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DEVELOPMENT OF PROCEDURES FOR THE DETERMINATION OF CATECHOLAMINES, INDOLEAMINES, RELATED METABOLITES, AND MONOAMINE OXIDASE ACTIVITY

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DEVELOPMENT OF PROCEDURES FOR THE DETERMINATION OF CATECHOLAMINES, INDOLEAMINES, RELATED METABOLITES, AND MONOAMINE OXIDASE ACTIVITY

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DEVELOPMENT OF PROCEDURES FOR THE DETERMINATION OF CATECHOLAMINES, INDOLEAMINES, RELATED METABOLITES, AND MONOAMINE OXIDASE ACTIVITY

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To Mom and Dad

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CHAPTER 1

REVERSE PHASE CHROMATOGRAPHIC THEORY AND PRACTICE

I. INTRODUCTION

Over the past five years, our laboratory has been particularly interested in the chromatographic separation and subsequent quantitation of catecholamine and indoleamine related compounds in biological fluids and tissues. A large variety of such procedures currently exist for the determination of these compounds.[66] However, most of these procedures can only be used to quantitate one to four of these many different compounds;[67-70] this can lead to an incomplete picture concerning the physiological profile of the associated tyrosine and tryptophan pathways. Also, these procedures require a considerably diverse array of mobile phases, a variety of chromatographic columns, and rather extensive sample manipulations prior to quantitation. In short, each of these assays requires a unique chromatographic system dedicated to that particular analysis. We have also recently developed a substantial number of different enzymatic assays in our laboratory by focusing on the chromatographic determination of products. Again, each of these procedures requires a distinct chromatographic system. In order to minimize the total number of chromatographs required, we decided to develop appropriate conditions to permit the simultaneous determination

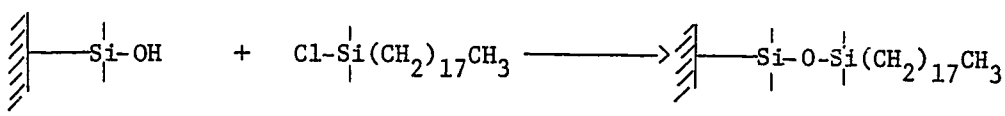
of as many catecholamine and indoleamine metabolites as possible. The compounds of interest are summarized in Table 1-1.

II. REVERSE PHASE LIQUID CHROMATOGRAPHIC THEORY

A. Fundamental Definitions

Modern partition chromatography began in the early 1940's.[75] At that time, liquid chromatographic columns were prepared by lightly coating silica beads with thin films of water or other polar compounds. This material was packed into a tube, and a nonpolar liquid was allowed to flow through the packed column. The silica beads were referred to as the stationary support, the polar coating was called the stationary phase, and the nonpolar liquid was known as the mobile phase. This general type of liquid chromatography was later labeled normal phase chromatography, NPC, and is schematically shown in Fig. 1-1A.

In contrast to NPC, the more commonly employed reverse phase chromatography (RPC) incorporates a nonpolar coating (see Fig. 1-1B). The stationary phase for modern reverse phase systems is typically prepared by reacting the exposed silicic acid groups on silica beads with alkyl chlorosilanes to produce a chemically bonded and uniformly distributed organic film over the surface of the particles



An entirely aqueous solution may be used as the mobile phase. However, an organic diluent is frequently added to the mobile phase to modify the chromatographic parameters.

The resulting chromatographic separation arises from differences

Table 1-1. Structures and Abbreviations of Some Catecholamines, Indoleamines and Related Metabolites

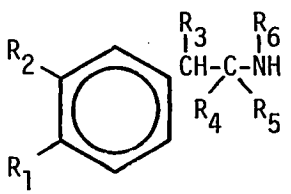
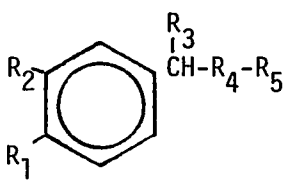
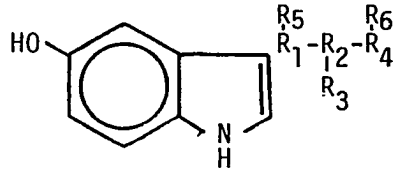
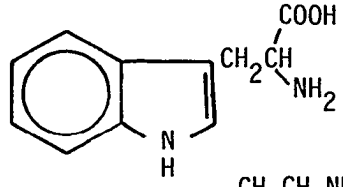
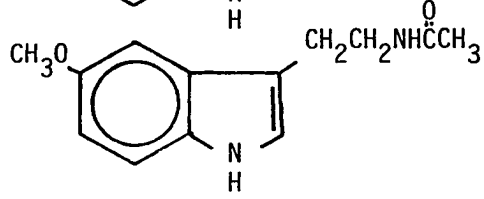
Compound	Structure	Abbreviation	R_1	R_2	R_3	R_4	R_5	R_6
Catecholamines and Related Metabolites								
Epinephrine		EPI	OH	OH	OH	H	H	CH_3
3-Methoxytyramine		3-MT	OH	OCH_3	H	H	H	H
Dopamine		DA	OH	OH	H	H	H	H
3,4-Dihydroxyphenylalanine		DOPA	OH	OH	H	H	COOH	H
Norepinephrine		NE	OH	OH	OH	H	H	H
Normetanephrine		NORMET	OH	OCH_3	OH	H	H	H
Tyrosine		TYR	OH	H	H	H	COOH	H
Homovanillic acid		HVA	OH	OCH_3	H	COOH	-	-
3,4-Dihydroxyphenylacetic acid		DOPAC	OH	OH	H	COOH	-	-
3,4-Dihydroxybenzylamine		DHBA	OH	OH	H	NH_2	-	-
3,4-Dihydroxymandelic acid		DOMA	OH	OH	OH	COOH	-	-
Vanillylmandelic acid		VMA	OH	OCH_3	OH	COOH	-	-
3,4-Dihydroxyphenylglycol		DOPEG	OH	OH	OH	CH_2	OH	-
3-Methoxy-4-hydroxyphenethylglycol		MOPEG	OH	OCH_3	OH	CH_2	OH	-

Table 1-1 (continued)

Compound	Structure	Abbreviation	R_1	R_2	R_3	R_4	R_5	R_6
Indoleamines and Related Metabolites								
5-Hydroxytryptophol		5-HTOL	CH_2	CH_2	OH	-	-	-
5-Hydroxytryptophan		5-HTP	CH_2	CH	NH_2	COOH	-	-
5-Hydroxyindole-3-acetic acid		5-HIAA	CH_2	COOH	-	-	-	-
5-Hydroxytryptamine		5-HT	CH_2	CH_2	NH_2	-	-	-
5-Hydroxy-Nω-methyltryptamine		nMET	CH_2	CH_2	-	NH	-	CH_3
5-Hydroxyindole-3-carboxylic acid		5-HICA	COOH	-	-	-	-	-
N-acetyl-5-hydroxytryptamine		N-Ac-5-HT	CH_2	CH_2	-	NH	-	$COCH_3$
Tryptophan		TP						
Melatonin		MEL						

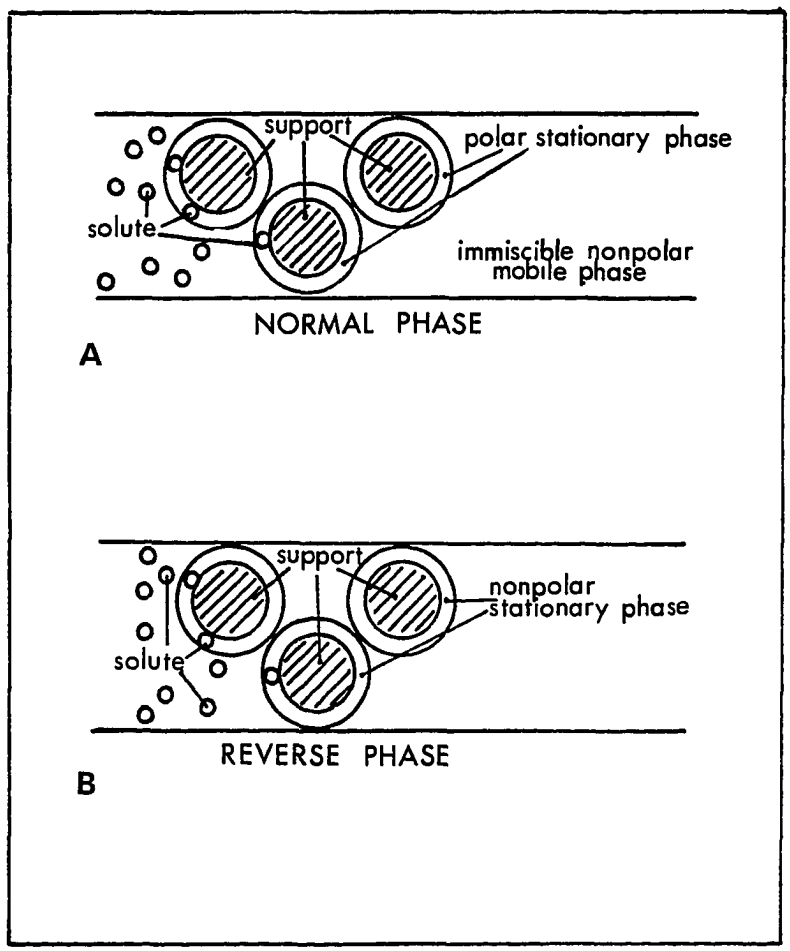


Figure 1-1. Illustration Comparing Normal Phase with Reverse Phase Chromatography. Normal phase (Figure A) consists of a polar stationary phase adsorbed onto a silica support. This is in contrast to reverse phase chromatography (Figure B) which consists of a nonpolar stationary phase chemically bonded onto a silicia support.

in the equilibrium distribution of the solutes, or sample components, between the mobile and stationary phases. The greater the difference in solubility for two solutes in the stationary phase, the greater the degree of separation. Consider, for example, the distribution of solute i between two immiscible phases, o and a, at constant temperature and pressure:

$$i_a \rightleftharpoons i_o \tag{1-1}$$

where o represents a nonpolar, organic stationary phase and a represents an aqueous mobile phase. The ratio of solute concentrations in the two phases is given by the equilibrium distribution constant, K:

$$K = \frac{[i]_o}{[i]_a} \tag{1-2}$$

where $[i]_o$ and $[i]_a$ are the molar concentrations of solute i in the organic and aqueous phases, respectively. Simply put, the larger the value of K, the greater the fraction of solute i in the stationary phase at any given instant and the longer the time i will spend in the chromatographic column. Equation 1-2 can also be expressed as:

$$K = \frac{[i]_o}{[i]_a} = \frac{n_o V_a}{n_a V_o} \tag{1-3}$$

where n_o and n_a are the number of millimoles of the solute in the organic and aqueous phases, respectively. V_a is the total volume of the mobile phase within the column at any given time (ml) and is commonly called the dead volume or void volume. V_o is the volume occupied by the coating on the stationary phase.

Rearranging eq. 1-3 yields

$$k' = K\frac{V_o}{V_a} = \frac{n_o}{n_a} \tag{1-4}$$

where k' is an important parameter termed the capacity factor. For a given column, V_o and V_a are constant, or

$$k' \alpha K \tag{1-5}$$

Thus, measurements of capacity factors for various solutes under fixed chromatographic conditions provide direct evaluations of the distribution coefficients for these solutes.

Experimentally the determination of the column void volume, V_a, is obtained from

$$V_a = (F)(t_o) \tag{1-6}$$

where F is the average flow velocity in ml/min and t_o is the retention time of a non-retained component, i.e., a solute which experiences no interaction with the stationary phase. V_o, the volume of the coating on the stationary phase, can also be written as:

$$V_o = \frac{(F)(t_r - t_o)}{K} \tag{1-7}$$

where t_r is the retention time for a retained solute.

Combining eqs. 1-4, 1-6, and 1-7, we obtain

$$k' = \left(\frac{t_r - t_o}{t_o}\right) \tag{1-8}$$

Therefore, k' for any solute can be conveniently determined by simply measuring the retention time of the solute and that of a nonretained component. These measurements are illustrated in Fig. 1-2. Because

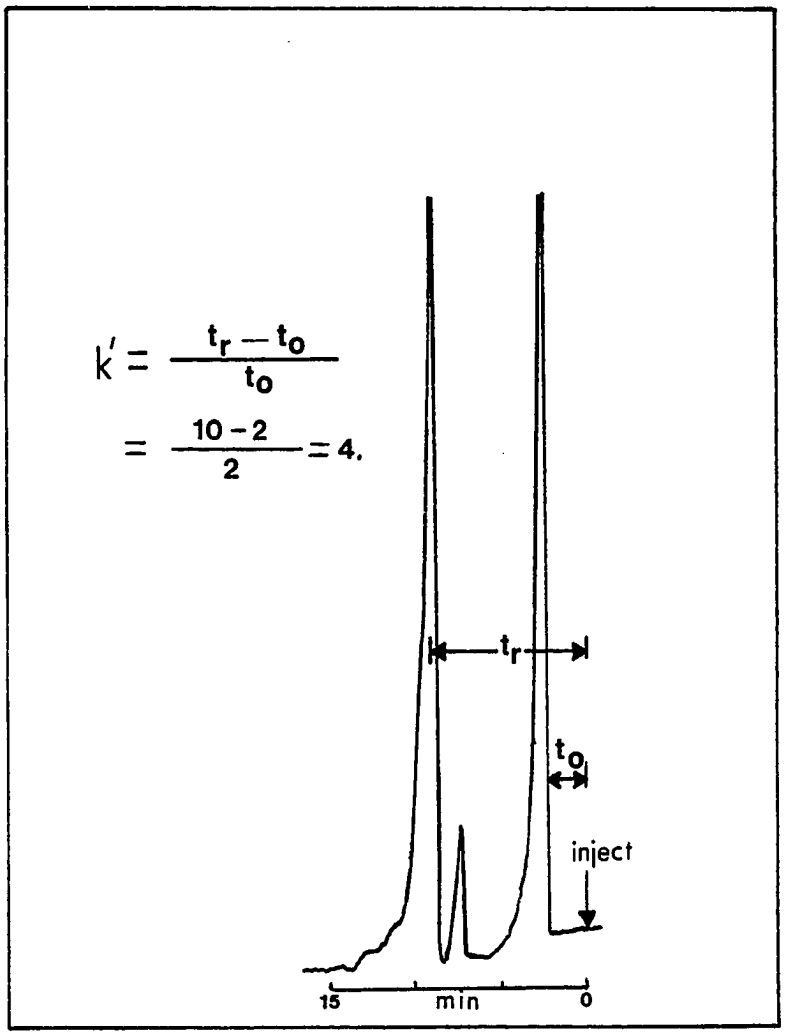


Figure 1-2. Calculation of the Capacity Factor, k', from a Chromatogram. t_r and t_o can be measured in units of time or distance.

k' is directly related to K, it is independent of the column length and flow rate to a first approximation. This makes k' a far more useful parameter than the retention time for chromatographic investigations.

Another useful parameter which allows the chromatographer to quantitatively evaluate a separation between two solutes is the separation factor, α:

$$\alpha = \frac{k_2'}{k_1'} \tag{1-9}$$

where k_1' and k_2' refer to the capacity factors for solutes 1 and 2 respectively. By convention, k_2' is always greater than k_1'. The separation factor is an indirect measure of the distance between band centers for two neighboring peaks; the larger the value for α, the better the separation. The essential parameters and the associated calculation of the separation factor for two well-separated solutes is exhibited in Fig. 1-3.

A third experimental parameter that is important in evaluating the performance of a chromatographic system is the number of theoretical plates, N. It is defined as:[71,73]

$$N = \left(\frac{t_r}{\sigma}\right)^2 \tag{1-10}$$

where t_r is the retention time for the solute and σ is the band variance in units of time. N is, thus, a dimensionless quantity which is empirical in nature and provides some measure of band spreading. The extensive and exclusive use of N to completely characterize band broadening and efficiency of a chromatographic system has been seriously questioned due to its dependence on a particular solute. However, as a general rule, the larger the reported value for N, the greater the

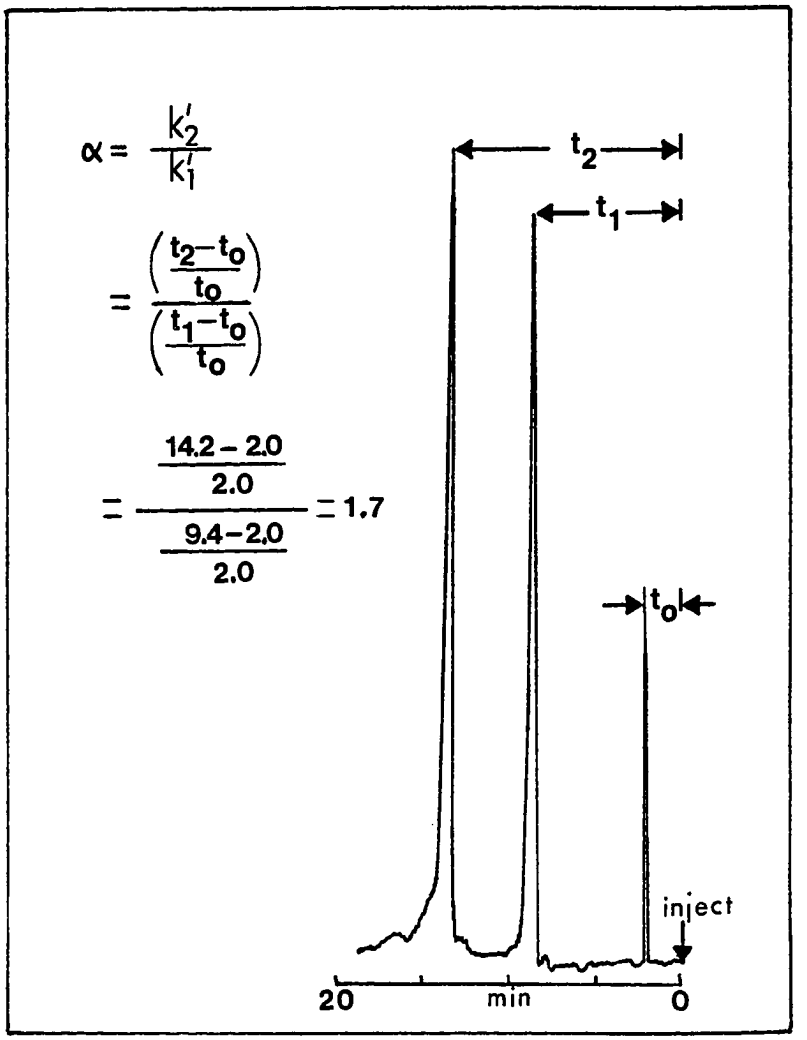


Figure 1-3. Calculation of the Separation Factor, α, from a Chromatogram. t_2, t_1, and t_o can be measured either as units of time or distance. The chromatogram illustrates the separation of two well-resolved peaks.

overall column efficiency. To derive N from an actual chromatogram, eq. 1-10 will have to be altered. Assuming Gaussian-shaped peaks, the width measured at the point where the peak is one-half of its maximum value, $W_{b/2}$, can be shown to be:[76]

$$W_{b/2} = 2\sigma\sqrt{2\ln 2} \qquad (1\text{-}11)$$

Solving for σ and substituting into equation 1-10 we arrive at:

$$N = 5.54 \left(\frac{t_r}{W_{b/2}}\right)^2 \qquad (1\text{-}12)$$

This equation is preferred since the resulting value of N is not significantly affected by the tailing observed for some peaks (see, e.g., Fig. 1-5A). Thus, equation 1-12 provides more uniformity in comparing the efficiencies of different columns. A calculation of N is illustrated in Fig. 1-4.

B. The Nature of Band Broadening.

Band broadening is the phenomenum which results from an increase in the width of a solute band as it migrates down the column. Excessive band broadening represents a degradation of the narrow zone of solute molecules initially introduced in the injection. This degradation is a major contributor to overlapping peaks and poor separations. There are many characteristics which contribute to band broadening in liquid chromatography, although only two are of primary importance: mass transfer and mobile phase effects.

Mass transfer contributions to band broadening are determined by two fundamental factors, the rate of diffusion of the solute within the stationary phase and the rates of absorption into and desorption out of the stationary phase. The mechanism of mass transfer can be

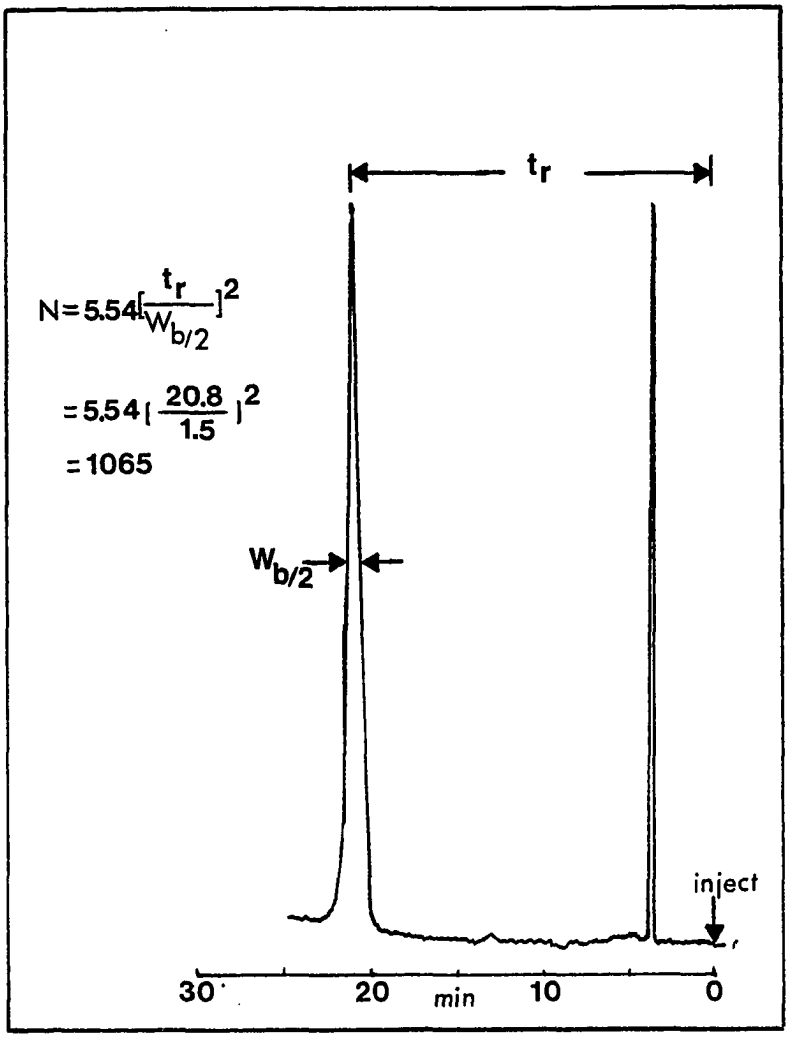


Figure 1-4. Calculation of the Number of Theoretical Plates, N, for a Chromatographic Column. $W_{b/2}$ is the width of the peak at one half the peak height. $W_{b/2}$ and t_r can be measured in units of time or distance.

described as follows. When a solute molecule is absorbed into the stationary phase, it immediately begins to fall behind the other solute molecules as they proceed with the mobile phase down the column. Diffusion within the stationary phase may enhance or detract from this falling behind as the solute randomly migrates in the chemically bonded coating. After some time, the molecule is desorbed back into the solvent stream. It resumes its journey down the column until it reabsorbs into another stationary particle. This percolating effect results in some molecules lagging behind, while others migrate ahead of, the central portion of the solute zone. Narrow solute zones are preserved by very small solute diffusivities within the stationary phase along with rapid absorption and desorption.

