typical chromatogram for mouse striatum, 10 µl injection.

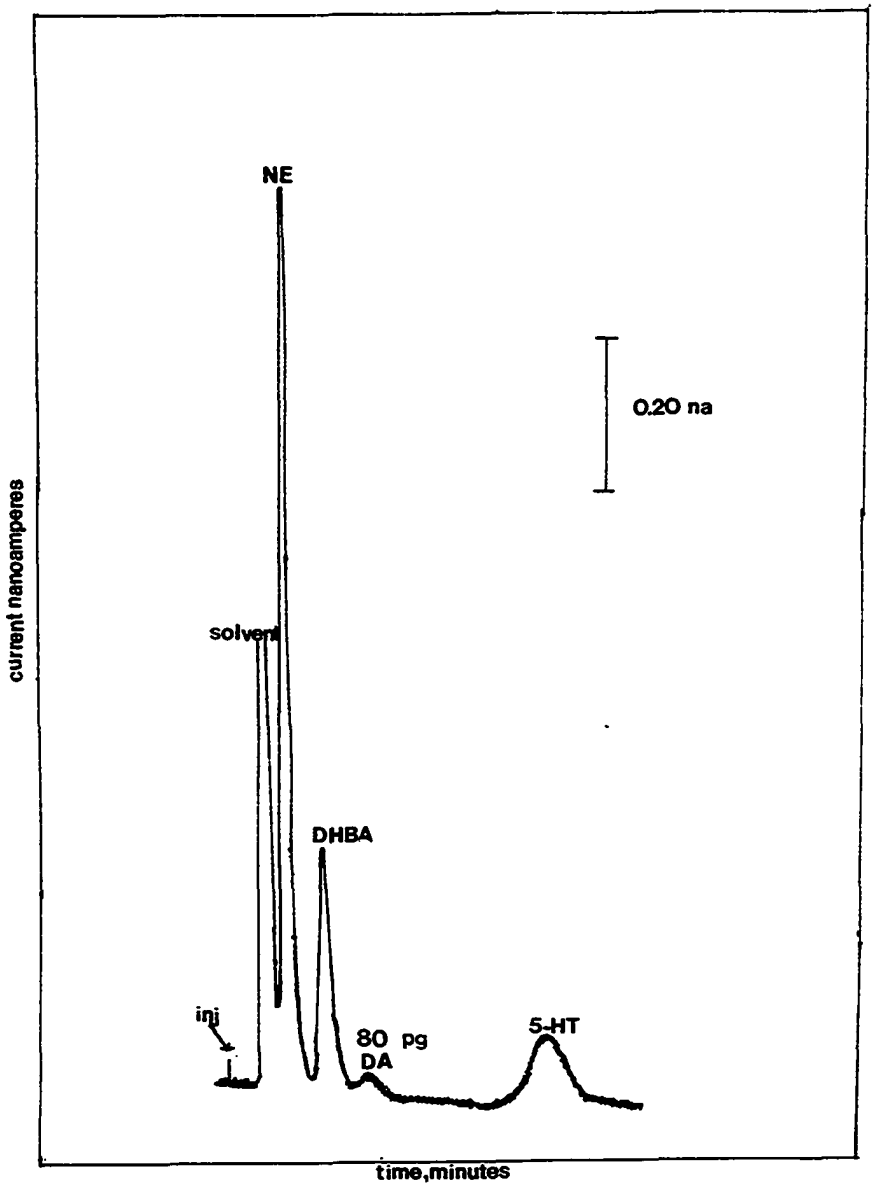

FIGURE 5-3. A typical chromatogram of mouse cerebellum, 30 µl injection.

References

1. T. Nagatsu, Biochemistry of Catecholamines, University Park Press, Baltimore, 1973.
2. Orthomolecular Psychiatry, D. Hawkins, and L. Pauling, eds., W. H. Freeman and Co., San Francisco, 1973.
3. M. M. Rapport, A. A. Green, and I. H. Page, J. Biol. Chem., 176, 1243 (1948).
4. M. M. Rapport, J. Biol. Chem., 180, 961 (1949).
5. S. Garattini and L. Valzelli, Serotonin, Elsevier, Amsterdam, 1965, p. 241.
6. J. P. Cooper, F. E. Bloom, and R. H. Roth, The Biochemical Basis of Neuropharmacology, Oxford Press, 1974, p. 180.
7. U. S. von Euler, Acta Physiol. Scand., 11, 168 (1946).
8. P. Holtz, K. Crender, and Kroneberg, Arch. Exp. Path. Pharmakol., 204, 288 (1947).
9. P. Holtz, K. Credner, C. Stubing, Arch. Exp. Path. Pharmakol., 199, 145 (1942).
10. McC. Goodall, Acta. Chem. Scand., 4, 550 (1950).
11. McC. Goodall, Nature, 166, 738 (1950).
12. H. Blaschko, J. Physicl., 96, 50P (1939).