Using a random walk model, Giddings[72] calculated the contribution of mass transfer to column efficiency as

$$N_S = \frac{LD_s(1+k')^2}{qk'd^2\nu} \qquad (1\text{-}13)$$

where L is the column length (cm), D_s is the solute diffusivity in the stationary phase (cm^2/sec), q is a particle shape factor (typically 2/3 for a uniform liquid film), ν is the flow velocity (cm/sec) and d is the particle diameter of the packing material (cm).

Mobile phase contributions to band broadening are partly determined by flow phenomena which result from the tortuous paths the solution must follow before emerging from the column. As the solution is forced through the column, its velocity at different points in the column varies. Consequently, molecules being carried in such a stream will have their individual velocities altered as they pass from one

interstitial space to another. Some molecules, by following relatively open passageways, will have a somewhat faster overall velocity. Others will become entangled in restricted zones and fall behind, Giddings[72] has also estimated the contribution of flow-profile effects to column efficiency:

$$N_F = L/\Lambda d \tag{1-14}$$

where Λ is a function of the packing structure. As in the stationary phase, diffusion can also occur within the mobile phase. Its contribution is expressed as:[71]

$$N_D = \frac{LD_m}{\Omega \nu d^2} \tag{1-15}$$

where D_m is the diffusivity of the solute in the mobile phase and Ω is a function of the packing structure.

The combined expression for the total number of theoretical plates is then the sum of eqs. 1-13, 1-14, and 1-15,

$$N = N_D + N_F + N_s = \left(\frac{LD_m}{\Omega d^2 \nu} + \frac{L}{\Lambda d} + \frac{LD_s(1+k')^2}{qk'd^2\nu}\right)_i \tag{1-16}$$

Thus, to increase the total number of theoretical plates and, thereby, the efficiency of the column, one can increase the column length, (L), decrease the particle size of the packing material (d), or decrease the flow velocity (ν) of the chromatographic system.

C. Resolution.

The previous discussion describes the factors which create band spreading of the injected solute molecules. As mentioned earlier, this phenomenon is of concern because it may lead to serious over-

lapping of chromatographic peaks. Fig. 1-5 very clearly illustrates the importance of minimizing this problem. Fig. 1-5A was obtained from component mixture using a highly inefficient column (N=600), whereas Fig. 1-5B is a chromatogram of the same mixture run on a high efficiency column (N=3000). Results such as those seen in Fig. 1-5A are undesirable for three primary reasons. First, the total number of individual compounds that can be determined in a single chromatographic run is considerably decreased. Secondly, the elution of other, unsuspected solutes may be obscured under the observed peaks. Finally, overlapping peaks are extremely difficult to quantitate. Quantitative measurements are frequently obtained by measuring the area underneath each peak, and it is extremely difficult to assign an appropriate fraction of the total area to a specific solute. Thus, measurements of low resolution peaks may require the use of complex, computer-aided deconvolution techniques[74,79] to obtain an acceptable level of precision.

The degree to which two adjacent bands are separated is termed resolution and is defined as the distance between band centers divided by the average band width:

$$R = \frac{t_2 - t_1}{1/2(W_1 + W_2)} \qquad (1\text{-}17)$$

Since the values of W for closely adjacent bands are approximately equal, and assuming the peaks are Gaussian, it can be shown that

$$W_2 \cong W_1 = 4\sigma$$

Substituting into eqn. 1-17, we arrive at

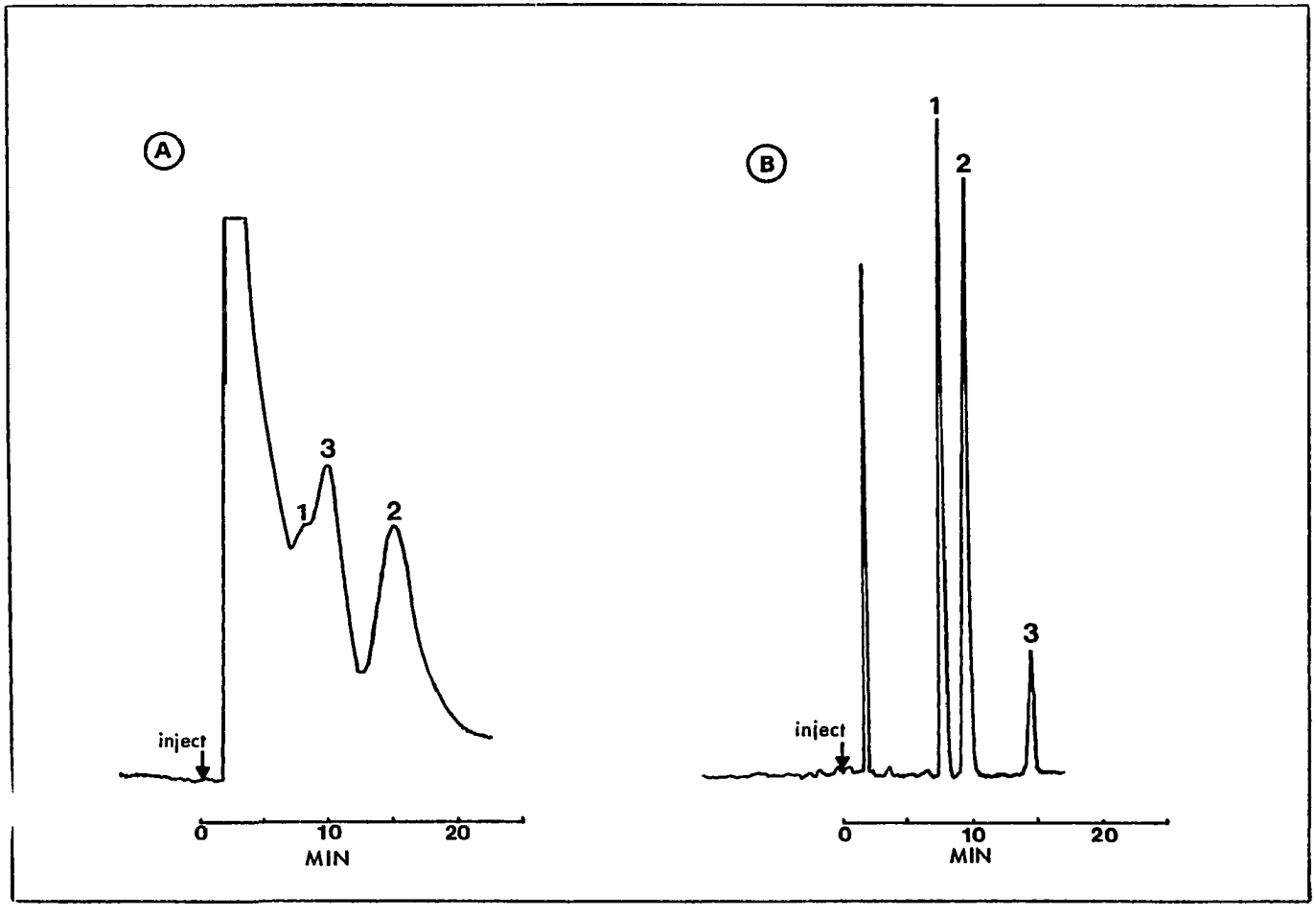


Figure 1-5. The Effect of Band Broadening on the Separation of a Three Component Mixture. Chromatogram A (left) was obtained from a low efficiency column where extensive band spreading occurred. Chromatogram B (right) was obtained from a high efficiency column employed to minimize band broadening. Peaks: DOPAC (1), 5-HIAA (2), and 5-HICA (3).

$$R = \frac{t_2 - t_1}{4\sigma} \tag{1-18}$$

An example illustrating how R is obtained is presented in Fig. 1-6. For optimal analytical purposes, very little overlapping should occur. This corresponds to a minimum resolution of 1.5, for which the purity of neighboring peaks exceeds 99%.[71]

The primary goal of any chromatographic procedure is to adequately separate and resolve the individual compounds of interest in a complex mixture. The primary task of the chromatographer is to establish the chromatographic parameters necessary to provide this resolution. A modified form of equation 1-18 more conveniently exhibits the experimental parameters involved. This can be derived from the equations already discussed. Assuming that the efficiencies are approximately equal for two adjacent peaks, eqs. 1-10 and 1-18 are combined to yield

$$R = \left(\frac{\sqrt{N}}{4}\right)\left(1 - \frac{t_1}{t_2}\right) \tag{1-19}$$

The ratio of retention times, t_1/t_2, for two different solutes can be obtained from eq. 1-8:

$$\frac{t_1}{t_2} = \frac{(k_1'+1)}{(k_2'+1)} \tag{1-20}$$

Substituting this into equation 1-19 we obtain

$$R = \left(\frac{N}{4}\right)\left(\frac{k_2' - k_1'}{k_2'+1}\right) \tag{1-21}$$

Finally, using equation 1-9, we arrive at the following important relationship:

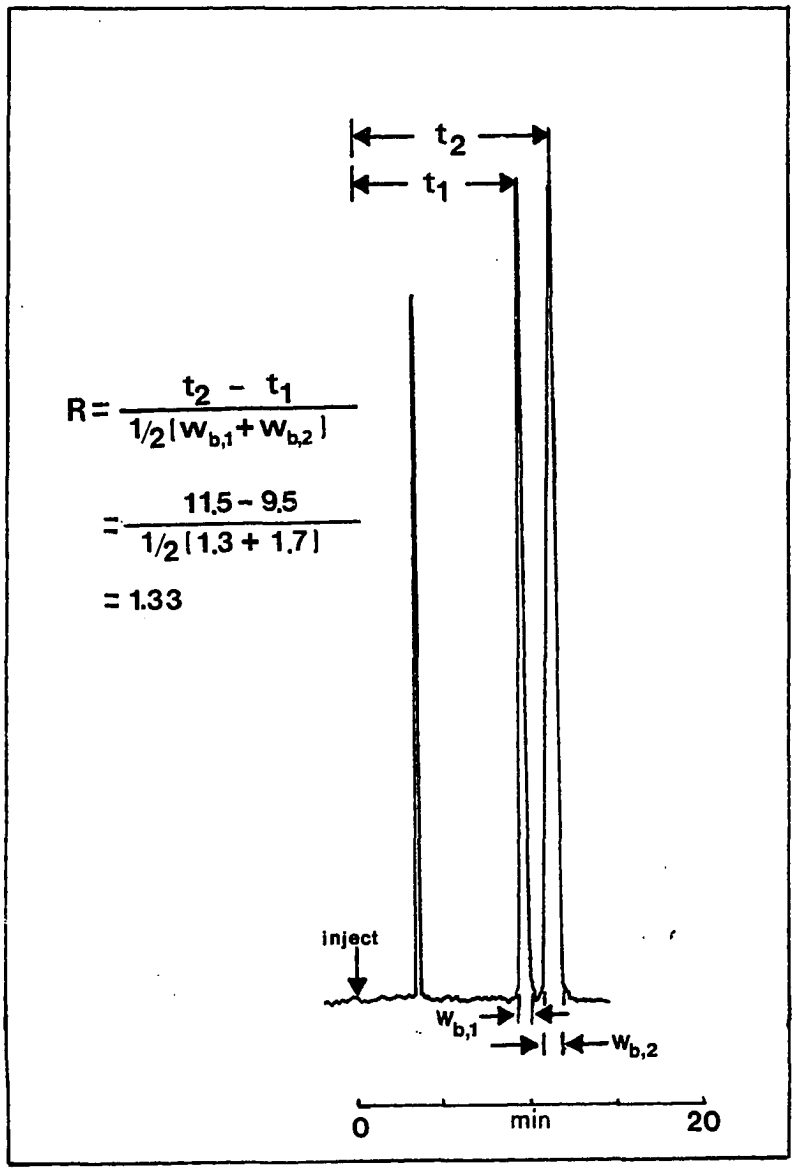


Figure 1-6. Calculation of the Resolution, R, of Two Neighboring Chromatographic Peaks. The width of the peak, W_b, was obtained by drawing tangential lines down each side of a peak until they intersect the baseline. W_b is the distance between these two lines measured at the baseline.

$$R = \left(\frac{\sqrt{N}}{4}\right)\left(\frac{\alpha-1}{\alpha}\right)\left(\frac{k'_2}{1+k'_2}\right) \qquad (1\text{-}22)$$

From eq. 1-22, we see that the resolution is a rather direct function of three experimental parameters: N, k' and α. An increase in any one of these parameters should, therefore, increase the resolution between adjacent chromatographic peaks. It follows that lengthening the column, reducing the particle size, or decreasing the flow rate should cause an improvement in R. Capacity factors and separation factors can be altered by changes in the nature of either the stationary phase or the mobile phase. In the following pages, we report adjustments of these two items which were undertaken in an attempt to separate and resolve ten biologically important catecholamine and indoleamine related compounds.

III. MATERIALS AND METHODS

A. Liquid Chromatographic System.

Pump. A Waters (Milford, MA.) Model 6000 double reciprocating pump was used for solvent delivery.

Injector. A Waters Model U6K was fitted with a 20 microliter fixed volume loop.

Column. This was a 150x4.6 mm reverse phase Ultrasphere column packed with 5 micron, octadecyl-coated spherical particles. It was purchased from Beckman (Irvine, CA) as part no. 235330.

Detector. The detector was homemade by our local machine shop from one-half inch thick plexiglass sheets (Fig. 1-7). A 5 mil teflon spacer was placed between the two machined plastic blocks. A notch cut in the spacer served as solvent path. This unit was obtained from

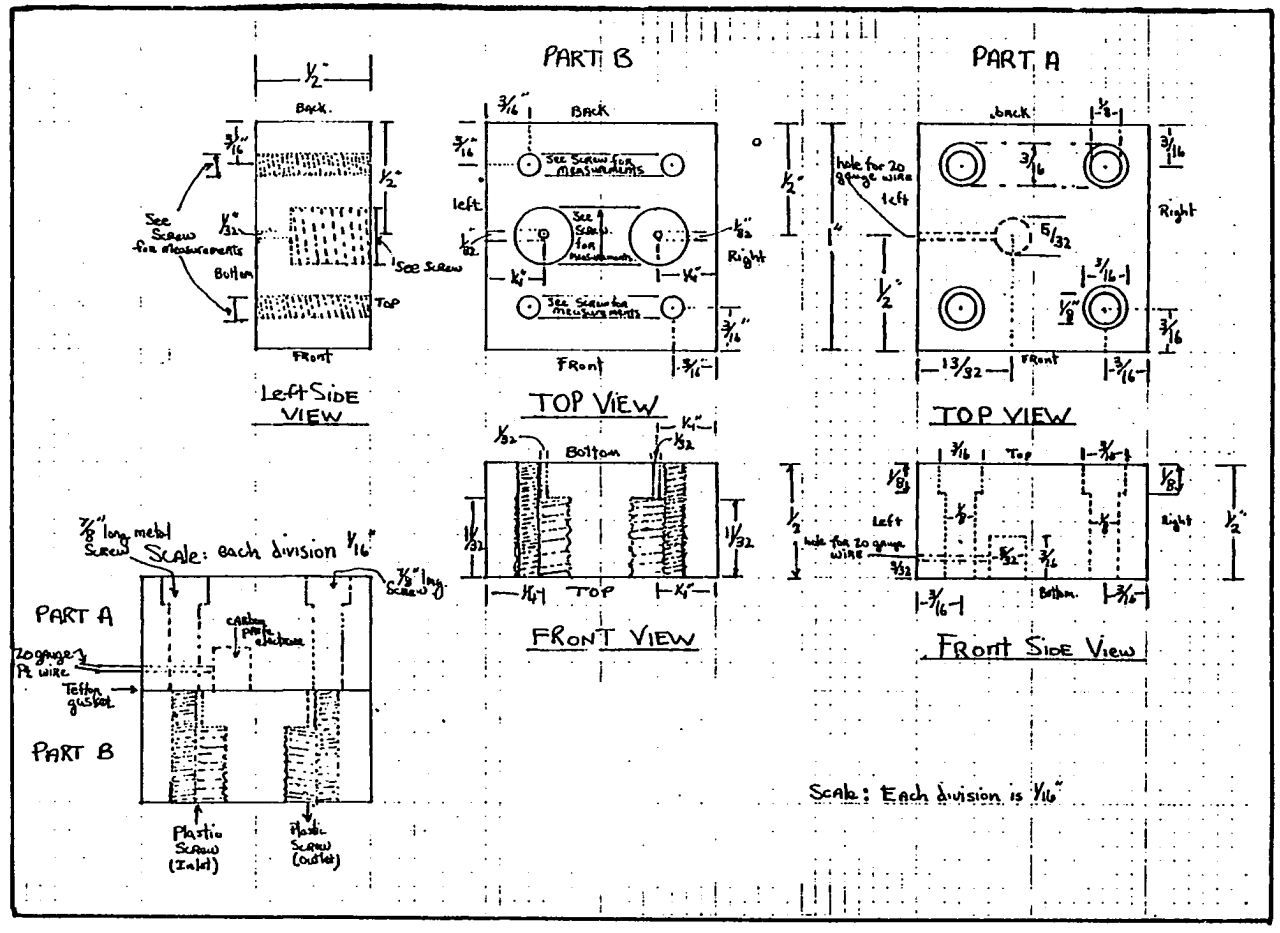


Figure 1-7. Diagram for the Construction of an Electrochemical Detector.

BioAnalytical Systems (W. Lafayette, IN) as part no. TG-SM. The electrochemical detector was packed with carbon paste and operated at a potential of +0.80 volts vs. SCE.

Carbon Paste. 3 grams of carbon, obtained from Ultra Carbon (Bay City, MI) as part no. UCP-1-M, and 2 ml of mineral oil were placed into a 100 ml 24/40 ground glass round bottom flask. 50 ml of benzene were added and the solution was mixed well for ca. 10 min. The flask was placed on a rotary evaporator and the benzene removed. The last traces of solvent were removed by placing the flask containing the carbon paste on a high vacuum line for 1 hour.

Electronics. The potential controller and current monitoring unit was identical to that previously described.[4]

Reference electrode. A saturated calomel electrode (SCE) was prepared in a similar fashion to that described in reference 5. The SCE was separated from the chromatographic stream by a piece of 3 mm Vycor rod held onto the tip of the electrode with a piece of teflon tubing. The holder for the SCE was obtained from BioAnalytical Systems (W. Lafayette, IN) as part no. RC-1.

Syringe. A 50 µl P/S Pressure-Lok syringe, series B-100, with a 0.020 inch o.d. needle was obtained from Supelco (Bellefonte, PA).

Strip Chart Recorder. An Omniscribe dual pen recorder was obtained from Houston Instruments (Austin, TX).

Filtration Device. A Millipore 47 mm filtering apparatus fitted with a stainless steel screen was obtained from Millipore (Bedford, MA) as part no. XX10 047 30. The filters used for purifying the chromatographic solvent were also obtained from Millipore as part

no. HAWP 047 00.

B. Reagents.

All reagents were purchased at the highest available purity and used without subsequent purification. The individual compounds were obtained from the following sources:

From Aldrich Chemical Co. (Milwaukee, WI):

3,4-dihydroxyphenylacetic acid (DOPAC)

3-hydroxytyramine hydrochloride (DA)

3-(3,4-dihydroxyphenyl)-DL-alanine (DOPA)

3-methoxy-4-hydroxyphenylacetic acid (homovanillic acid) (HVA)

serotonin(5-hydroxytryptamine)creatinine sulfate monohydrate (5-HT)

5-hydroxyindole-3-acetic acid (5-HIAA)

DL-5-hydroxytryptophan (5-HTP)

norepinephrine hydrochloride (NE)

epinephrine (EPI)

3-O-methyltyramine hydrochloride (3-MT)

3,4-dihydroxybenzylamine hydrobromide (DHBA)

5-hydroxy-N_{ω}-methyltryptamine oxalate (nMET)

3,4-dihydroxyphenylglycol (DOPEG)

From Sigma Chemical Co. (St. Louis, MO):

normetanephrine hydrochloride (NORMET)

5-hydroxytryptophol (5-HTOL)

From Pfaltz and Bauer (Flushing, NY):

3-methoxy-4-hydroxymandelic acid (VMA)

3,4-dihydroxymandelic acid (DOMA)

From Eastman Kodak (Rochester, NY):

Sodium octyl sulfate (SOS)

Structures for all the above compounds except SOS can be found in Table 1-1 (vide supra). All other reagents were purchased at the highest available purity from commercially available sources. The water used was double distilled from an all glass apparatus.

C. Solutions.

Mobile Phase. The chromatographic buffer was prepared by dissolving 117.64 g of trisodium citrate dihydrate (0.050 M) and 84.04 grams of citric acid monohydrate (0.050 M) into 8.0 liters of distilled water. Portions of this bulk solution were used to prepare the various mobile phases that were tested. The pH was adjusted by the dropwise addition of 1 M NaOH or 2 M HCl while monitoring with a pH meter. Methanol was added, as appropriate, for various test solutions. Prior to use, the solution was passed through a 0.45 micron Millipore filter.

Stock solutions. Solutions of the catecholamine- and indoleamine-related compounds were individually prepared by dissolving ca. 2 mg of each compound into 100 ml of deoxygenated water.[78] Portions of these solutions were stored in small vials at -4°C for future use.

IV. RESULTS

A. Optimization of N.

Our initial work with the various metabolites of catecholamines and indoleamines began in 1978 with an attempt to quantitate DOPAC and 5-HIAA in brain tissue. A 27-35 μm anion exchange packing material

called Zipax SAX (DuPont, Wilmington, DE) was selected for this work. The resin was hand-packed into glass columns according to the method of Kirkland.[81] A typical chromatogram resulting from an ethyl acetate extract of brain tissue is shown in Fig. 1-8A. Note the extremely broad and overlapping peaks. Also note that the peak for DOPAC is only barely discernible as a shoulder on the peak for the internal standard, 5-HICA. The value for 5-HIAA in whole mouse brain determined using these conditions was 435±111 ng/gram wet weight (n=11). Although this value was in fairly close agreement with those reported by others using alternative procedures,[69,85] it was felt that an improvement could certainly be made to increase the amount of available data from the chromatogram. As luck would have it, a somewhat used reverse phase column was found and inserted into our chromatographic system. The reasons for considering reverse phase chromatography were: 1) the rapidly expanding availability of microparticulate (5-10 μm particle diameter, reverse phase) columns, 2) the excellent stability of the columns toward organic-aqueous solvents, 3) the capability of reverse phase columns to operate as either cation or anion exchange columns through the addition of ion-pairing reagents, and 4) the capability of these columns to withstand a pH range of 1 to 7.

1. Effect of Particle Size on N.

Our first reverse phase column was a 300 x 4.6 mm stainless steel unit packed with octadecyl-coated, 10 μm silica particles. A typical chromatogram obtained from an ethyl acetate extract of whole mouse brain tissue is shown in Fig. 1-8B. Note the considerable improvement in peak capacity for the 10 μm reverse phase column as

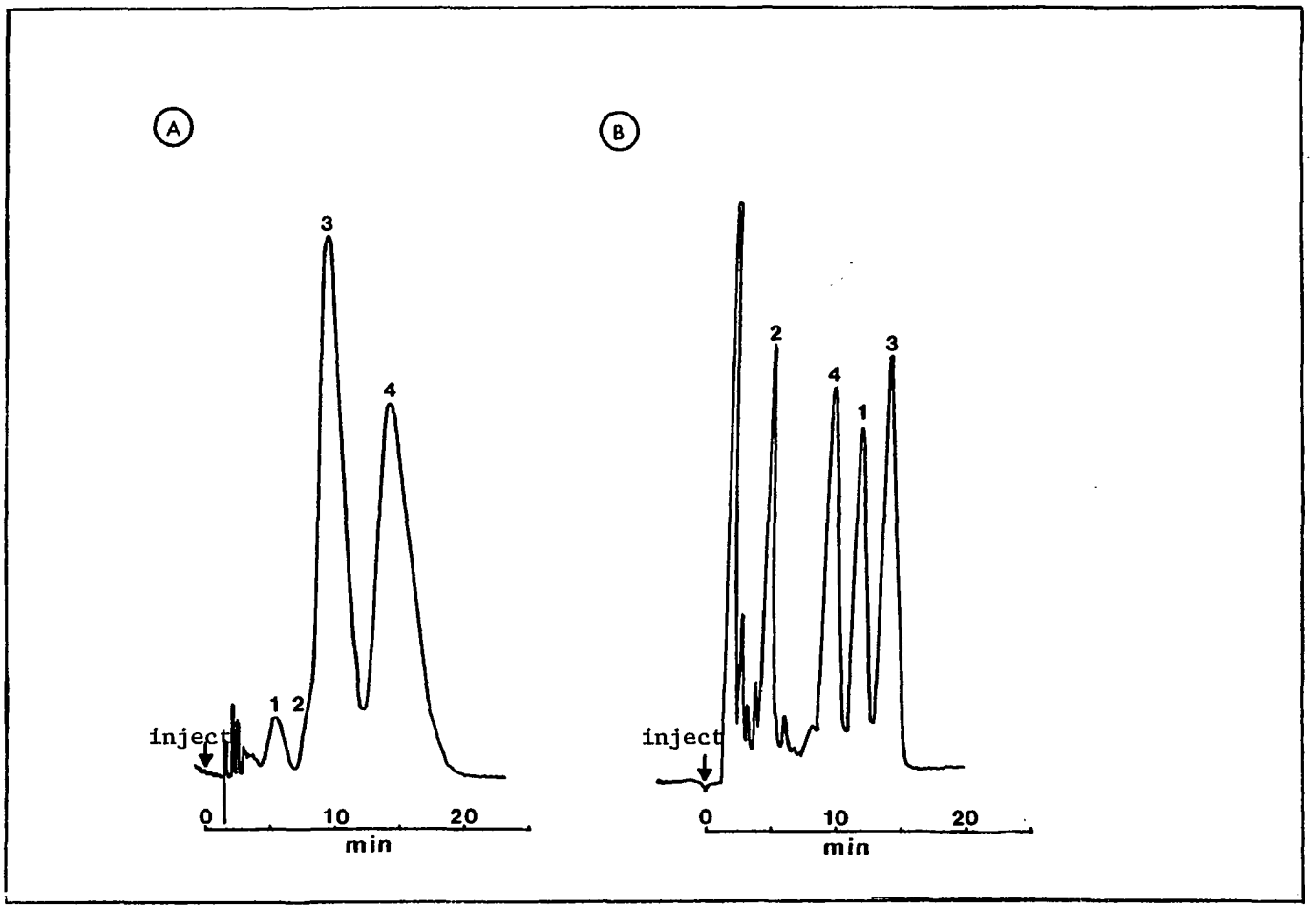


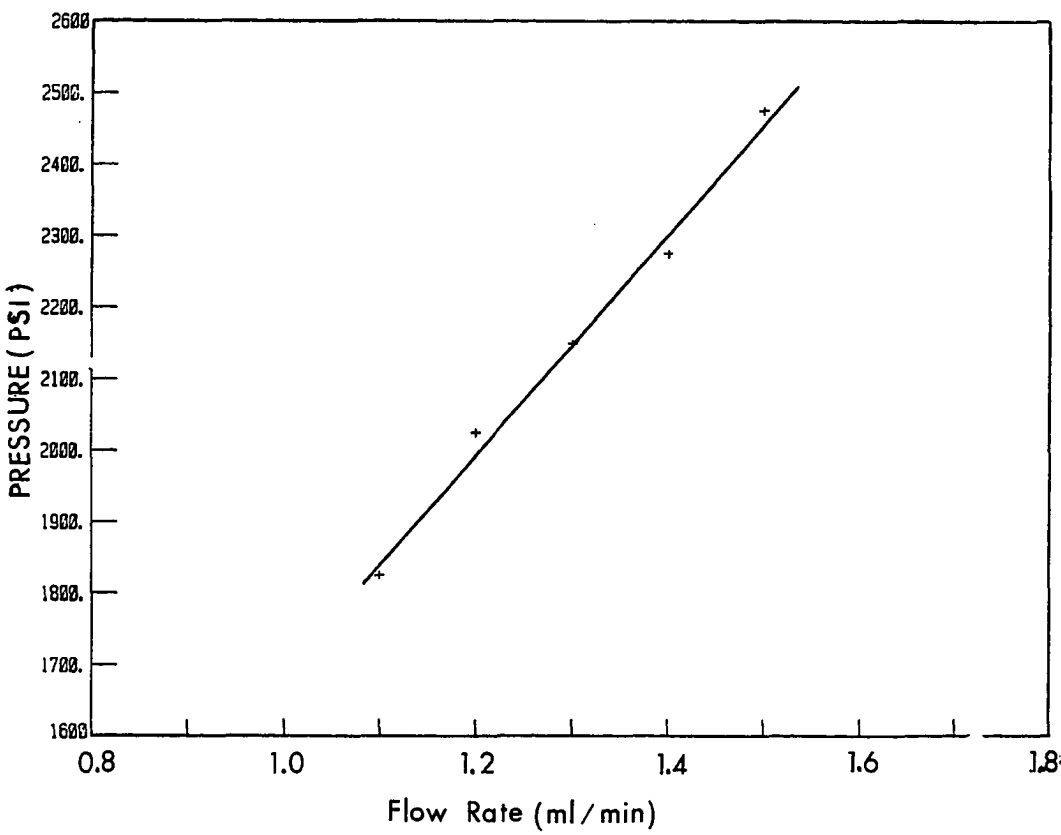
Figure 1-8. The Effect of Particle Size on Chromatographic Resolution. The figure illustrates chromatograms obtained from two columns packed with stationary supports of different sizes. Fig. A is from a hand packed 30-40 micron bed. Fig. B is from a commercially prepared reverse phase column containing 10 micron particles. N, as calculated from peak four, is 450 and 900 for Fig. A and B, respectively. Peaks: HVA (1), DOPAC (2), 5-HICA (3), and 5-HIAA (4).

compared to the previously employed Zipax resin of Fig. 1-8A. Four compounds are now easily resolved. Although this separation was obviously encouraging, we still had some reservations about using these columns. The disadvantages of such reverse phase columns are: 1) higher operating pressures require the use of all-metal fittings and tubings, 2) the cost of a typical reverse phase column is considerably higher than the hand-packed glass columns, and 3) microparticulate columns are much more easily clogged by particulate matter. However, after careful consideration, we felt that the potential increase in data obtained per sample clearly outweighed these relatively minor problems.

2. Limitations of Flow Rate and Column Length.

Both N and R, as seen in equations 1-16 and 1-22, increase with increased column length. But, extensive increases in L eventually lead to unacceptably large back pressures. Increasing the flow rate, as seen in Fig. 1-9, similarly leads to excessive operating pressures. Thus, increases in either length or flow rate rapidly reach the pressure limitations of the equipment. In particular, the noise dampening component of the system is rated at a maximum pressure of only 3000 p.s.i.

It should also be noted that there exists an optimal flow velocity, ν_{opt}, at which the system exhibits the maximum number of theoretical plates for the components of interest. However, it is usually found that the retention times for the most strongly retained components are excessively large at ν_{opt}. The increased time


2600
2500.
2400.
2300.
2200.
2100.
2000.
1900.
1800.
1700.
1600
PRESSURE (PSI)
0.8
1.0
1.2
1.4
1.6
1.8
Flow Rate (ml / min)

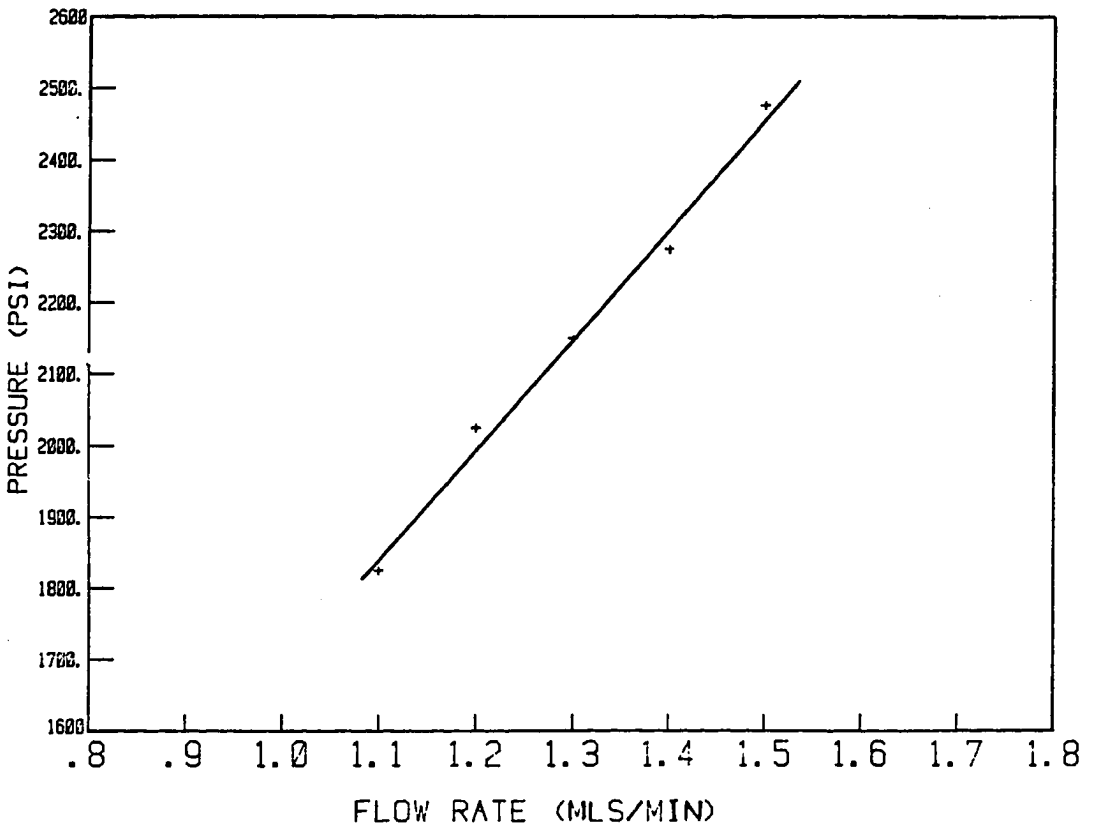


Figure 1-9. The Effect of Flow Rate on Chromatographic Operating Pressures. The data was obtained from a 30 cm μBondapak ODS column packed with 10μ particles.

required for each injection drastically restricts the number of samples which can be conveniently run in a single day. Thus, optimal resolution must be partially sacrificed for greater separation speed by using higher flow rates. The compromise for these conflicting needs was a 150 x 4.6 mm column with 5 μm particles operated at greater flow rates than the 300 x 4.6 mm column with 10 μ particles. This unit contained enough plates to adequately separate the components of interest, and, by increasing the flow from 1.1 to 1.5 ml/min, a considerable savings in total analysis time was achieved.

B. Optimization of Separation by Changes in the Mobile Phase.

Three mobile phase parameters were extensively investigated to find the optimal k' values for the individual components of interest. These were the solvent strength, the pH and ion-pairing agents. After adjustment of the tested parameter, the system was allowed time to reestablish equilibrium. This required one hour for changes in solvent strength and pH and six hours for adjustments in the ion-pairing agent. Subsequent assessment of retention time, t_r, and half-peak width, $W_{b/2}$, values were employed in equations 1-8, 1-9, 1-12, and 1-22 to determine k', α, N, and R. The following discussion focuses on k' since this is the fundamental value which causes alterations in α, N, and R.

1. Solvent Strength.

Changes in k' can be readily produced by the addition of miscible organic solvents to aqueous buffers used as the mobile phase. Adding organic solvents generally leads to a decrease in k' with reverse phase systems. This is primarily due to an increase in

solubility of the partially hydrophobic solute molecules in the mobile phase. Using methanol as the mobile phase organic modifier, we obtained the results shown in Figs. 1-10 and 1-11. For all the compounds examined, the k' values asymptotically approached zero with increasing concentrations of MeOH. It is important to note that the observed elution order was independent of the alcohol concentration. An increase in methanol only served to simultaneously decrease the retention times for all the components. The maximum limit for MeOH when using a carbon paste electrode was judged to be 10% on a volume/volume basis. Higher concentrations destroy the electrode by dissolving the Nujol used as a binder.

2. pH Adjustments and Ion Suppression.

Hydrogen bonding between the aqueous mobile phase and a charged solute results in a greater solubility of the solute in the mobile phase. The degree of hydrogen bonding can be very precisely adjusted by using buffered solvents having a pH within ±0.5 units of the pK_a of the solute. The effect of the mobile phase pH on k' values is illustrated for a number of relevant compounds in Fig. 1-12. The pH range of 3.5 to 5.5 produces dramatic changes in k' for the acid metabolites, while leaving the amine, neutral, and zwitterionic metabolites virtually unaffected. These results can be explained with the aid of Fig. 1-13. The pK_a values for carboxylic acids like the investigated metabolites are approximately 4.5.[82] As the pH is lowered, these compounds become protonated and, consequently, lose their negative charge. This causes a decrease in hydrogen bonding between the solute and the mobile phase.

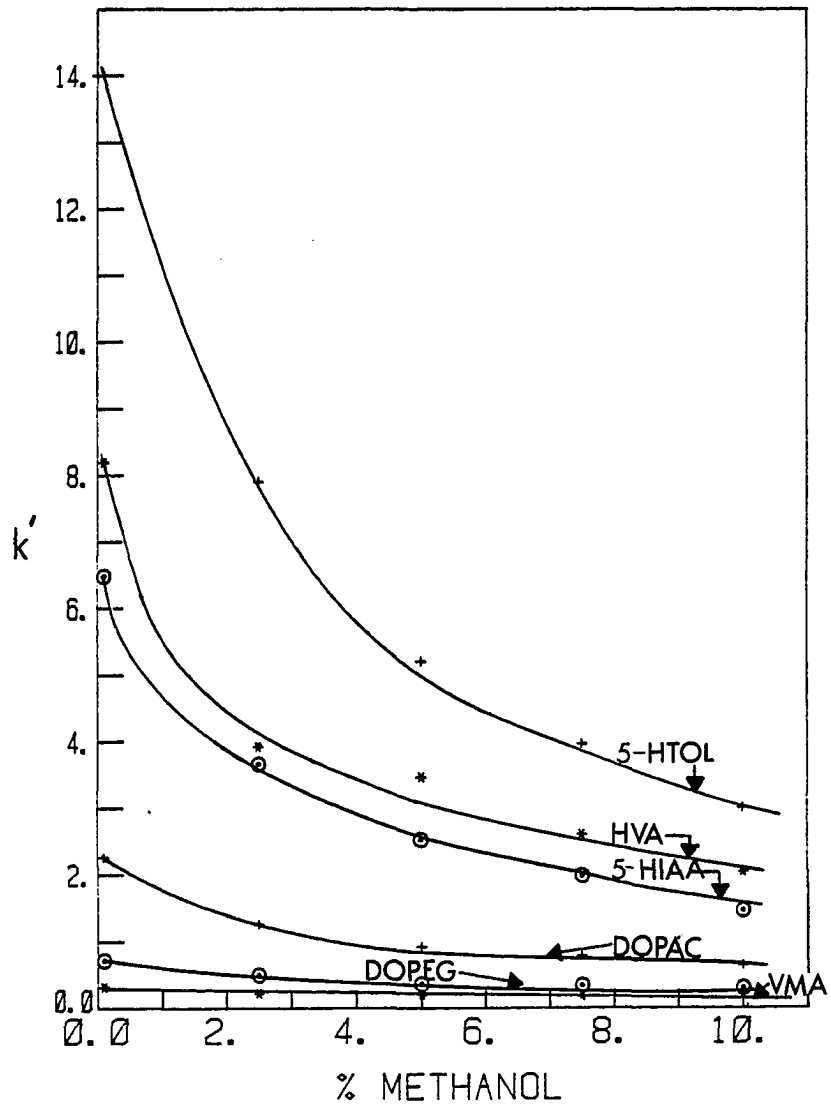


Figure 1-10A. The Effect of Methanol on k' for Various Acids and Alcohols. Chromatographic conditions: flow rate = 1.3 ml/min, citrate buffer (0.050 M, pH=5.0).

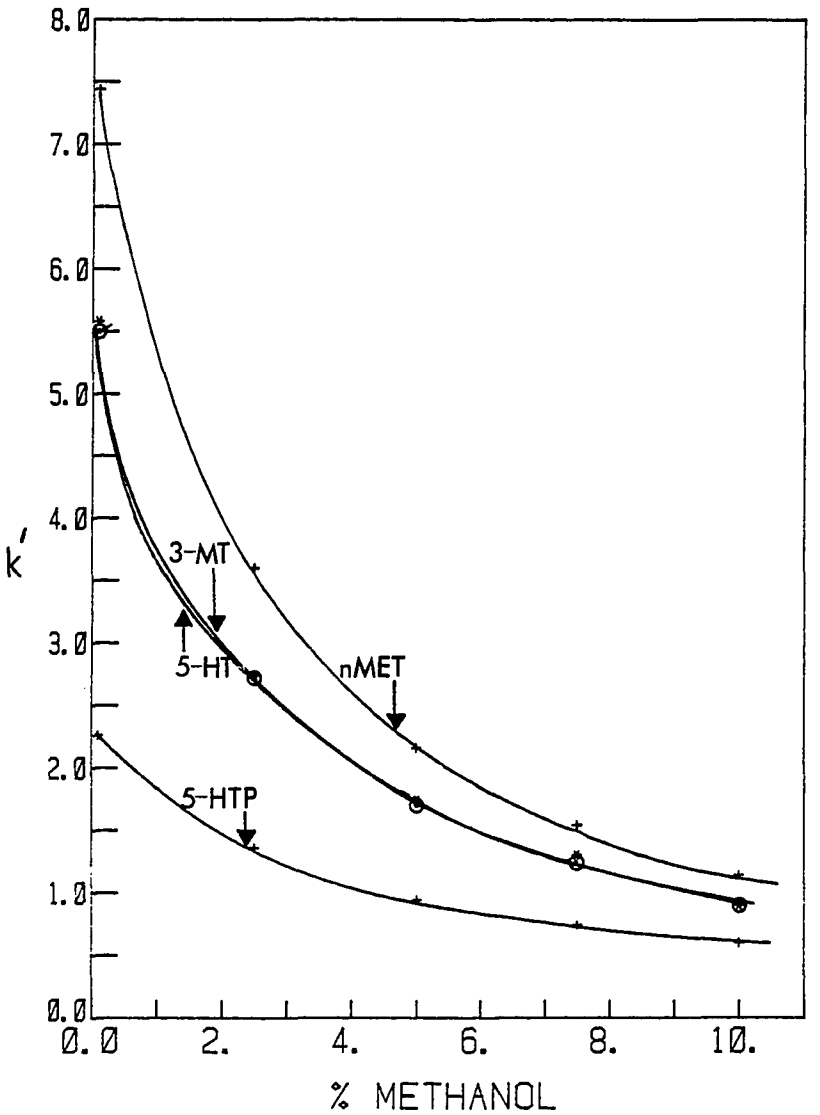


Figure 1-10B. The Effect of Methanol on k' for Various Amines and Amino Acids. Chromatographic conditions: flow rate = 1.3 ml/min, citrate buffer (0.050 M, pH=5.0).

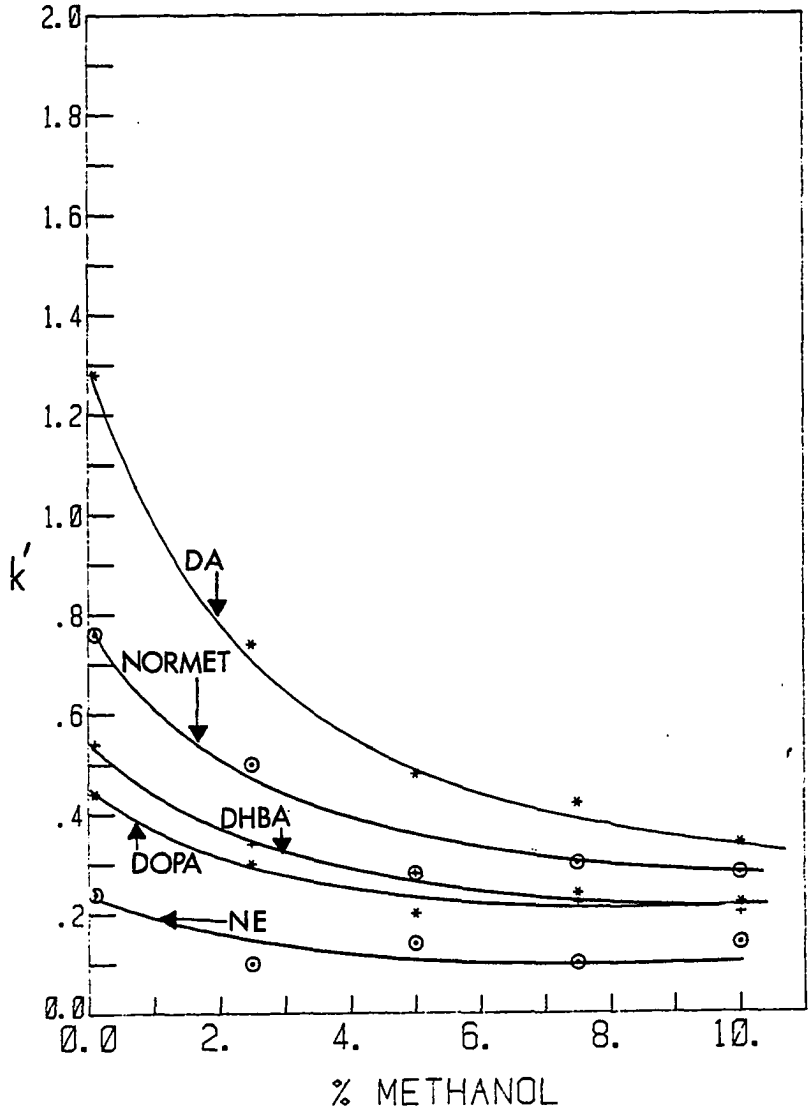


Figure 1-10C. The Effect of Methanol on k' for Various Amines and Amino acids. Chromatographic conditions: flow rate = 1.3 ml/min, citrate buffer (0.050 M, pH=5.0).