13. T. Nagatsu, M. Levitt, and S. Udenfriend, J. Biol. Chem., 239, 2910 (1964).

14. U. S. von Euler, Clin. Chem., 18, 1445 (1972).

15. A. Coppen, Brit. J. Psychiat., 113, 1237 (1967).

16. D. M. Shaw, F. E. Camps, and E. G. Eccleston, Brit. J. Psychiat., 113, 1407 (1967).

17. S. T. Breisch. F. P. Zemilan, and B. G. Hoebel, Science, 192, 384 (1976).

18. J. Barchas and E. Usdin, Serotonin and Behavior, 1973, p. 567.

19. ibid, p. 565.

20. G. C. Cotzias, New Engl. J. Med., 278, 630 (1968).

21. L. Stein and G. D. Wise, Science, 171, 1032 (1971).

22. P. L. Carlton, Psychol. Rev., 70, 19 (1963).

23. B. A. Campbell, L. D. Lytle and H. C. Fibiger, Science, 166, 635 (1969).

24. A. K. Swonger and R. H. Rech, J. Comp. Physiol. Psychol., 81, 509 (1972).

25. J. H. Gordon and M. K. Shellenberger, Neuropharmacology, 13, 129 (1974).

26. A. S. Welch and B. L. Welch, Anal. Biochem., 30, 161 (1969).

27. K. M. Taylor and R. Laverty, J. Neurochem., 16, 1367 (1969).

28. G. B. Ansell and M. F. Beeson, Anal. Biochem., 23, 169 (1968).

29. K. M. Shellenberger and J. H. Gordon, Anal. Biochem., 39, 356 (1971).

30. R. P. Maickel, R. H. Cox, J. Saillant, and F. P. Miller, Int. J. Neuropharmacol., 7, 725 (1968).

31. J. W. Vanable, Jr., Anal. Biochem., 6, 393 (1963).

32. U. S. von Euler, Pharmcol. Rev., 11, 262 (1959).

33. D. F. Bodanski, A. Pletscher, B. B. Brodie, and S. Udenfriend. J. Pharmacol. Exp. Ther., 117, 82 (1956).

34. E. L. Arnold and R. Ford, Anal. Chem., 45, 85 (1973).

35. I. L. Martin and G. B. Ansell, Biochem. Pharmacol., 22, 521 (1973).

36. S. H. Koslow, G. Racagni and E. Costa, Neuropharmacology, 13, 1123 (1974).

37. C. Refshauge, P. T. Kissinger, R. Dreiling, L. Blank, R. Freeman, and R. N. Adams, Life Sci., 14, 311 (1974).

38. P. A. Shore and J. S. Olin, J. Pharmacol. Exp. Ther., 122, 295 (1958).

39. A. C. Cuello, R. Hiley, and L. L. Iversen, J. Neurochem., 21, 1337 (1973).

40. J. M. Saavedra, B. Brownstein, and J. Axelrod, J. Pharmacol. Exp. Ther., 186, 508 (1973).

41. A. H. Anton and D. F. Sayre, J. Pharmacol. Exp. Ther., 138, 360 (1960).

42. G. Zweig and J. Sherma. Anal. Chem., 44, 42R (1972).

43. P. T. Kissinger, C. Refshauge, R. Dreiling, R. N. Adams, Anal. Letters, 6, 465 (1973).

44. P. T. Kissinger, L. J. Felice, R. M. Riggin, L. A. Pachla, and D. C. Wanke, Clin. Chem., 20, 992 (1974).

45. C. L. Blank, J. Chrom., 117, 35 (1976).

46. R. J. Borgman, M. R. Baylor, J. J. McPhillips, and R. E. Stitzel, J. Med. Chem., 17, 427 (1974).

47. C. Refshauge, Ph.D. Dissertation, University of Kansas, Lawrence, Kan., 1974.

48. R. N. Adams, Electrochemistry at Solid Electrodes, Marcel Dekker, Inc., New York, 1969.

49. P. Albrecht, M. B. Vischer, J. J. Bittner, and F. Halberg, Proc. Soc. Exp. Biol. Med., 92, 702 (1956).

50. D. Wassil, M.Sc. Thesis, University of Okla., Norman, Oklahoma, 1977.

51. Louis Levine, Biology of the Gene, 2nd ed., C. V. Mosely Co., St. Louis, Mo., p. 84, 1973.

52. R. E. Majors, American Laboratory, 7, 13 (1975).

53. R. L. Pike, M.Sc. Thesis, University of Oklahoma, Norman, Okla., 1976.

54. Application Highlight 343, Waters Association, July (1974).

55. T. B. B. Crawford and C. M. Yates, Brit. J. Pharmacol., 38, 56 (1968).