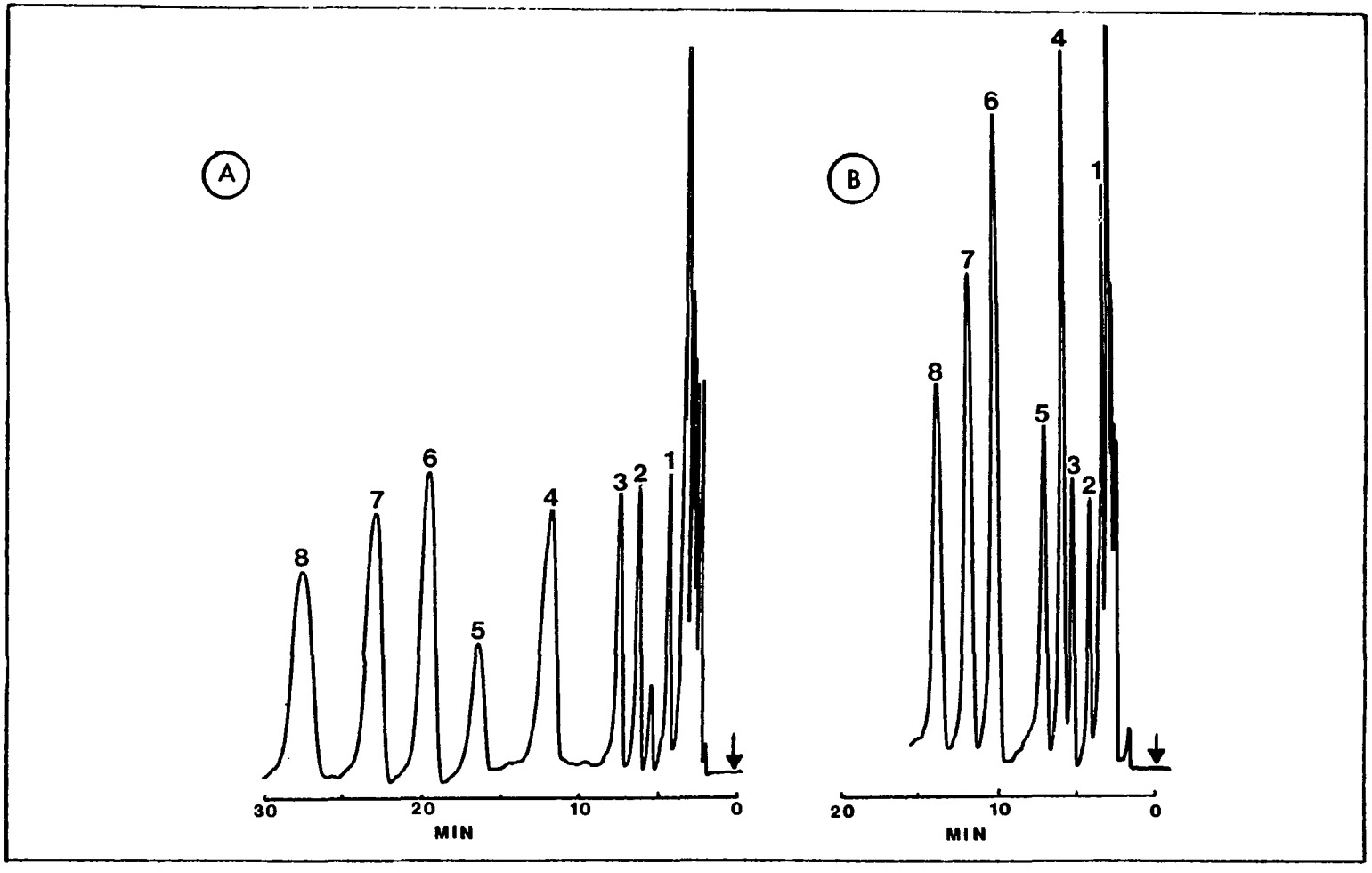


Figure 1-11. Typical Chromatograms Illustrating the Effect of Increasing the Strength of the Mobile Phase by the Addition of Methanol. Chromatographic conditions: 150 x 4.6mm Ultrasphere ODS column, flow rate = 1.3 mls/min, 0.10 M citrate buffer. Figure A - no methanol. Figure B - 10% methanol. Peaks: DA (1), 5-HTP (2), DOPAC (3), 5-HT (4), nMET (5), 5-HIAA (6), 5-HICA (7), and HVA (8).

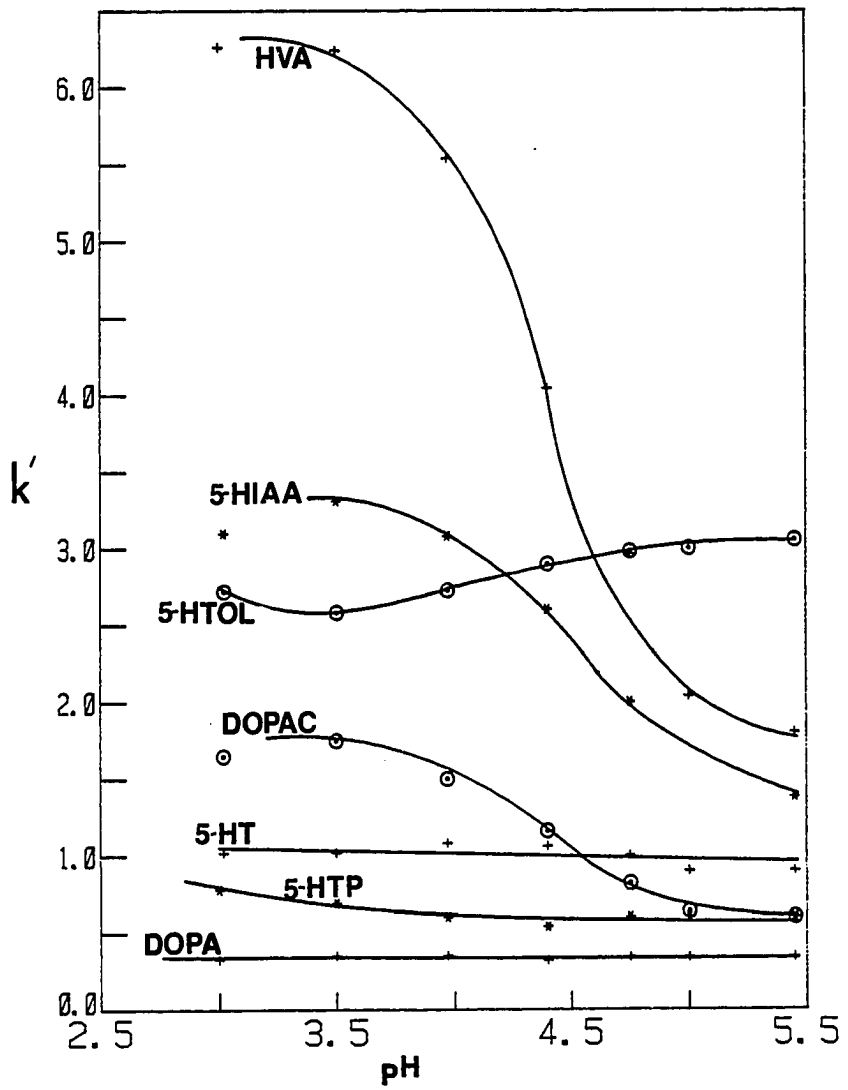


Figure 1-12. The Effect of pH on the k' of Selected Acids, Alcohol, Amines and Amino Acids. Chromatographic conditions: 10% methanol, flow rate = 1.3 ml/min, citrate buffer (0.10 M), pH = 4.6.

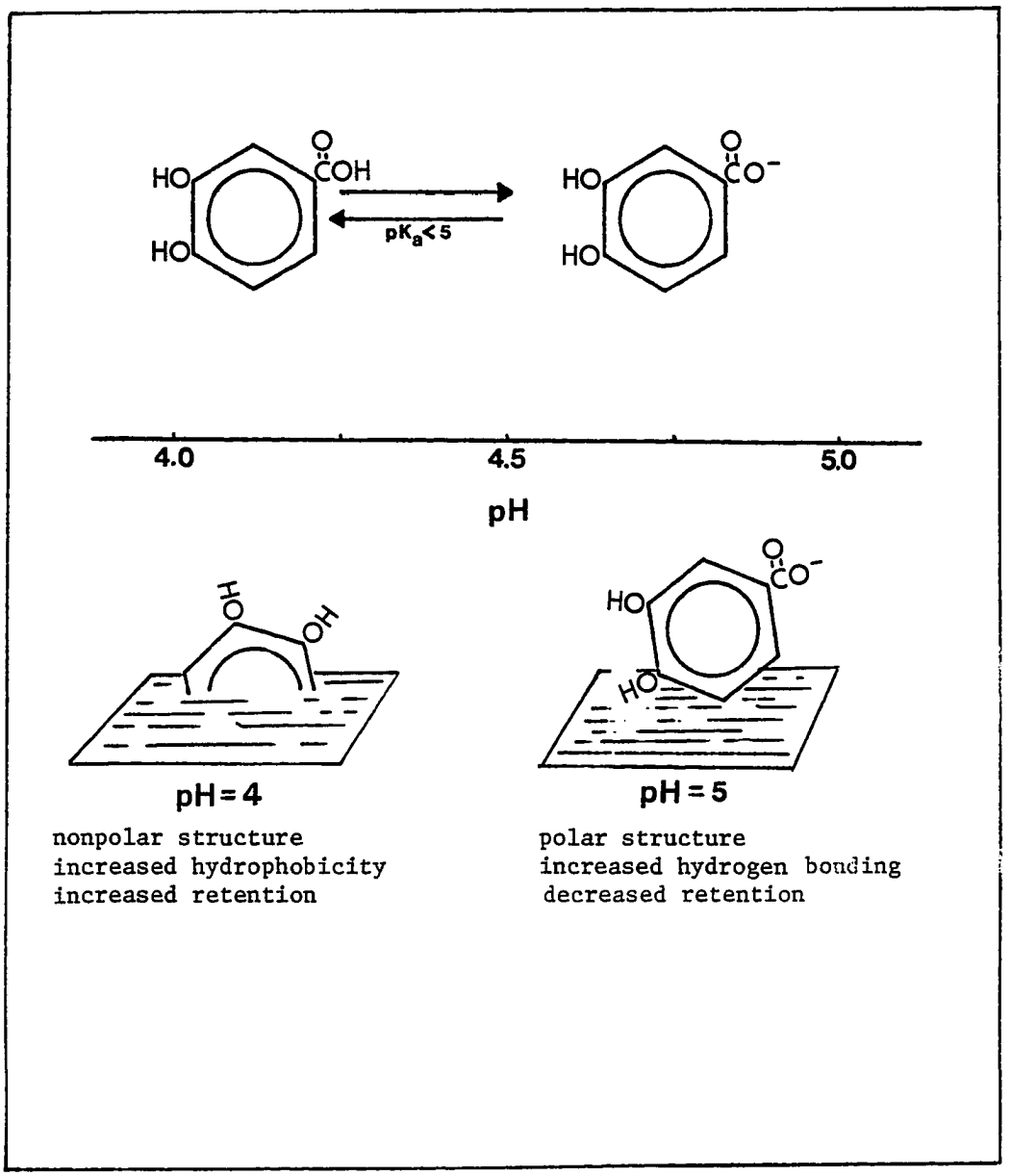


Figure 1-13. An Illustration Describing the Retention Mechanism of Ion Suppression for Weak Acids. At low mobile phase pH's(left), the acids are predominately protonated, creating an increase in their adsorption into the stationary phase. Removal of the acidic hydrogen by increasing the mobile phase pH, decreases the retention of the acid, primarily through an increase in hydrogen bonding of the solute with the mobile phase.

The resultant increase in hydrophobicity of the solute also leads to an increased solubility in the nonpolar stationary phase. The overall effect of lowering the pH is, thus, to drive the acidic compounds from the mobile phase into the stationary phase with a corresponding increase in k'. Controlling the capacity factor in this fashion is often referred to as ion-suppression.[83]

The implications of these results are fairly straightforward: we now have a means to adjust k' values for the acidic solutes while keeping k' virtually constant for the other components. A specific example of pH control is given in Fig. 1-14. In the chromatogram of Fig. 1-14A, 5-HIAA, 5-HICA, and HVA exhibit excessive retention times at a pH of 4.2. HVA is not shown in this diagram because of its extreme retention time. Increasing the pH of the mobile phase to 4.6 greatly reduces the overall time of analysis (Fig. 1-14B), while the separation of the non-acidic components remains essentially unchanged.

Although k' adjustments for the amines could also theoretically be achieved by pH variations in the range of 8-11, this practice is not recommended for silica-based columns. Literature supplied by the manufacturers suggests that these columns should not be operated with neutral or basic eluting solvents. The stationary phase is degraded by direct dissolution under these conditions as well as by chemical attack of the silyl bonds holding the alkyl group onto the stationary support. Therefore, alternative means had to be developed for manipulating the amine compounds.

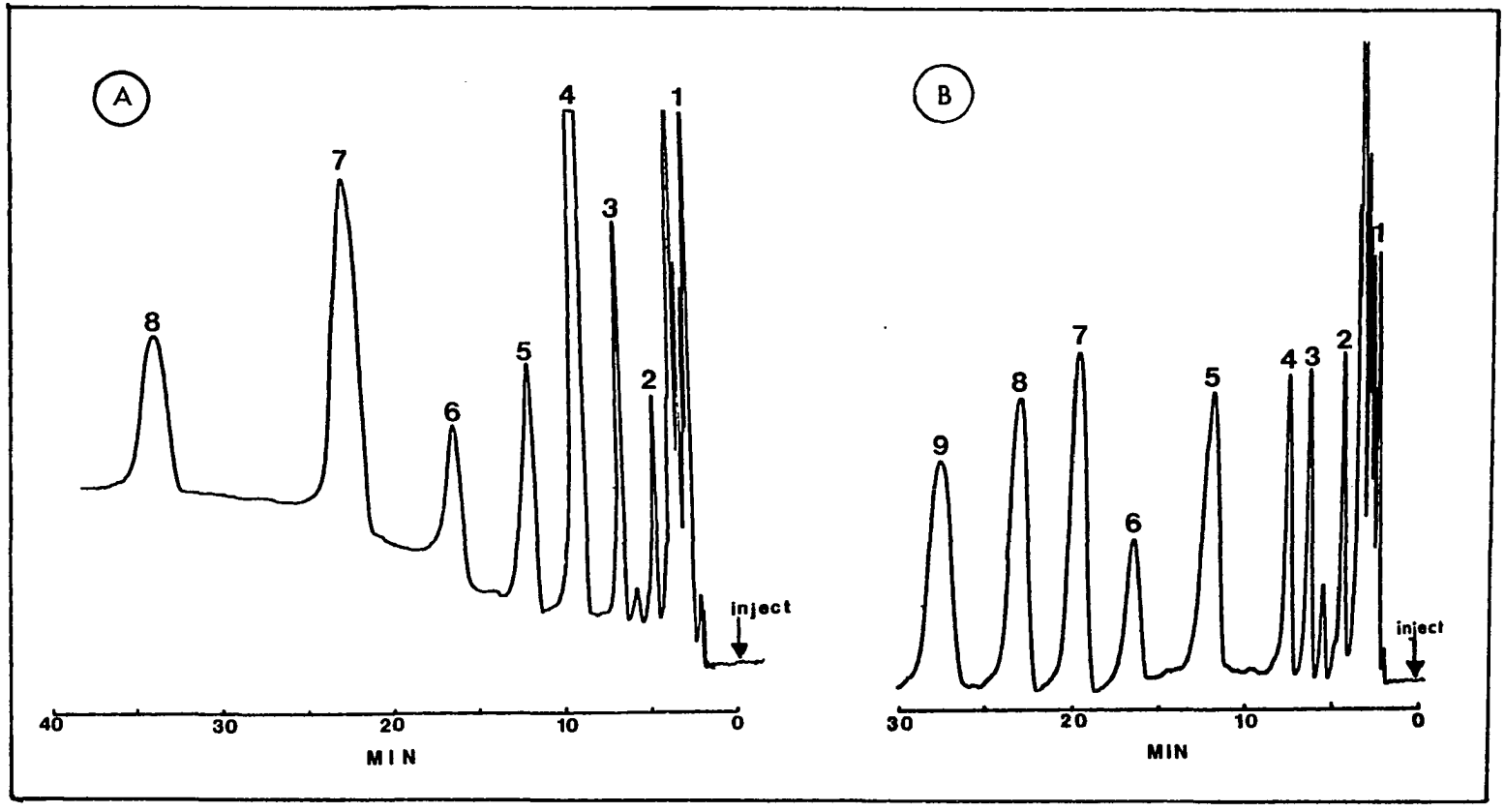


Figure 1-14. Typical Chromatograms Illustrating the Effect of the Mobile Phase pH on the Retention of Various Acids, Amines and Amino Acids. A pH shift from low to high values causes the carboxylic acids to shift from hydrophilic to hydrophobic species. Chromatographic conditions: 150 x 4.6 mm. Ultrasphere ODS column, flow rate = 1.3 mls/min, 0.10 M citrate buffer. Fig. A: pH = 4.2; Fig. B: pH = 4.6. Peaks: NE (1), DA (2), 5-HTP (3), DOPAC (4), 5-HT (5), nMET (6), 5-HIAA (7), 5-HICA (8), HVA (9).

3. Reverse Phase Ion-Pairing.

Ion-pairing is a chromatographic technique in which a reverse phase column is, in essence, transformed into an ion exchange column by the addition of ion-pairing agents to the mobile phase. These agents are typically long chain aliphatic species which have a highly polar ionic head group coupled to a nonpolar alkyl tail. Because of the extensive employment of similar compounds in detergents and cleaning solutions, this technique is sometimes referred to as "soap" chromatography.

The use of the term ion-pairing to describe this technique is believed to be a misnomer. This expression originated from early attempts to describe the retention mechanism involved with ion-pairing agents. The formation of ion-pairs between the solute and the agent within the mobile phase was presumably followed by extraction of the neutral complex into the stationary phase (Fig. 1-15).[86,87] However, work by Bidlingmeyer and others[83,84] has produced strong evidence against this model. The model which currently best explains the retention mechanism in ion-pairing chromatography incorporates formation of a charged layer of lipophilic ions (ion-pair agents) on the surface of the stationary phase (Fig. 1-15B). Associated with this primary ionic layer is an oppositely charged layer of counterions. Solute molecules having a charge similar to these counterions are attracted to the surface of the chromatographic support where they exchange with the counterions. Their retention by the modified stationary phase involves a combination of both electrostatic and dispersion forces. Increasing the concentration of ion-pairing agents in the mobile phase leads to an increased concentration of these agents in the stationary

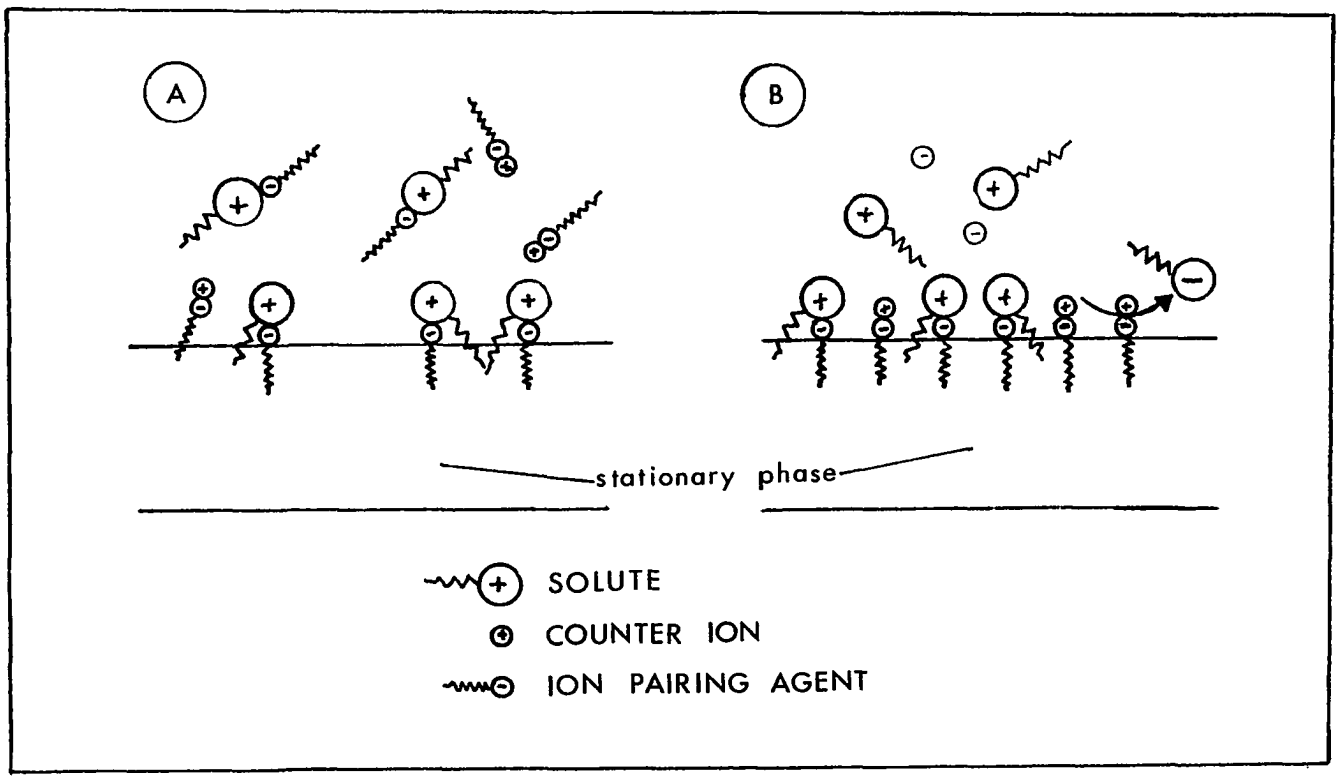


Figure 1-15. Diagram Depicting Two Theories on the Retention Mechanism of Ion Pair Chromatography. Figure A represents the ion pair model. Figure B illustrates the ion interaction model proposed by Bidlingmeyer.[84]

phase. This increases the charge density on the surface of the stationary phase and the electrostatic attraction of the oppositely charged solutes. The opposite, although less dramatic, effect occurs for ionic solutes having the same charge as the ion-pairing agent. Electrostatic repulsion of these solutes will drive them away from the stationary phase, resulting in a reduction of k' values. Common ion-pairing agents include n-alkylsulfonic acids and n-alkyl-quaternary ammonium salts, which are used to enhance retention of cations and anions, respectively.

The effect of sodium octyl sulfate (SOS) on k' values is shown in Figs. 1-16A and 1-16B. As can easily be seen, retention is greatly enhanced for the amines with increasing concentrations of SOS; the k' values for the amino acids are slightly increased, while those for the neutral and acidic metabolites are not affected or slightly decreased. It should be noted that this study was conducted at a mobile phase pH of 2.55. The acidic metabolites, exhibiting only slightly decreased retention times with increasing concentrations of SOS, would experience somewhat greater decreases at a moderately higher pH. The zwitterionic amino acids, on the other hand, do show substantial k' increases with increasing SOS concentrations. These compounds possess pK_a values of ca. 2.5 for deprotonation of the cation to form the zwitterion. Thus, a significant fraction of these species exist in the cationic form. At considerably higher pH values, they would be reconverted to the zwitterionic form almost exclusively and be only minimally affected by changes in SOS concentration. The presence of SOS greatly alters the effect of pH on the amino

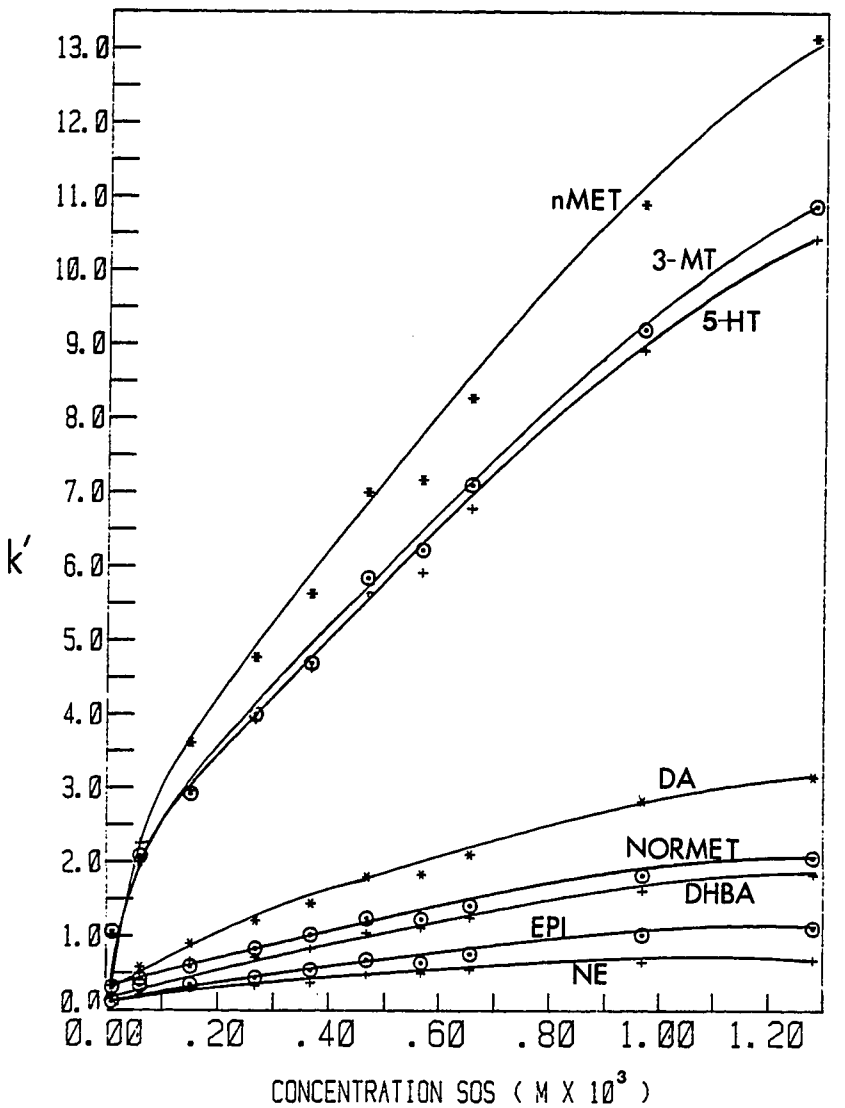


Figure 1-16A. The Effect of Sodium Octyl Sulfate (SOS) on k' for Various Amines. Chromatographic conditions: 150 x 4.6mm Ultrasphere ODS column, flow rate = 1.1 ml/min, 0.10 M citrate buffer, pH = 2.55, 10% methanol.

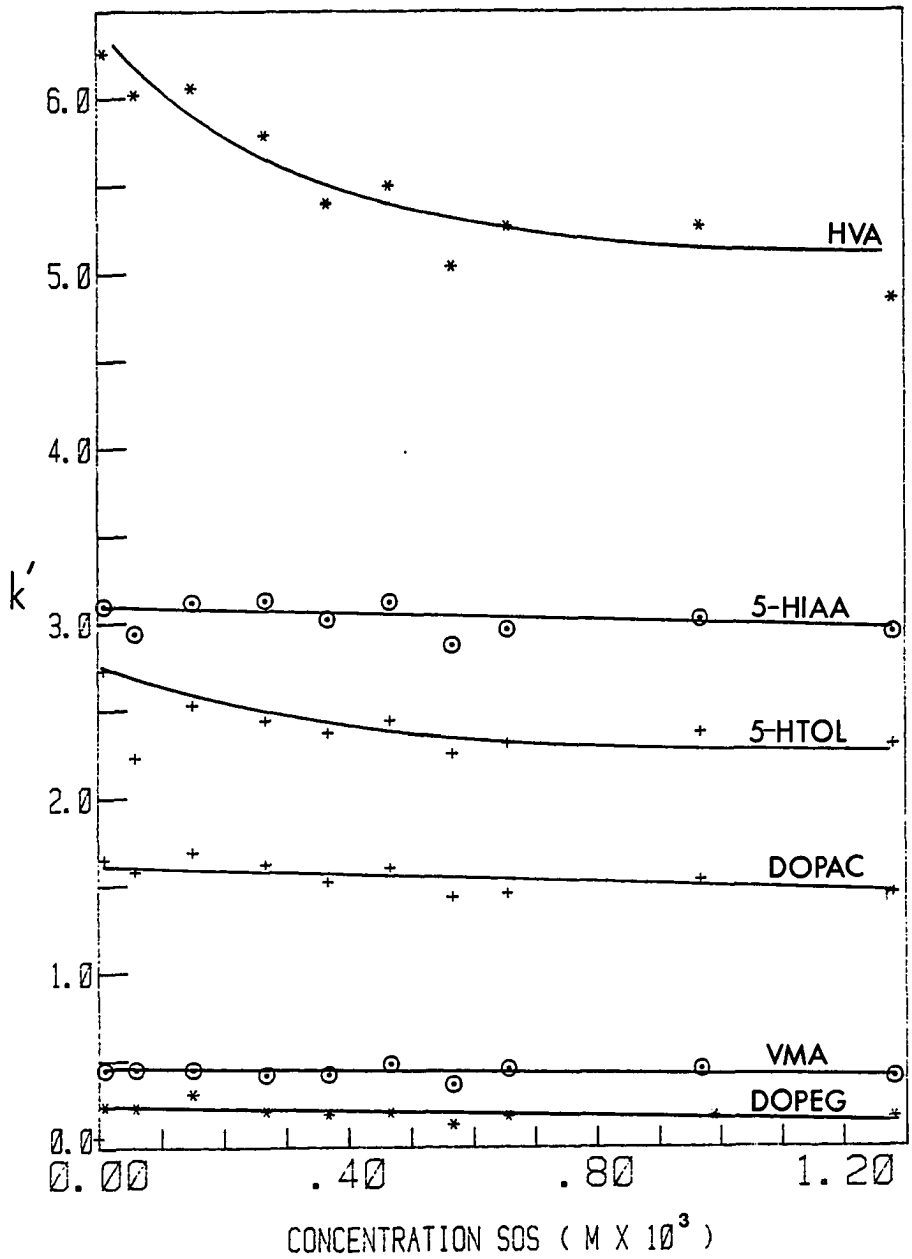


Figure 1-16B. The Effect of Sodium Octyl Sulfate (SOS) on k' for Various Carboxylic Acids and Alcohols. Chromatographic conditions: 150x4.6 mm Ultrasphere ODS column, flow rate = 1.1 ml/min, 0.10 M citrate buffer, pH = 2.55, 10% methanol.

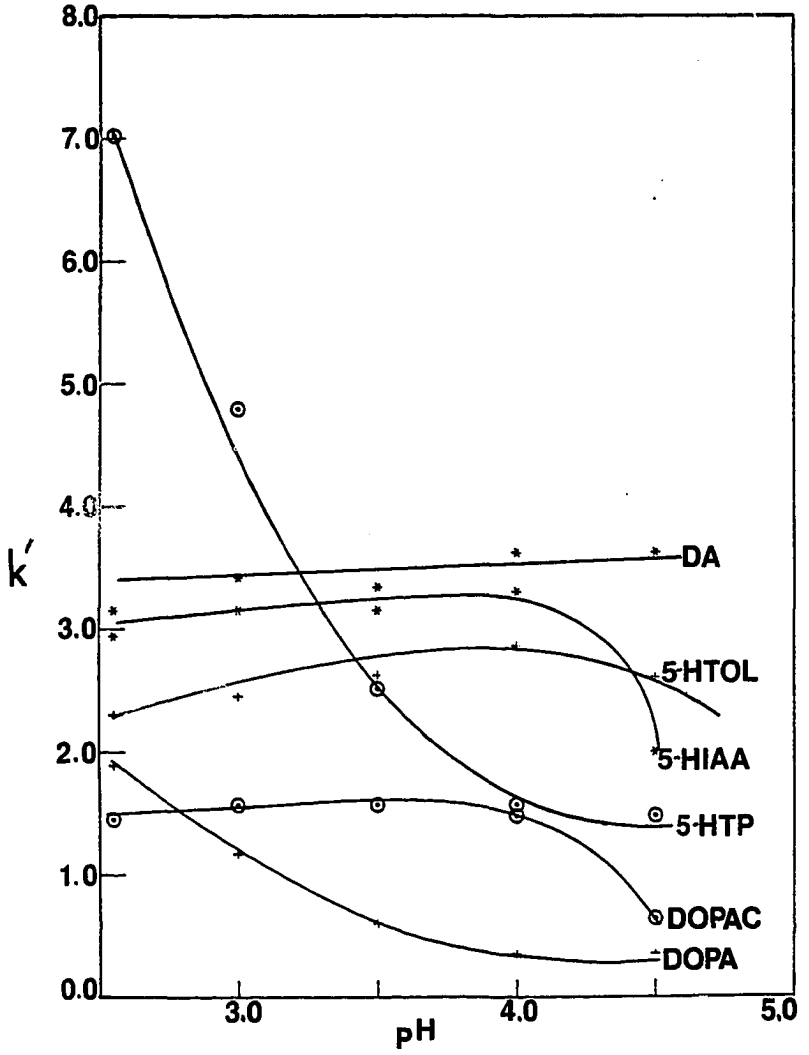


Figure 1-17. The Effect of pH on the Retention of Various Acids, Alcohols, Amines, and Amino Acids in Ion Pair Reverse Phase Chromatography. Chromtographic conditions: 150 x 4.6mm Ultrasphere ODS column, flow rate = 1.3 ml/min, 0.10 M citrate buffer, 1.28 mM SOS, 10% methanol.

acids and amines as shown in Fig. 1-17. The added SOS causes a significant increase in k' for the amino acids at low pH values where they exist as cations. Likewise, the higher pH values lead to a decrease in k' values for the acids since these species are shifted to their anionic form and, thus, repulsed by the negatively charged surface of the SOS-modified stationary phase.

V. CONCLUSION

Once the effects of these mobile phase parameters on k' values were established, the chromatographic conditions were varied to produce a rapid separation for the various catecholamine and indoleamine metabolites. Our efforts proceeded in approximately the following order.

1) A pH (ca. 4.5) was selected which separated the acids from one another aided by the ion suppression effect. No MeOH or SOS was present at this point in the study. This left many of the amines unresolved and very close to the solvent front.

2) SOS was added to the mobile phase to provide a separation of the amines. Small increements (1-3 ml) of a 0.030 M SOS solution were added to 2 liters of the eluting solvent until a satisfactory separation was achieved. This resulted in extremely long retention times for some of the desired compounds.

3) Methanol was added in small increments to decrease the total time required for each chromatogram.

4) Slight alterations of the mobile phase were performed in order to "fine tune" the resolution of any overlapping peaks.

A chromatogram obtained using the optimum mobile phase is shown in Fig. 1-18. The complete chromatographic conditions used

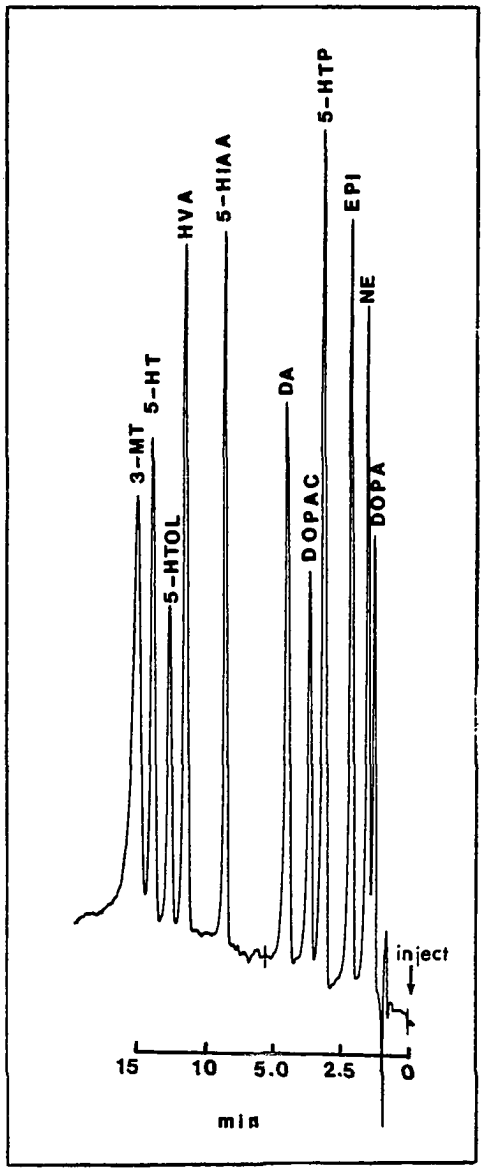


Figure 1-18. Chromatogram Obtained Using the Optimal Conditions for Separating Various Catecholamine and Indoleamine Related Metabolites.(Note change in time axis at 5.0 minutes.) Chromatographic conditions: 150 x 4.6 mm Ultrasphere ODS column, 5 micron particle size; the mobile phase consisted of 0.050 M citric acid, 0.050 M sodium citrate, 7.5% methanol, pH = 4.8; flow rate = 1.3 ml/min.

to produce this separation were:

Mobile Phase: 0.10 M citrate buffer, pH 4.8, containing 7.5% (volume/volume basis) methanol and 0.180 mM SOS.

Flow Rate: 1.3 ml/min.

Column: 150 x 4.6 mm Ultrasphere ODS, 5 μm particle diameter.

All commercially obtained columns are not exactly the same. Similarly, individual columns may deteriorate with use. Adjustment or readjustment of the various parameters to establish the appropriate resolution of individual components should rely on the data presented in this chapter. Modifications useful in separating compounds of a particular type from others of the same type or a different type are summarized in Table 1-2.

Table 1-2. Mobile Phase Adjustments for Separating Specific Groups of Catecholamines, Indoleamines, and Related Metabolites by Reverse Phase Liquid Chromatography

Compound Group	Compound Group			
	Acids	Amines	Alcohols	Amino Acids
Acids	1. pH change[a] (4-5)	1. pH change[a] (4-5)	1. pH change[a] (4-5)	1. pH change[a,b] (2-5)
	2. ion-interaction[c]	2. ion-interaction[c,d]	2. ion-interaction[c]	2. pH change[b] (2-3.5)
Amines		1. ion-interaction[d]	1. ion-interaction[d]	1. ion-interaction[d]
				2. pH change[b] (2-3.5)
Alcohols			1. solvent strength	1. pH change[b] (2-3.5)
Amino Acids				1. pH change[b] (2-3.5)

[a]Lower pH (appropriate range shown in parentheses) increases retention of acids. [b]Lower pH (appropriate range shown in parentheses) in combination with sulfonic acid ion-interaction agent increases retention of amino acids. [c]Addition of an aliphatic quaternary ammonium salt might be useful to increase the retention of acidic metabolites. [d]Addition of aliphatic sulfonic acid increases retention of amines.

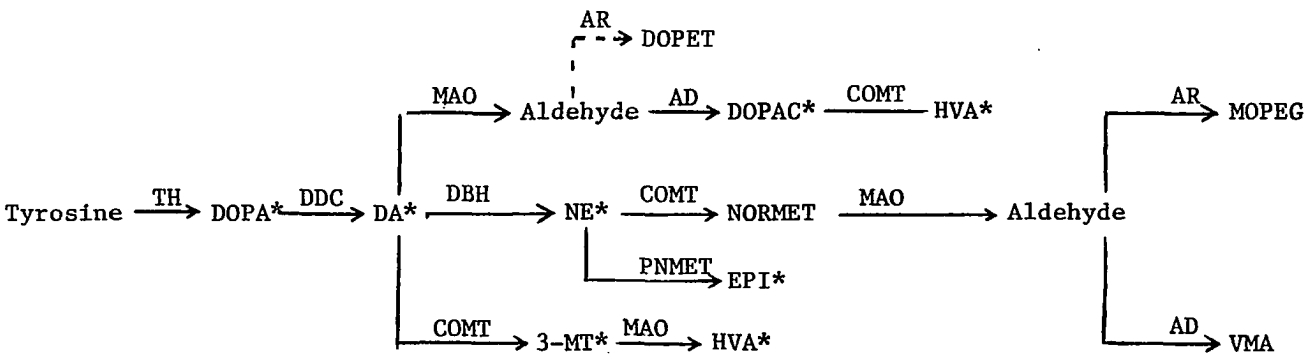
CHAPTER 2

THE DETERMINATION OF CATECHOLAMINES, INDOLEAMINES, AND RELATED METABOLITES IN TISSUE SAMPLES

I. INTRODUCTION

The wide interest in catecholamines, indoleamines, and related species by neuroscientists can be directly attributed to their functions as neurochemical transmitters, modulators, and hormones. Levels of these compounds in the body have been related to metabolic disorders associated with pathological states such as neuronal tumors,[105] hypertension,[104] and certain psychic syndromes.[51] The detailed involvement of these compounds in the area of mental disorders is currently under active investigation by many groups.

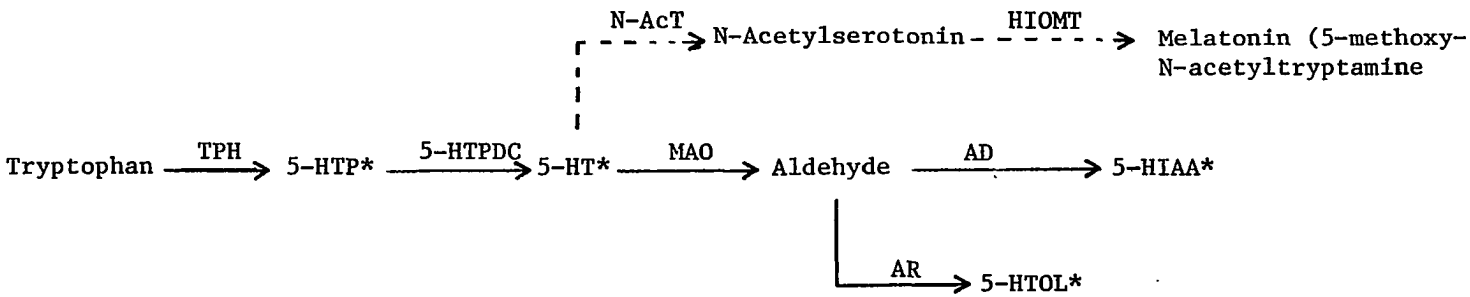
The biosynthesis of catecholamines and indoleamines originates with the dietary amino acids tyrosine (TYR) and tryptophan (TP), as shown in Figs. 2-1 and 2-2. The rate limiting step in the conversion of these amino acids to their bioactive amine derivatives is, in both cases, represented by the initial hydroxylation of the aromatic ring. The facile decarboxylation of the hydroxylated products directly yields dopamine (DA) and 5-hydroxytryptamine (serotonin, 5-HT), respectively. Subsequent side chain hydroxylation of dopamine by dopamine-β-hydroxylase affords norepinephrine (NE), while N-methylation of norepinephrine pro-



ENZYME LEGEND

TH	Tyrosine Hydroxylase	AD	Aldehyde Dehydrogenase
DDC	DOPA Decarboxylase	AR	Aldehyde Reductase
MAO	Monoamine Oxidase	PNMT	Phenethanolamine-N-Methyl-transferase
DBH	Dopamine β-Hydroxylase	COMT	Catechol-O-Methyltransferase

Figure 2-1. Biosynthesis and Degradation of Catecholamines. The bold arrows represent primary pathways. Complete names for compounds shown may be found in Table 1-1. Compounds with asterisk (*) are accessible for quantitation by the newly proposed method (vide infra).



ENZYME LEGEND

TPH	Tryptophan Hydroxylase	AR	Aldehyde Reductase
5-HTPDC	5-Hydroxytryptophan Decarboxylase	HIOMT	Hydroxyindole-O-Methyltransferase
		N-AcT	N-Acetyltransferase
MAO	Monoamine Oxidase		
AD	Aldehyde Dehydrogenase		

Figure 2-2. Biosynthesis and Degradation of Serotonin. Bold arrows represent primary pathways. Complete names for compounds shown may be found in Table 1-1. Compounds with asterisk (*) are accessible for quantitation by the newly proposed method (vide infra).

vides epinephrine (EPI). These biogenic amines are catabolized by a variety of neuronal enzymes. Oxidation of the side chain amine group and methoxylation of a ring hydroxyl group, however, represent the two primary degradative pathways.

Since these amines, as well as their precursors and metabolites, are normally found in brain tissues at only nanogram/gram or picogram/gram levels, analytical techniques having very low detection limits are fundamental to their quantitation. Techniques found to be adequate for this purpose include fluorescence,[77,96,100] gas chromatography with electron capture detection,[97] gas chromatography combined with mass spectrometry,[88,98] and radiochemical methods.[99] These techniques, unfortunately, suffer from a lack of selectivity, the need for chemical derivatization, or cost requirements which are prohibitive for routine analyses. The combination of liquid chromatography with electrochemical detection (LCEC), introduced by Adams and coworkers[63,78] in 1973, offered a considerable improvement in this situation. LCEC provided excellent selectivity through both chromatographic separation and the requirement for electrochemical activity at the chosen working electrode potential; it did not require derivatization and it was relatively inexpensive. Further, the electrochemical detector exhibited a very wide linear dynamic range[106] and low limits of detection.[102] While the initially reported LCEC procedures were capable of quantitating only a few of the compounds of interest,[63,94,95,108] they were readily adopted by a variety of research groups.[78,91-93,101-102]

The limitation of a single LCEC procedure to only a few compounds, however, has recently been significantly modified by the intro-

duction of high efficiency reverse phase columns. The reduced size of the microparticulate support material and improvements in packing techniques have almost increased the peak capacity by a factor of ten (c.f. Chapter 1). The dramatic improvement in separation substantially reduces the necessity for extensive sample cleanup prior to injection. It also markedly increases the number of compounds accessible for quantitation by a single procedure. Reports have already appeared which have taken advantage of these advances in LCEC. Thus, Mefford and Barchas[102] have developed appropriate conditions for the determination of tryptophan and a number of its metabolites, and others[91,93] have produced a procedure for dopamine and three of its metabolites.

The following, newly proposed, LCEC method permits the simultaneous determination of as many as eleven catecholamines, indoleamines, and related metabolites in as little as 10 mg of brain tissue. Only one chromatographic column and one eluting solvent are required for this procedure. Direct injection of the supernatant fraction from a deproteinized brain sample provides quantitation of 5-hydroxytryptophan (5-HTP), 5-hydroxyindoleacetic acid (5-HIAA), homovanillic acid (HVA), 5-hydroxytryptophol (5-HTOL), serotonin (5-HT), and 3-methoxytyramine (3-MT). Following the application of a simple isolation of step to the remaining supernate, injection into the same column provides quantitation of 3,4-dihydroxyphenylalanine (DOPA), norepinephrine (NE), epinephrine (EPI), 3,4-dihydroxyphenylacetic acid (DOPAC), and dopamine (DA).

II. EQUIPMENT AND REAGENTS

A. Apparatus

1. Liquid Chromatographic System. The chromatographic equipment and electrochemical detector have been previously described in Chapter 1.