56. F. M. Bumpus and I. H. Page, J. Biol. Chem., 212, 111 (1955).

57. B. Higgins and J. H. Richards, J. Pharm. Pharmacol., 19, 641 (1967).

58. H. Tamir, A. Klein, and M. M. Rapport, J. Neurochem., 26, 871 (1976).

59. R. M. Fleming, W. G. Clark, G. D. Fenster, and J. E. Towne, Anal. Chem., 37, 692 (1965).

60. R. G. Wigand and J. E. Perry, Biochemical. Pharmacol., 7, 181 (1961).

61. C. B. Smith, J. Pharmacol. Exp. Ther., 142, 343 (1963).

62. H. C. Agrawal, S. N. Glisson, and W. A. Himwich, Int. J. Neuropharmacol., 7, 97 (1968).

63. S. T. Weintraub, W. B. Stavinoha, R. L. Pike, W. W. Morgan, A. T. Modak, S. H. Koslow, and L. Blank, Life Sci., 17, 1423 (1975).

64. H. Alliger, Am. Lab., 7, 75 (1975).

65. W. B. Stavinoha, B. Pepelko, and P. W. Smith, Pharmacologist, 12, 257 (1970).

66. W. B. Stavinoha, A. T. Modak, and S. T. Weintraub, J. Neurochem., 20, 361 (1973).

67. D. J. Jones, M. A. Medina, D. H. Ross, and W. B. Stavinoha, Life Sci., 14, 1577 (1974).

68. K. Knieriem and M. A. Medina, Trans. Am. Soc. of Neuro-Chem., 6, 170 (1975).

69. M. A. Medina, D. J. Jones, W. B. Stavinoha, and D. H. Ross, J. Neurochem., 24, 223 (1975).

70. E. Costa, in Brain, Nerves and Synapses, G. H. Acheson, ed., v. 4, Karger, Basel, p. 215, 1973.

71. J. Glowinski, ibid, p. 205.

72. J. J. Kopin, ibid, p. 227.

73. A. Groppatti, S. Algeri, F. Cattabeni, A. B. DI Guilio, C. L. Galli, F. Ponzio, and P. F. Spano, J. Neurochem., 28, 193 (1977).

74. D. G. Grahame-Smith in Serotonin and Behavior, J. Barchas, and E. Usdin, eds., Academic Press, p. 5, 1973.

75. G. Jonsson and C. Sachs, J. Neurochem., 21, 117 (1973).

76. G. R. Siggins, D. S. Forman, F. E. Bloom, K. L. Sims, and R. N. Adams, Fed. Proc., 32, 692 (1972).

77. C. L. Blank, E. Murrill, R. N. Adams, Brain Research, 45, 635 (1972).

78. A. Oke, R. Freeman, R. N. Adams, Eur. J. Pharmacol., 26, 125 (1974).

79. C. C. Porter, J. A. Totaro, and C. A. Stone, Pharmacologist, 4, 149 (1966).

80. J. P. Tranzer and H. Thoener, Experientia, 29, 314 314 (1973).

81. C. A. Stone, Chem. Abstr., 61, 6953e.

82. E. P. Miller, R. H. Cox, Jr., and R. P. Maickel, Science, 162, 463 (1968).

83. R. C. Hanig and M. H. Aprison, Life Sci., 10, 279 (1971).

84. M. K. Shellenberger, Neuropharmacology, 10, 347 (1971).

85. M. K. Shellenberger, J. Pharmac. Exp. Ther., 177, 481 (1971).

86. R. F. Butterworth, F. Landreville, M. Guitard, and A. B. Barbeau, Clin. Biochem., 8, 298 (1975).

87. L. E. Smith, J. D. Lane, P. A. Shea, W. J. McBride, and M. H. Aprison, Anal. Biochem., 64, 149 (1975).

88. R. H. Cox, Jr. and J. L. Perhach, Jr., J. of Neurochem., 20, 1777 (1973).

89. D. R. Haubrich and J. S. Denzer, Anal. Biochem., 55, 306 (1973).

APPENDIX I

Abbreviations

Substance	Abbreviation
S-Adenosylmethionine	SAM
b-Aminodopamine	6-ADA
Catechol-O-methyltransferase	COMT
Dihydroxybenzylamine	DHBA
Dihydroxyphenylalanine	DOPA
DOPA decarboxylase	DDC
Dopamine	DA
Epinephrine	E
5-Hydroxyindoleacetic acid	5-HIAA
5-Hydroxytryptamine (serotonin)	5-HT
5-Hydroxytryptophan	5-HTP
Metanephrine	M
3-Methoxytyramine	3-MT
α-Methyl-6-aminodopamine	α-Me-6-ADA
α-Methyldopamine	α-MeDA
Monamine oxidase	MAO
Norepinephrine	NE
Normetanephrine	NMN
Tryptophan hydroxylase	TPH