2. Tissue Homogenizer. Tissue samples were homogenized with an ultrasonic cell disruptor, Model W-200P (Branson Ultrasonics, Plainview, NY), equipped with a long probe tip. This was operated at a 50% duty cycle and a medium power setting of 6.

3. Centrifuge. A Sorvall model RC2-B refrigerated unit fitted with an SM-24 rotor was used.

4. Centrifuge Tubes. Polypropylene centrifuge tubes (1.5 ml capacity) from KEW Scientific, Columbus, OH, were used for processing of individual samples. When subjected to centrifugation, the attached caps were removed by cutting with a pair of wire cutters so these tubes could be placed inside the standard 15 ml centrifuge tubes accepted by the Sorvall SM-24 rotor.

B. Animals

Adult male albino mice of the ARS HA/ICR strain were used as the source of tissue samples. Obtained from Gibson Laboratories of Madison, WI, these animals were typically 6 to 12 weeks old and weighed ca. 35 grams at the time of sacrifice. They were maintained on a 12 hr light/12 hr dark schedule and allowed access to food and water ad libitum.

C. Chromatographic Conditions

The routinely employed chromatographic conditions for this

procedure were presented in Chapter 1. They are summarized here for convenience.

Mobile Phase	0.10 M citrate buffer, pH 4.8, containing 7.5% methanol (v/v) and 0.180 mM SOS. (See below for detailed preparation.)
Flow Rate	1.3 ml/min.
Column	150 x 4.6 mm Ultrasphere ODS, 5 μm particle diameter.
Detector Potential	+0.700 V vs. SCE.
Operating Pressure	typically 2200-2500 p.s.i.

D. Reagent Sources

The chemicals employed and their commercial sources have been described in Chapter 1.

E. Solutions

1. Stock Standard Solutions. Each stock standard solution was prepared by dissolving the appropriate amount of material into 100.0 ml of previously deoxygenated double distilled water containing 1.0×10^{-4} M ascorbic acid (see Table 2-1). These solutions were stored at 4°C and discarded after 2-3 days.

2. Working Standard Solution. On the day of analysis, appropriate amounts of each stock standard solution were combined and diluted to 5.00 ml with deaerated water containing 1×10^{-4} M ascorbic acid (see Table 2-2). This resulted in a solution containing the appropriate compounds in concentrations similar to those found in whole mouse brains and brain parts.

3. Internal Standard Solution. A solution containing

Table 2-1. Preparation of Stock Standard Solutions*

Compound	mg/100 ml	Compound	mg/100 ml
NE	0.70	5-HIAA	1.30
EPI	0.75	5-HT	3.70
DOPAC	1.00	3-MT	2.50
DA	1.50	HVA	3.50

*The weights in this table refer to the commercially available form of the compound. For example, the entry for NE refers to 0.70 mg of norepinephrine hydrochloride.

Table 2-2. Preparation of Working Standard Solutions

Compound	Amount of Stock Solution Added (µl)*				
	Whole Brain and Cortex	Cerebellum	Diencephalon, Midbrain, and Medulla-pons	Hippocampus	Striatum
NE	500	100	200	100	100
EPI	25	25	25	25	25
DOPAC	250	20	50	25	300
DA	1000	50	100	20	2500
5-HIAA	250	50	100	100	100
HVA	500	50	50	25	200
5-HT	750	100	350	100	250
3-MT	100	25	25	25	250

*Final combination of stock solutions diluted to 5.00 mls with 1.0×10^{-4} M ascorbic acid.

ca. 1.0×10^{-5} M DHBA and nMET was prepared by dissolving 1.60-2.00 mg of the commercially obtained salts of these compounds into 100.0 ml of deaerated 0.010 M HCl. 1.00 ml of this solution was then further diluted to 10.00 ml with the same 0.010 M HCl.

4. Mobile Phase. A buffer solution was prepared by dissolving 21.01 grams citric acid monohydrate (0.050 M), 29.41 grams trisodium citrate dihydrate (0.050 M), 175 ml anhydrous, reagent grade methanol (7.5% v/v), 6.00 ml of 0.030 M sodium octyl sulfate (0.180 mM) and 10.0 ml of 1.0 M sodium hydroxide in water and diluting to 2.00 liters. The final concentration of each reagent is displayed in parentheses. Further sodium hydroxide was added, as necessary, to adjust the pH to 4.8. The final buffer was filtered through a 0.45 micron Millipore filter and vacuum deaerated prior to use.

5. Homogenization Solution. 6.4 ml of glacial acetic acid (1.0 M), 4.9 grams of sodium perchlorate (0.40 M) and 2.0 grams NaOH (0.50 M) were diluted to 100.0 ml with double distilled water, yielding a solution with a pH of 4.80. Prior to use, this solution was deaerated on crushed ice for 15 min with oxygen-free nitrogen.[78]

F. Tissue Preparation

The mice were sacrificed by cervical dislocation. The brains were removed as rapidly as possible, weighed, frozen by immersion in liquid nitrogen, and stored on dry ice. Brain parts were prepared from whole brains using the dissection procedure described by Wassil.[8]

III. EXPERIMENTAL PROCEDURE

A. Standard Procedure for Determination of Catecholamines, Indoleamines, and Related Metabolites in Tissues.

The standard procedure is summarized in Fig. 2-3. Appropriate volumes of homogenization solution, internal standard solution, and ascorbic acid are initially added to individual portions of tissue or working standard solution to form tissue samples and standard samples, respectively (see Table 2-3). Typically, 3-5 standard samples are prepared for each separate group of tissue samples. Individual samples, whether tissue or standard, are subjected to ultrasonic homogenization, briefly mixed on a vortex mixer, and centrifuged for 30 min at 30,000 x g and 4°C. The supernatant fraction of each sample is carefully transferred to a new 1.5 ml centrifuge tube and stored on dry ice. Any supernatant fractions which remain cloudy are centrifuged for an additional 15 min. Care must be exercised here to prevent injection of particulate matter into the LCEC, since this will cause subsequent breakdown of the system. After melting the sample by warming in the hand, ca. 20 µl are injected into the chromatograph for quantitation of the species not containing a catechol moiety. The output of the LCEC is connected to both terminals of a dual pen recorder and settings of 5 namp and 50 namp are typically employed for full scale deflection.

The catechol containing compounds are determined by the procedure previously described by Mefford et al.[93] approximately 250 µl of the supernatant fraction mentioned above is added to 20 mg of acid washed alumina and 500 µl of deoxygenated TRIS buffer (3.0 M,

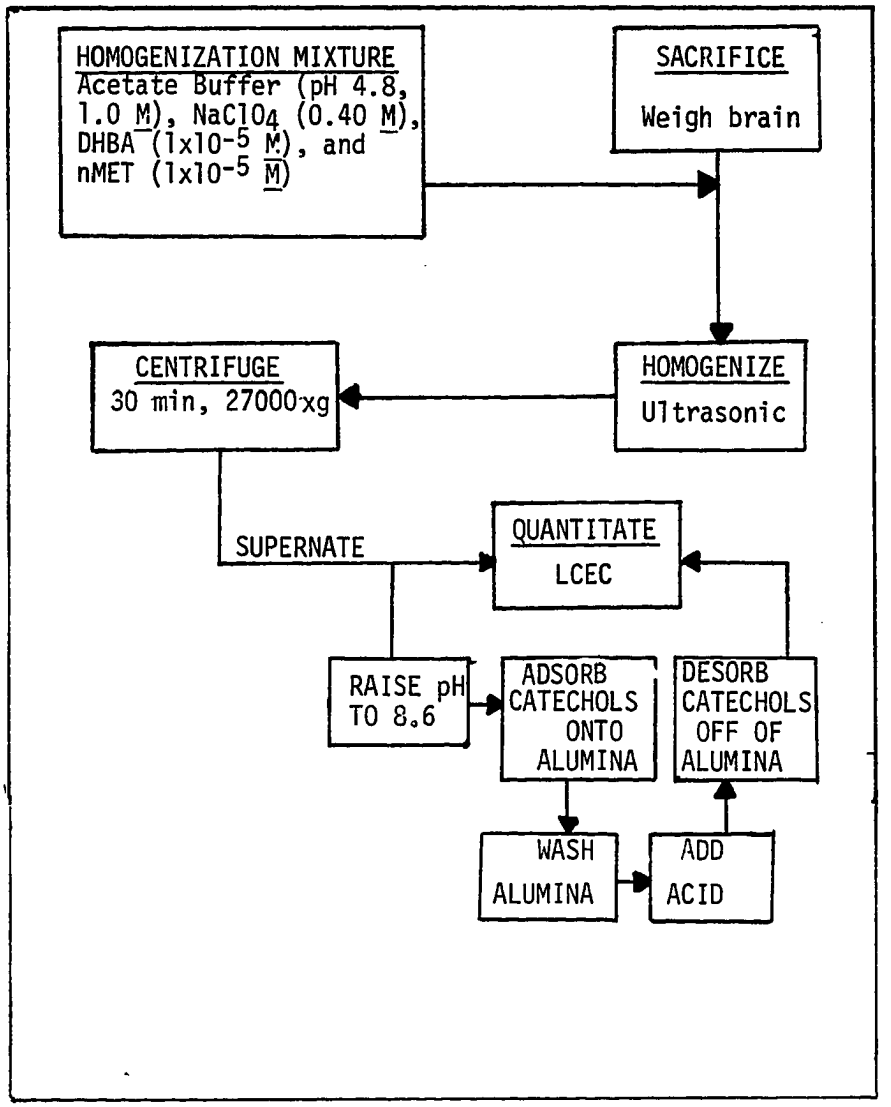


Figure 2-3. Flow Chart for Determination of Catecholamines, Indoleamines, and Related Metabolites in Brain Tissue.

Table 2-3. Preparation of Tissue and Standard Samples

Solution	Amount Added to Individual Samples, µl			
	Tissue Samples		Standard Samples	
	Whole Brain or Cortex	Other Brain Parts[a]	Whole Brain or Cortex	Other Brain Parts[a]
Internal Standard	200	50	200	50
Working Standard	---	---	100	50
Homogenization Solution	750	250	750	250
Ascorbic Acid, 1.0×10^{-4} M[b]	100	50	350	50

[a]Refers to all brain parts except cortex.

[b]Added to compensate for the working standard solution used in standard samples and the brain tissue used in tissue samples.

pH 8.6). After shaking for 15 min, the alumina is allowed to settle. The liquid is carefully aspirated by suction with a disposable pipet having a finely drawn tip. Care should be taken to remove as much liquid as possible without removing any of the very small alumina particles. The alumina is washed once with a 250 µl aliquot of TRIS (6.0 mM, pH 8.6) and once with a 250 µl aliquot of water. Both washings are followed by aspiration. After the final wash, the catechols are desorbed by addition of 200 µl of 0.20 M $HClO_4$ and brief mixing on a vortex mixer. Finally, after light centrifugation(1000xg, 5 min), ca. 20 µl of the supernate is injected into the LCEC for quantitation of the catechol species. Again, recorder settings of 5 and 50 namp are typically employed.

Calculations of the results for individual compounds are analogous to those previously reported by Sasa and Blank:[67]

$$ng/g = \left(\frac{R_{tissue}}{R_{std,\ ave}}\right)\left(\frac{ng\ compound\ in\ std}{wt.\ tissue,\ g}\right)$$

where

$$R_{tissue} = \left(\frac{peak\ height\ of\ compound\ in\ tissue\ sample}{peak\ height\ of\ internal\ std\ in\ tissue\ sample}\right)$$

$$R_{std,ave} = \left(\frac{peak\ height\ of\ compound\ in\ standard\ sample}{peak\ height\ of\ internal\ std\ in\ standard\ sample}\right)_{ave}$$

The internal standard is nMET for all the compounds determined by direct injection of the original supernatant (5-HIAA, HVA, 5-HTOL, 5-HT, and 3-MT), while DHBA serves as the internal standard for the catechols (DOPA, NE, DOPAC, and DA). All results for multiple determinations

are expressed as the mean ± the standard error of the mean (SEM). Statistical comparisons were performed by use of the Student t-distribution.[7]

B. Determination of Per Cent Recovery of Individual Compounds from Tissue Samples

The per cent recovery of individual components was obtained by subtracting the results for tissue homogenates from those for similar samples with a standard addition and comparing the result to untreated standard samples. The absolute per cent recovery focused on the peak heights obtained for the individual components only, while the relative per cent recovery examined the ratio of the peak heights of the desired components to the appropriate internal standards.

The three sample groups for the employed procedure were labelled according to their composition: brain homogenate (BH), brain homogenate plus standard addition (BHP), and no brain homogenate (NB). A pooled homogenate was prepared by adding three whole mouse brains to 750 µl of homogenization solution in a 15 ml polypropylene centrifuge tube. Following sonication for 10 sec, a further 1.50 ml of homogenization solution were added and the mixture was again sonicated until homogeneous. Aliquots of the homogenate were immediately dispensed into prelabeled, individual sample tubes according to Table 2-4. After adding the remaining solutions, the sample tubes were briefly mixed on a vortex mixer. Those samples containing brain homogenates (BH and BHP) were subjected to the standard procedure outlined above in Section III.A.

The per cent recoveries of 5-HIAA, 5-HT and 3-MT were

Table 2-4. Preparation of Samples for Determination of Per Cent Recoveries.

Solution	Amount Added to Individual Samples, µl		
	Sample Type		
	BH	BHP	NB
Brain Homogenate	250	250	--
Ascorbic Acid[a]	50	--	250
Working Standard[b]	--	50	50
Internal Standard[b]	50	50	50
Total Volume	350	350	350

[b]The ascorbic acid ($1x10^{-4}$ M) substitutes for brain homogenate in NB samples and for working standard in BH samples.

[c]See sections II.D.2 and II.D.3 above.

separately calculated as:

$$\text{Absolute Recovery (\%)} = \frac{(\text{peak height cmpd in BHP sample} - \text{peak height cmpd in BH sample})}{(\text{peak height compd in NB sample})} x100$$

$$\text{Relative Recovery (\%)} = \frac{R_{BHP} - R_{BH}}{R_{NB}} x100$$

where

$$R_{BHP} = \frac{\text{peak height of compd in BHP sample}}{\text{peak height of internal std in same BHP sample}}$$

$$R_{BH} = \frac{\text{peak height of cmpd in BH sample}}{\text{peak height of internal std in same BH sample}}$$

$$R_{NB} = \frac{\text{peak height of cmpd in NB sample}}{\text{peak height of internal std in same NB sample}}$$

The per cent recoveries for catechol containing compounds also employed the standard procedure outlined in Section III.A. However, the

determination of absolute recoveries for these species requires that the volume of original supernatant fraction taken for alumina isolation must be carefully controlled at 250 µl rather than using the usual approximation of this quantity. Since desorption from alumina uses only 100 µl of acid eluent, the calculations of absolute recoveries must be slightly modified from that outlined above. This is accomplished by multiplying the results by (100/250); otherwise, the calculations are exactly as presented. It should also be noted that injection volumes are assumed to be constant throughout these determinations; appropriate corrections are required if this is not the case.

During the development of the standard procedure, a solvent extraction was investigated as a possible single step isolation procedure for acidic metabolites. For these studies, a 250 µl aliquot of buffer tartrate or acetate), brain homogenate, or brain homogenate with standard addition was thoroughly shaken with 500 µl of a nonaqueous extraction solvent for 15 min. A 250 µl aliquot of the extraction solvent was placed in a small vial and blown dry with a stream of nitrogen. The residue was taken up in 250 µl of water and 10 µl of this solution was injected into the chromatograph. The absolute per cent recoveries of individual compounds were determined by directly comparing the difference in peak heights obtained from the two types of brain homogenate samples to those from a 5 µl injection of the original, unextracted buffer solution.

IV. RESULTS AND DISCUSSION

A. Preliminary Studies with Low Resolution Chromatograph

Our initial efforts in the area of multicomponent deter-

minations were, in fact, only concerned with three acidic metabolites, i.e., DOPAC, HVA, and 5-HIAA. Using a hand-packed column containing a large particle diameter anion exchange resin (DuPont Zipax SAX), we incorporated 5-hydroxyindole-3-carboxylic acid (5-HICA) as an internal standard. A typical chromatogram of a simple aqueous solution of these four compounds is shown in Fig. 2-4A. The resulting peaks are quite broad and the separation affords only marginal resolution (R = 1.05) for 5-HIAA and 5-HICA. The separation proved to be even less desirable when acidified, centrifuged supernatant fractions of brain homogenates were employed as shown in Fig. 2-4B. The very large 'solvent' peak almost totally obscured the HVA and DOPAC in these samples. Determination of whole mouse brain 5-HIAA yielded a typical value of 427±47 ng/g, although the severely sloping baseline under the 5-HIAA peak makes these results somewhat questionable.

Attempts at eliminating the rather substantial 'solvent' peak were undertaken to improve the results. We, thus, focused our attention on a simple isolation step requiring only extraction of the brain homogenate with a non-aqueous solvent. The compounds would be recovered from the non-aqueous solvent by taking this solution to dryness and then reconstituting in an aqueous buffer for injection into the LCEC. Initial studies employed only standard solutions of the compounds in a 0.50 M phosphate buffers. Since these compounds and related species were known to have pK_a values around 4.5, a pH of 4.1 was selected to maintain the acids in their protonated, uncharged forms. As can be seen in Table 2-5, ethyl acetate yielded the best results and this solvent was used in all subsequent experiments.

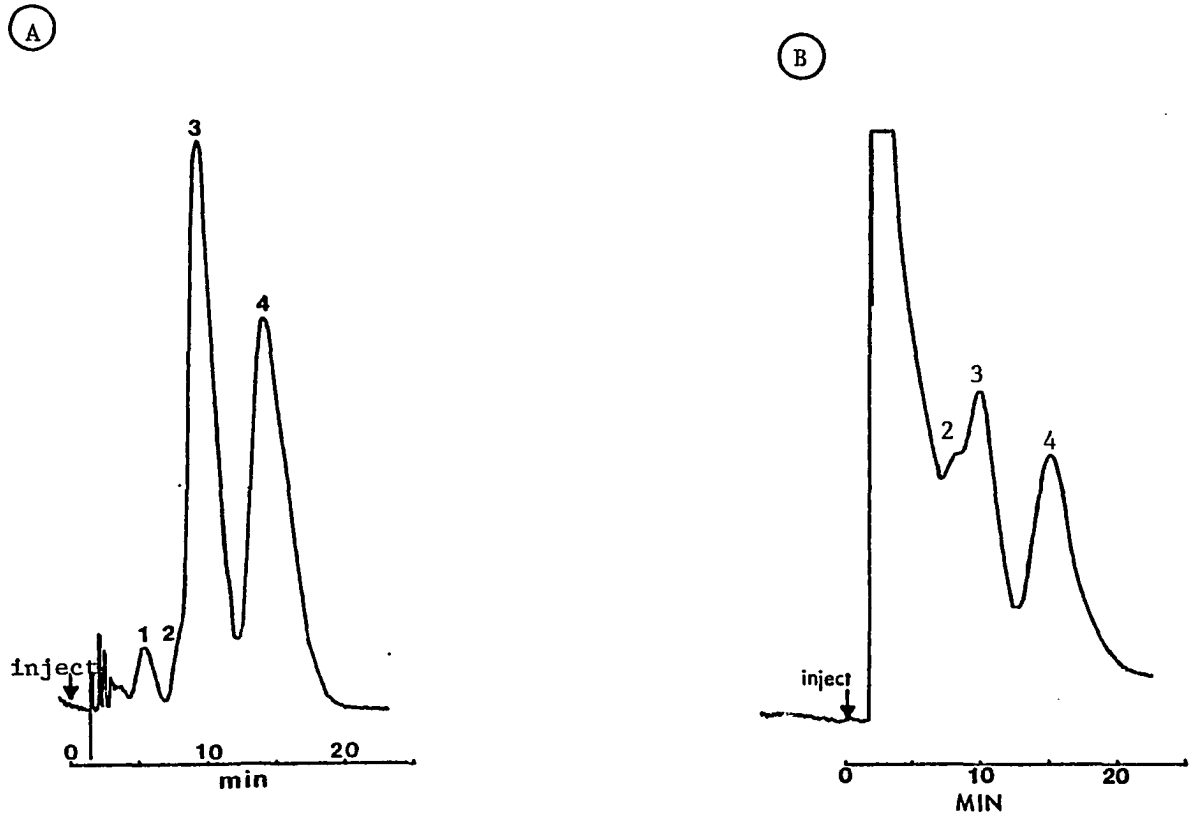


Figure 2-4. Typical Chromatograms Obtained from Zipax SAX Resin. Fig. A illustrates the low resolution separation of acidic compounds from an aqueous standard solution. Fig. B was obtained by injecting an acidic extract of brain tissue. Peaks are: HVA (1), DOPAC (2), 5-HICA (3), 5-HIAA (4). Chromatographic conditions: acetate buffer (0.080 M), pH = 5.0; flow rate = 1.7 ml/min.

Table 2-5. Absolute Per Cent Recovery of Acid Metabolites from Tartrate Buffer Using Various Extraction Solvents

Extraction Solvent	Absolute Per Cent Recovery			
	DOPAC	5-HIAA	HVA	5-HICA
Ethyl acetate	52	42	64	102
Chloroform	25	1.2	8.9	2.9
Ethyl ether	24	29	37	55
Benzene	0	0	3.0	0
Carbon tetrachloride	0	0	0	0
Dichloroethane	0	0	0	0
Heptane	0	0	0	5.7
Toluene	0	0	0	0

Since the pH value of 4.1 was rather arbitrary, the affect of pH on the extraction was examined more closely. As seen in Fig. 2-5, the per cent recovery for the three endogenous components was highly dependent on the pH of the aqueous media. A fixed value between 4.0 and 4.5, however, would appear to provide an acceptable recovery for all the compounds of concern.

But, buffering tissue homogenates to a constant pH proved to be an impossible task. The homogenates were prepared by sonicating a mixture of brain tissue with 1.0 M HCl which was prepared by adding 1.0 ml

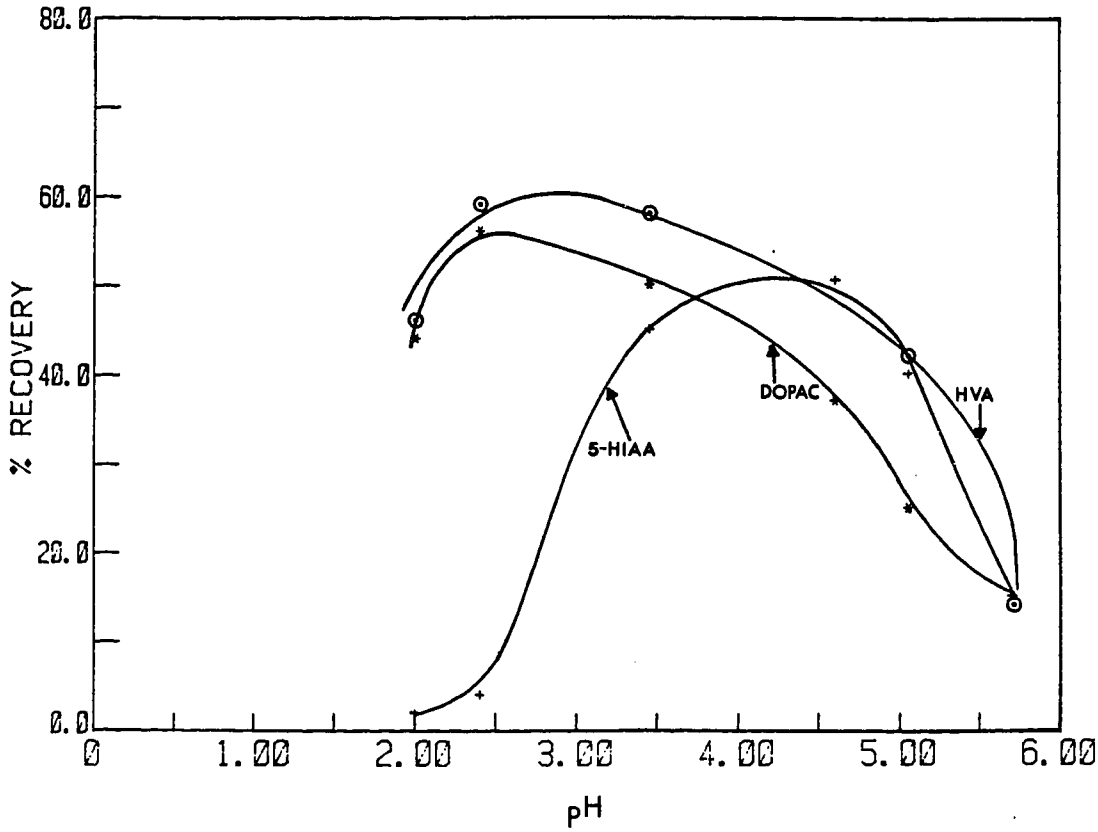


Figure 2-5. Absolute Per Cent Recovery for Various Acid Metabolites as a Function of the pH of the Extracted Medium. The solution extracted contained 0.50 M phosphate buffer for pH control. The extracting solvent was ethyl acetate.

of acid per 1.0 g of tissue. 250 µl of the supernatant fraction from the centrifuged homogenate was mixed with 100 to 1500 µl of a saturated solution of sodium tartrate (ca. 1.0 M) for extraction with ethyl acetate. However, the resulting aqueous mixture exhibited pH fluctuations as great as ±0.5 for individual samples. Similar results were obtained when acetate buffers of various pH values and molarities were attempted.

The dramatic success reported for the separation of biogenic amines and related species using high resolution columns about this time,[91,93] as well as the difficulties encountered in the above approach, caused us to abandon our attempts at solvent extraction coupled with separation by the strong anion exchange resins. Instead, we began to investigate the use of reverse phase chromatography to provide the determination of the originally desired compounds and many others at the same time.

B. Determinations Employing Reverse Phase Chromatography.

The development of appropriate chromatographic conditions for the high efficiency, reverse phase separation of catecholamines, indoleamines, and related metabolites has been covered in Chapter 1. Using the derived conditions, simple injection of the supernate from an acid homogenized, centrifuged tissue sample leads to a result similar to that presented in Fig. 2-6 for mouse brain striatum. The 'solvent' front noticeably trails into the chromatogram for the first 5 minutes or so. This makes quantitation of the more rapidly eluting components difficult at best. Fortunately, however, most of these species contain a catechol group in their structure. Thus, we originally attempted

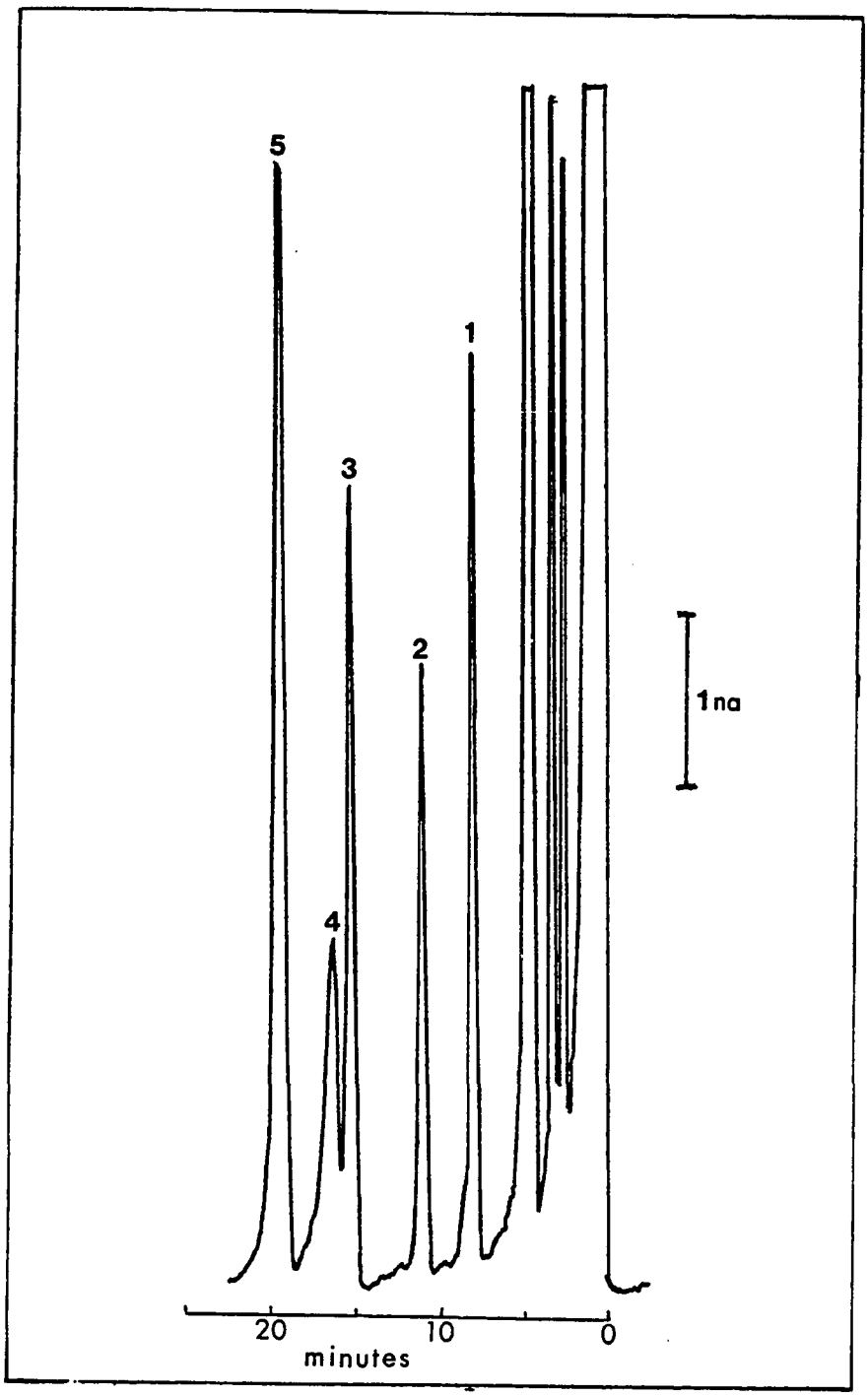


Figure 2-6. Typical Chromatogram of Mouse Striatum Direct Injection of Centrifugate. Mobile phase: 0.10 M citrate buffer, pH 4.8, containing 7.5% methanol and 0.18 mM sodium octyl sulfate. Flow rate = 1.3 ml/min. Peaks are: 5-HIAA (1), HVA (2), 5-HT (3), 3-MT (4), nMET (5).

to employ direct injection of the homogenate supernate only for quantitation of those compounds eluting after dopamine ($t_r \cong 4$ min); the remaining supernatant fraction would be subjected to a standard catechol isolation on alumina[8,68,93] with subsequent injection of the alumina eluate into the same LCEC for determination of the earlier eluting species. As shown in Fig. 2-7, the alumina step leads to a substantially decreased 'solvent' front with a corresponding increase in the ease of quantitating the catechols.

The procedure originally used here was virtually identical to that described in Section III.A, the only difference being that 0.10 M $HClO_4$ was used as the homogenization medium. While this is a relatively straightforward procedure, we wanted to make sure it was appropriate for general application before subjecting it to routine utilization. Thus, we proceeded to examine the linear dynamic range, detection limits, possible interferences, and composition of the homogenization solution before employing it with typical tissue samples.

1. Linear Dynamic Range and Detection Limits. Solutions containing various amounts of the compounds of interest and a fixed amount of the internal standards, DHBA and nMET, were prepared for injection into the LCEC. The concentrations were selected to adequately cover those expected from the analysis of typical tissue samples. A linear regression of the plots of peak height ratio (peak height of the compound of interest/peak height of the internal standard) vs. the concentration produced correlation coefficients very close to 1.00 for all the compounds investigated (see Table 2-6). Detection limits for the individual compounds, defined as the amounts which pro-

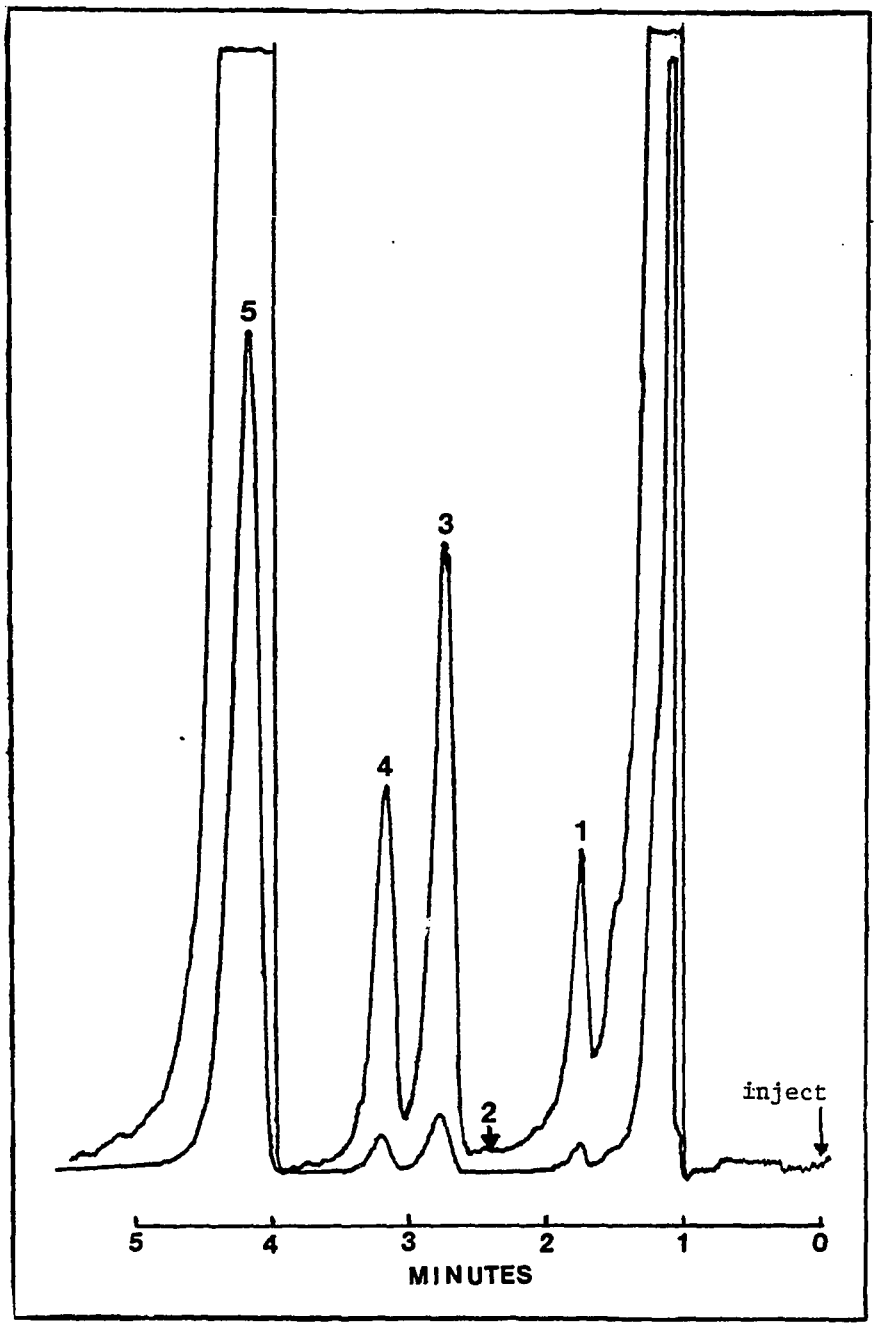


Figure 2-7. Chromatogram of Sample from Fig. 2-6 after Alumina Isolation of Catechols. Chromatographic conditions: see Fig. 2-6. Peaks are: NE (1), EPI (2), DHBA (3), DOPAC (4), DA (5).

Table 2-6. Linear Dynamic Range and Limits of Detection for Various Compounds Determined by Reverse Phase LCEC Procedure.

Compound	Internal Standard	Range (pmol)	Correlation Coefficient
NE	DHBA	0.05-200	1.00
EPI	DHBA	0.10-150	1.00
DOPAC	DHBA	0.10-200	0.998
DA	DHBA	0.07-350	1.00
5-HIAA	nMET	0.15-250	1.00
HVA	nMET	0.40-500	1.00
5-HT	nMET	0.10-300	1.00
3-MT	nMET	0.040-500	0.997
TYR	DHBA	50-50,000	1.00
TP	nMET	150-50,000	1.00
DOPA	DHBA	0.05-100	1.00
5-HTP	nMET	0.10-150	1.00
5-HTOL	nMET	0.15-300	0.999

duced peak heights twice the level of baseline noise, are also represented in Table 2-6 as the lower value of the range investigated.

2. Possible Interferences. While the electrochemical detector adds a great deal of selectivity to this procedure, complete assurance of peak purity is certainly not guaranteed by employment of LCEC. Thus, we decided to investigate the chromatographic and electro-

chemical characteristics of a number of compounds which might interfere with this technique. Further proof of peak identity is provided later by the agreement between tissue results obtained using LCEC and alternative techniques (vide infra). The capacity factors and peak potentials for species which might interfere as well as the compounds of interest are presented in Table 2-7.

3. Homogenization Solution. The homogenization solution represents virtually the only parameter which should effect the per cent recoveries for individual components. We initially anticipated that this parameter would have only a minimal effect on the results. In fact, we expected 100% absolute recoveries for, especially, the components determined by direct injection. Using classical precipitating reagents, i.e., $HClO_4$ and HCl, however, we were surprised to find some recoveries to be well below 100%. This is exhibited in the evaluation of the relative per cent recoveries presented in the first three columns of Table 2-8. This result was most notable for the acidic metabolites, 5-HIAA and HVA. These compounds are neutral, relatively hydrophobic species at low pH, possibly leading to strong interactions with the more hydrophobic components of the centrifugal pellet. Thus, we decided to increase the pH of the homogenization solution above the pK_a values for these acids. We also decreased the polarity of the homogenization solution by employing organic acids in place of the mineral acids. Perchlorate anion, as $NaClO_4$, was added to aid in macromolecular precipitation. The results, shown in the last three columns of Table 2-8, exhibit an adequate yield of the desired compounds using either acetate or proprionate buffers. Similar results are

Table 2-7. Capacity Factors and Voltammetric Peak Potentials for Catecholamines, Indoleamines, Metabolites, and Possible Interfering Species

Compound	k'[a]	E_p[b] (Volts vs. SCE)
Vanillylmandelic acid (VMA)	0.28	0.64
3,4-Dihydroxyphenylalanine (DOPA)	0.52	0.51
3,4-Dihydroxyphenethylglycol (DOPEG)	0.80	0.54
Norepinephrine (NE)	0.85	0.61
Tyrosine (TYR)	0.93	0.81
Epinephrine (EPI)	1.4	0.57
3,4-Dihydroxybenzylamine (DHBA)	2.1	0.38
3-Methoxy-4-hydroxyphenethylglycol (MOPEG)	2.1	0.68
5-Hydroxytryptophan (5-HTP)	2.2	0.50
Normetanephrine (NORMET)	2.3	0.64
3,4-Dihydroxyphenylacetic acid (DOPAC)	2.9	0.50
3,4-Dihydroxyphenethylamine (DA)	3.8	0.51
Metanephrine (MET)	4.2	0.70
5-Hydroxyindoleacetic acid (5-HIAA)	7.3	0.48
Tryptophan (TP)	7.5	0.89
Homovanillic acid (HVA)	9.7	0.62
5-Hydroxytryptophol (5-HTOL)	11.4	0.47
5-Hydroxytryptamine (5-HT)	12.6	0.50
3-Methoxytyramine (3-MT)	13.1	0.64
5-Hydroxy-N_ω-methyltryptamine (nMET)	16.0	0.50
N-Acetyl-5-Hydroxytryptamine (N-Ac-5-HT)	20.1	0.43
5-Methoxytryptamine (5-MT)	40.0	0.78
Melatonin (MEL)	40.0	0.75

[a] $k' = (t_r - t_o)/t_o$ where t_r is the retention time of the compound of interest and t_o is the retention time of an unretained compound.

[b] Determined at a carbon paste electrode using the chromatographic mobile phase as the electrochemical solvent.

Table 2-8. Per Cent Relative Recoveries Obtained Using Various Homogenizing Solutions.[a]

Compound	Homogenization Medium					
	0.090 M $HClO_4$	0.44 M $HClO_4$	1.0 M HCl	1.0 M Formate Buffer with 0.40 M $NaClO_4$ (pH = 2.4)	1.0 M Acetate Buffer with 0.40 M $NaClO_4$ (pH = 4.8)	1.0 M Propionate Buffer with 0.40 M $NaClO_4$ (pH = 4.6)
NE	101	95	104±13	100	106±4	113±5
EPI	90	110	95±4	98	98±3	109±3
DOPAC	118	120	120±10	150	190±16[b]	151±9
DA	103	102	96±5	95	105±5	110±2
5-HIAA	60	75	66±6	74	90±2	94±2
HVA	78	77	87±1	98	110±3	102±4
5-HT	93	92	100±3	96	97±2	106±5
3-MT	59	54	97±3	108	91±10	102±3

[a]Results are presented as mean ±S.D. for the average values taken from three separate experiments. Each experimental average was derived from five individual determinations.

[b]This value becomes 97±8 if one corrects for the absolute recovery of DOPAC in the presence vs. the absence of brain tissue (see Table 2-10).

found through the calculations of the absolute per cent recoveries, as presented in Table 2-9. The absolute per cent recoveries obtained using either acetate or propionate approach 100% for the directly injected species. Since acetate is generally more readily available, it was adopted for all routine investigations. The values of ∿60% for the compounds isolated on alumina are completely comparable to those obtained by others. The only exception to this, i.e., the poor recovery of DOPAC from alumina, is not understood. However, other investigators have reported similar anomolous behavior with DOPAC.[93] Also disturbing is the fact that the absolute per cent recovery for DOPAC is greater in the presence of brain tissue than in the absence of brain tissue as shown in Table 2-10. Although this result cannot currently be explained, the final results for all brain samples were corrected to account for this by dividing the normally obtained answer by 0.51.

Table 2-10. Per Cent Absolute Recovery of DOPAC in Presence and Absence of Brain Tissue.[a]

	With Tissue	Without Tissue
% Recovery	33±3	16±4

[a]Homogenization medium was 1.0 M acetate buffer with 0.40 M $NaClO_4$ (pH = 4.8).

Table 2-9. Per Cent Absolute Recoveries Obtained Using Various Homogenizing Solutions.[a]

Compound	Homogenization Medium			
	1.0 M Formate Buffer with 0.40 M $NaClO_4$ (pH 2.4)	1.0 M HCl	1.0 M Acetate Buffer with 0.40 M $NaClO_4$ (pH 4.8)	1.0 M Proprionate Buffer with 0.40 M $NaClO_4$ (pH 4.6)
NE[b]	--	70	63±4	--
EPI[b]	--	70	59±6	--
DHBA[b]	--	65	61±2	--
DOPAC[b]	--	35	32±6	--
DA[b]	--	66	62±2	--
5-HIAA	76	67	95±5	83
HVA	98	94	106±6	92
5-HT	92	104	92±2	92
3-MT	113	95	97±22	93
nMET	98	105	92±9	103

[a]Results presented as mean ±S.D. for the average values taken from three separate experiments. Each experimental average was derived from five individual determinations.

[b]Results obtained following alumina isolation.

4. Analysis of Mouse Brains. Having developed and refined the procedure, we proceeded to employ it in the determination of the catecholamines, indoleamines, and related metabolites in tissue samples. The results are presented in Table 2-11. Although the chromatographic conditions are capable of separating many more species than those quantitated (see Section IV.B.3), such species are present, unfortunately, at levels below the detection limits of the current configuration. Likewise, some of the more difficult to oxidize compounds, like TYR and TP, were selectively ignored through the use of a moderate working electrode potential (+0.700 V vs. SCE). Nonetheless, we were capable of determining eight compounds in all or most of the eight different brain regions examined. This is a substantial improvement over previous procedures which could quantitate only one to, at most, four of these compounds on a routine basis.

Comparison of the currently obtained results to those previously reported by others is provided in Table 2-12. Since results for mice are relatively uncommon, we have taken the liberty of including some data for rats. In general, the current results are quite comparable to those previously reported.

To further demonstrate the applicability of the final procedure, we undertook a pharmacological investigation. One group of mice (experimentals) was injected with 500 µl of a pargyline solution, i.p., containing 1.0 mg/ml of the hydrochloride salt in isotonic saline (0.90% NaCl). A second group (controls) was injected with 500 µl of the isotonic saline only. Both groups were sacrificed 90 minutes later. The brains were removed and subjected to the described procedure.

Table 2-11. Tissue Content of Catecholamines, Indoleamines and Related Metabolites in Different Regions of Mouse Brain.[a]

Region	Average Weight of Region (mg)	Content, ng/g							
		NE	EPI	DOPAC	DA	5-HIAA	HVA	5-HT	3-MT
Cerebellum	54±4	173±25	--	--	--	130±10	--	85±8	--
Medulla-Pons	37±2	392±14	14±3	15±5	66±3	550±18	--	690±23	--
Midbrain	38±11	445±26	--	32±3	66±6	935±35	180±10	870±73	77±10
Diencephalon	63±1	494±24	17±4	113±23	434±105	797±16	321±23	835±61	97±12
Hippocampus	30±3	358±26	9±1	11±1	56±17	444±27	--	660±7	--
Striatum	41±4	116±11	--	416±4	7691±423	495±23	1132±23	732±7	1732±110
Cortex	135±7	242±13	--	76±13	452±20	327±45	197±16	613±35	--
Whole Brain	450±28	386±29	--	113±45	1174±109	401±53	266±54	767±89	239±108
Whole Brain (reconstructed from regional results)[b]	--	302	3.6	98	1031	480	251	717	201

[a]Results expressed as mean±SEM for at least five separate determinations.

[b]The reconstructed whole brain results were calculated by summing the product of the regional result times the average weight of that region for all regions.

Table 2-12. Comparison of Reported Values for Various Catecholamines, Indoleamines, and Related Metabolites in Rat and Mouse Brain Tissues

Whole Brain or Brain Tissue	Reported Value (ng/g±SEM)								Animal Type	Method[a]	Reference
	NE	EPI	DOPAC	DA	5-HIAA	HVA	5-HT	3-MT			
whole brain	--	--	--	--	526±81	--	526±81	--	rat	GCMS	69
whole brain	--	--	--	973±16	--	--	805±12	--	mouse	GCMS	101
whole brain	--	--	--	--	294±7	--	238±19	--	rat	LCEC	69
whole brain	--	--	--	--	253±26	106±17	--	--	mouse	FLUOR	109
whole brain	380±16	--	--	843±16	525±36	--	464±32	--	rat	FLUOR	90
whole brain	386±13	--	113±26	1174±50	401±50	266±25	761±40	239±25	mouse	LCEC	this report
cerebellum	296±14	--	--	28±4	--	--	103±8	--	mouse	LCEC	67
cerebellum	200±10	--	<50	100±30	--	<20	--	--	rat	FLUOR	85
cerebellum	190±18	--	--	29±9	144±6	--	78±15	--	rat	FLUOR	90
cerebellum	256±7	.58±.08	--	8±1	--	--	--	--	rat	FLUOR	107
cerebellum	173±25	--	--	--	130±10	--	85±8	--	mouse	LCEC	this report

Table 2-12 (continued)

Whole Brain or Brain Tissue	Reported Value (ng/g±SEM)								Animal Type	Method[a]	Reference
	NE	EPI	DOPAC	DA	5-HIAA	HVA	5-HT	3-MT			
striatum	--	--	1950±130	--	--	690±60	--	--	rat	LCEC	92
striatum	153±13	--	--	4777±380	--	--	579±25	--	mouse	LCEC	67
striatum	380±100	--	840±70	8300±420	--	630±50	--	--	rat	FLUOR	85
striatum	116±11	--	476±4	7691±423	495±23	1132±23	732±7	--	mouse	LCEC	this report

[a]Methods utilized are gas chromatography/mass spectrometry (GCMS), fluorescence (FLUOR), and liquid chromatography with electrochemical detection (LCEC).

Since pargyline is a well known blocking agent for monoamine oxidase (MAO), one would expect the treated animals to show increased levels of amines and decreased levels of their acid metabolites. As shown in Fig. 2-8, this is precisely what was observed. The fact that no abnormalities were observed for the treated samples also adds credence to the original assignment of peaks.

V. COMMON OPERATIONAL PROBLEMS

Prolonged application of the outlined procedure has demonstrated the occurrence of two rather distinctive problems: column plugging and loss of resolution.

A. Column Plugging.

The direct injection of centrifuged, homogenized brain supernatant material into the LCEC will eventually lead to clogging of the column inlet. This is due to the incomplete removal of particulate matter by this rather simple isolation step. Although such clogging requires a considerable increase in operating pressure, it does not usually alter the observed resolution of individual components. This situation can generally be remedied by simply replacing the 2 μm inlet end frit with a new unit. Attempts at cleaning the old frit have generally proved ineffective. If the particulate matter has penetrated into the top of the packing material, it may also be necessary to replace the top 2 mm or so with new packing material.[110] If this is required, the reassembled column should be briefly subjected to normal operation, i.e., pumping the eluting solvent through the column, for about 5 min. It should then be taken apart again to fill any re-

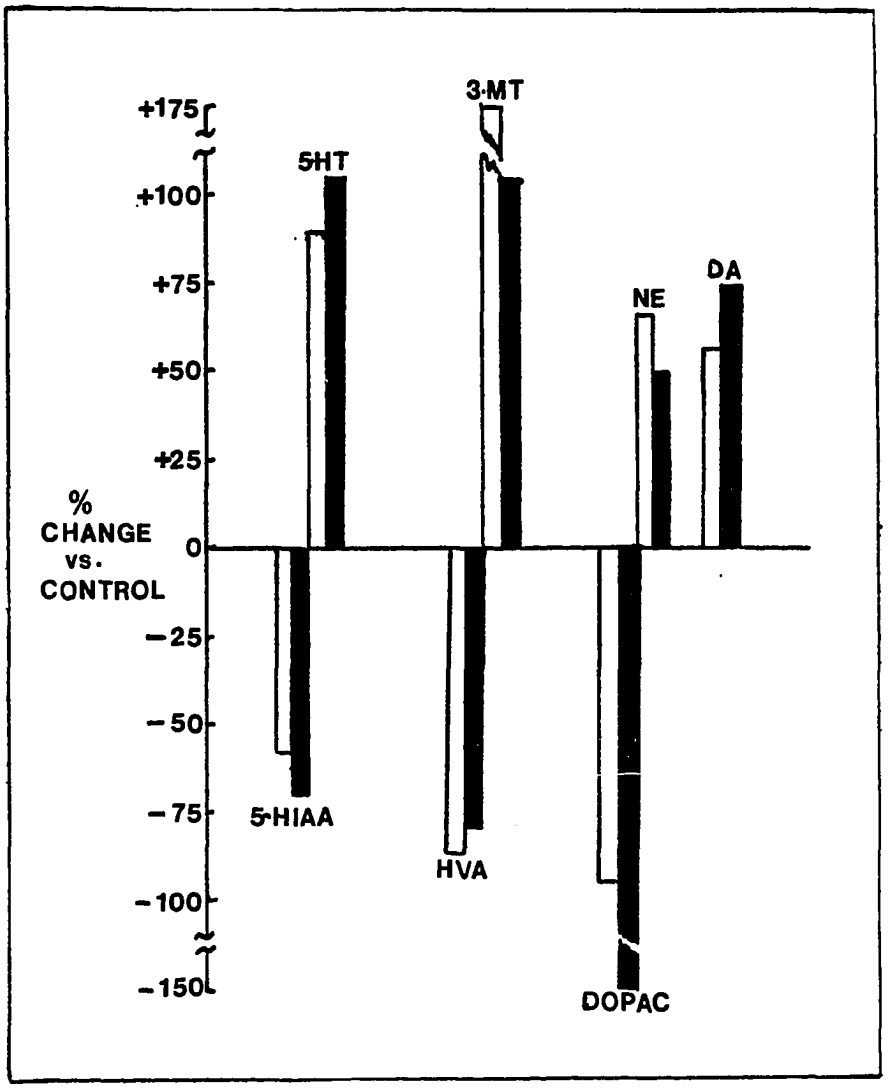


Figure 2-8. The Effect of Pargyline on Catecholamines, Indoleamines, and Related Metabolites in Mouse Striatum and Diencephalon. Mice were injected with pargyline-HCl in saline (150 mg/kg, i.p.), and were sacrificed 1 1/2 hrs after treatment. ▭ Striatum ▬ Diencephalon.

$$\% \text{ change} = \frac{\text{treated} - \text{control}}{\text{control}} \times 100$$

sulting voids at the top of the column. The repaired column should exhibit only minor losses in resolution with an operating pressure near that of a new column.

Clogging can be substantially decreased by filtering the sample prior to injection in the LCEC. Our laboratory now routinely utilizes filtration for all tissue homogenates.

B. Resolution Problems.

Injection of multiple samples into any liquid chromatograph and/or simple aging of the system will almost certainly lead to a loss in resolution. Likewise, one may be interested in modifying the conditions to focus on metabolites not included in the current procedure. If either of these situations should occur, there are three major alternatives available to the analyst. These are (1) alteration of the mobile phase, (2) adjustment of the electrode potential, and (3) installation of a new column. However, we would suggest the analyst carefully examine which solutes are essential for the particular experiment at hand before attempting any of these alternatives. A judicious choice will allow systematic investigations rather than the usually employed, and ultimately frustrating, trial and error approach.

Mobile phase parameters to be considered include pH, ionic strength, solvent strength, and ion-pairing agents.[111-113] These should all be considered for compounds not accessible by the current procedure. Also, in the current procedure, a loss of resolution can lead to a deterioration in the separation of, especially, 5-HT and 3-MT. An increase in the mobile phase concentration of the ion-pairing agent, sodium octyl sulfate (SOS), should provide improvement in such cases

(c.f. Fig. 1-16), although this solution may also require longer times for individual chromatograms.

The selection of the electrode potential is a very pertinent parameter to be considered.[89,102,108] Thus, if the peak potential (c.f. Table 2-6) of an undesirable component is at least 0.10 volt greater than the compounds to be determined, the former can typically be 'tuned out.' This alternative may also be applied to the routine determination described above. Thus, if a deteriorating column exhibits a poor separation for 5-HT and 3-MT, one may decide that the 3-MT information is not essential. Selecting a working electrode potential of +0.52 to 0.60 volt *vs.* SCE, the analyst would be able to quantitate 5-HT (E_p = +0.50 V) while 3-MT (E_p = +0.64 V) would pass through the detector virtually unnoticed.

Replacement of the column is, of course, always available to the analyst as an alternative. However, the cost of new columns generally makes this the last choice to be considered. For the determination of compounds other than those which are the subject of the current procedure, one should also consider the possible use of different packing materials.

VI. CONCLUSION

The currently employed procedure for the determination of catecholamines, indoleamines, and related metabolites is relatively simple, inexpensive, and rapid. Approximately 25 samples may be processed with this procedure by a trained analyst in a single day. Even faster throughput is available if one employs an automatic sample

injection device and computer controlled data acquisition/manipulation. Tissue samples as small as 1 mg may be analyzed, although brain tissue samples of $\geq$20 mg are typically required to provide adequate data for the majority of compounds of interest. Only minor modifications in the sample volume and preparation would allow the direct application of the described procedure to the analysis of biological fluids such as blood, serum, cerebrospinal fluid, or urine.

Since the current procedure is capable of quantitating a large number of compounds under simple isocratic conditions, the necessity for multiple chromatographic setups or multiple eluting solvents has been effectively eliminated. The system employed is also capable of quantitating virtually all the products of the primary enzymatic reactions involved in the biosynthesis and degradation of catecholamines and indoleamines. This expands the capability of the system to include its utilization in routine determinations of enzyme activities. Indeed, our laboratory is currently using the described LCEC conditions for the assay of tyrosine hydroxylase, DOPA decarboxylase, 5-hydroxytryptophan decarboxylase and monoamine oxidase.

CHAPTER 3

MONOAMINE OXIDASE - A GENERAL INTRODUCTION

I. INTRODUCTION

The previous two chapters have discussed the chromatographic separation and quantitation of low level concentrations of tyrosine and tryptophan metabolites in mouse brain tissue. In the following chapter, these chromatographic principles are applied to the quantitation of the enzymatic degradation products of biogenic amines by monoamine oxidase (MAO).

Monoamine oxidase [monoamine:oxygen oxidoreductase (deaminating), EC 1.4.3.4] is of considerable physiological importance in the brain, since it catalyzes the inactivation of neurochemical transmitters such as dopamine, serotonin and norepinephrine. Monoamine oxidase (MAO) promotes the oxidative deamination of monoamines according to the following reaction:

$$R\text{-}CH_2NH_2 + O_2 + H_2O \rightarrow RCHO + H_2O_2 + NH_3$$

The initial product from deamination of an amine is an aldehyde. This, in turn, can be converted by aldehyde dehydrogenase (AD) to the corresponding acid (Fig. 3-1). The aldehyde intermediate may also be reduced to form an alcohol by aldehyde reductase (AR).

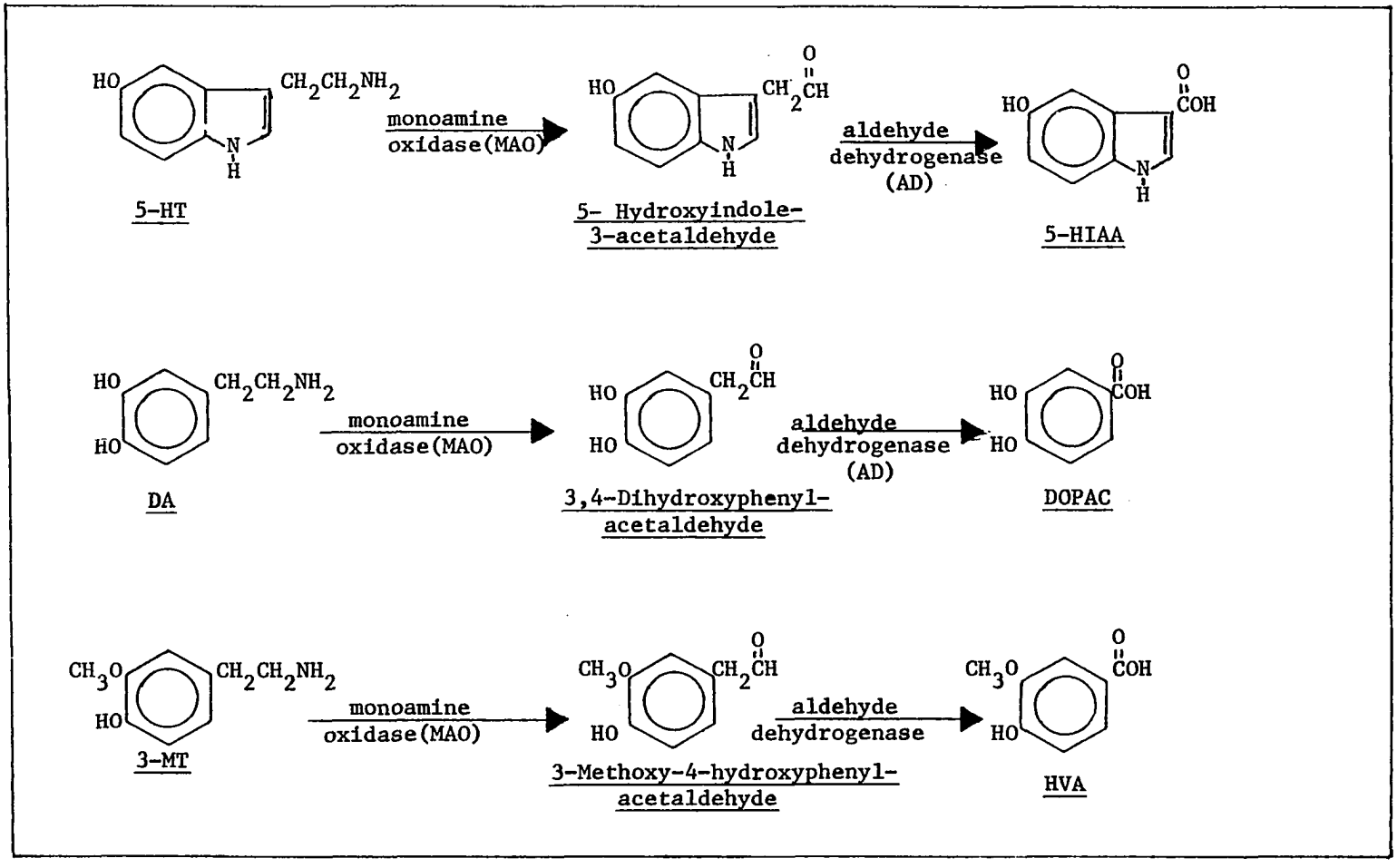


Figure 3-1. Metabolic Pathway of Primary Amines in Brain Tissue.

The amount of product produced by MAO-AD coupled systems (nmol/mg tissue/min) is an empirical value which directly represents the concentration of this enzyme in the examined tissue. The development of the analytical procedure for MAO is preceded, in this chapter, with a discussion of the biological properties of the enzyme and the previously employed methodology used to assess its activity.

Over the past thirty years, MAO has received considerable attention. It has been implicated in mental depression[39] and schizophrenia[40,45] for several years. Pharmacological interest in MAO is widespread due to the efficiacy of certain compounds which inhibit MAO in combating some forms of mental depression. Increased levels of serum MAO have also been reported in patients with chronic heart failure,[52] liver conditions,[53] diabetes mellitus[54] and hepatic fibrosis.[55] An extensive review of the biochemical and pharmacological properties of this enzyme is presented in the work of Costa et al.[44]

MAO is one of two enzymes that are primarily responsible for the degradation of catecholamines in neuronal tissue, the other being catechol-O-methyltransferase. It is generally nonspecific and will act on many monoamines, including the methoxylated metabolites 3-O-methyldopamine (also known as 3-methoxytyramine or 3-MT) and normetanephrine (NORMET). Measurements of MAO activity have employed a wide variety of primary amines as substrates.[15,26]

II. Characterization and Distribution of Monoamine Oxidase

Two forms of the enzyme have been tentatively identified in rat brain tissue.[41] These isozymes have been classified as MAO A and MAO B. This classification is based both on *in vitro* substrate specificity and inhibitor selectivity. Table 3-1 characterizes some

Table 3-1. Characterization of Monoamines as Substrates for Type A and Type B MAO[a]

Type A	Type A + B	Type B
5-hydroxytryptamine (5-HT)	p-tyramine	beta-phenethylamine (low concentration)
5-methoxytryptamine (5-MT)	m-tyramine	phenethanolamine (low concentration)
epinephrine (EPI)	dopamine (DA)	o-tyramine
norepinephrine (NE)	p-octopamine	1,4-methylhistamine
metanephrine (MET)	tyramine	
normetanephrine (NORMET)	m-octopamine	
	beta-phenethylamine (high concentration)	
	phenethanolamine (high concentration)	

[a]Data obtained from ref. 33.

biogenic amines as type A, type B, or Type A+B substrates. The MAO inhibitors commonly used for classifying substrates are clorgyline, which preferentially inhibits the A form and deprenyl, which preferentially inhibits the B form. These compounds are basically acetylenic amines as shown on the next page. A third compound, pargyline, is reported to be MAO B specific,[42] however it is neither as specific nor as commonly employed as deprenyl for MAO B inhibition. Typical inhibition curves are reproduced in Figs. 3-2A and 3-2B as an illustration of how the isozymes are classified.[43] The two curves show type A (Fig. 3-2A)

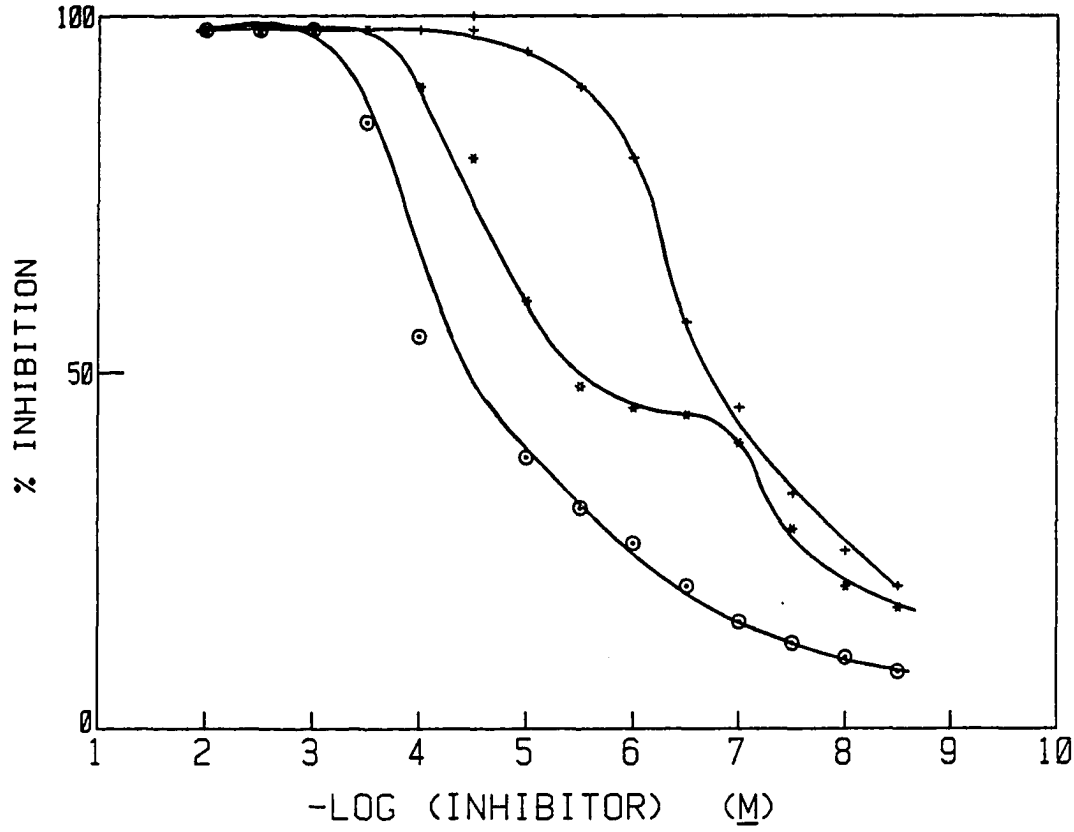


Figure 3-2A. The Effect of Deprenyl Inhibition on the Oxidation of Biogenic Amines. Substrate: 5-HT (o), DA (*), and beta-phenethylamine (+). Data from reference 33.

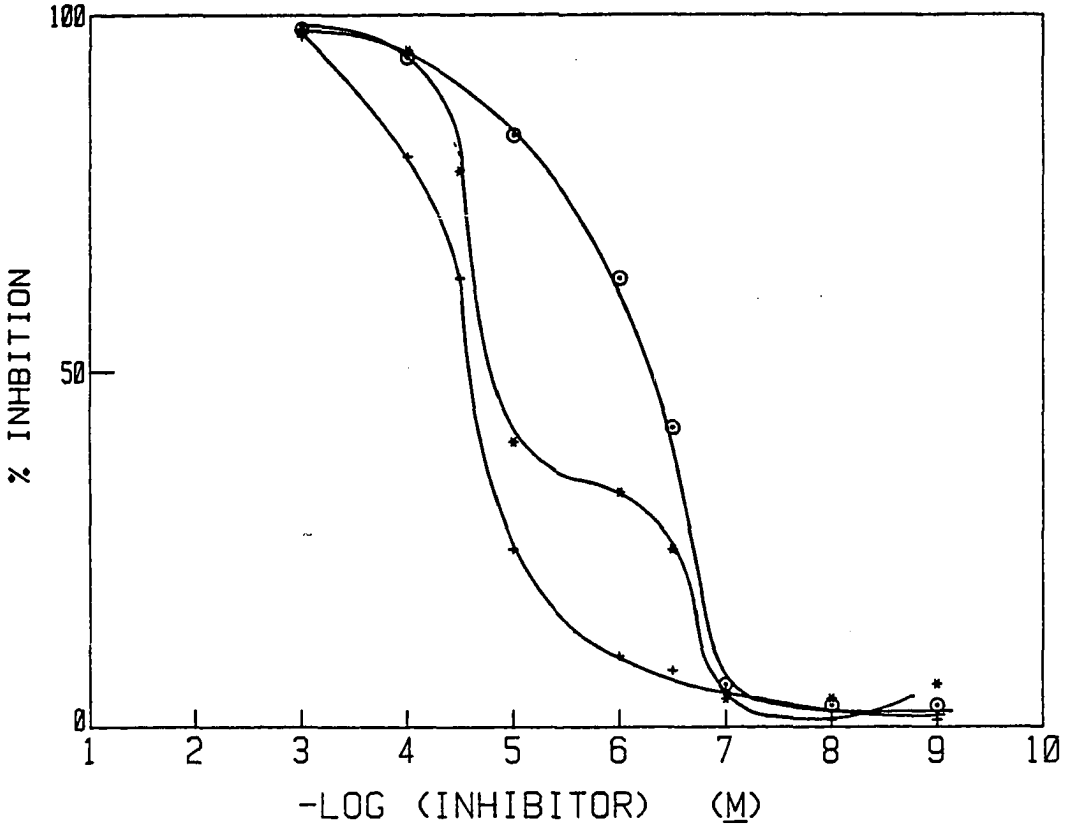
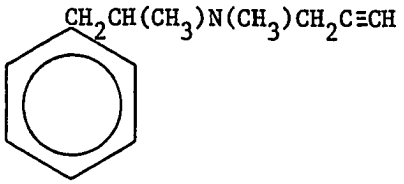
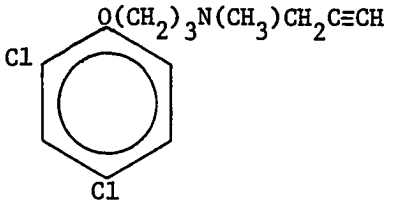


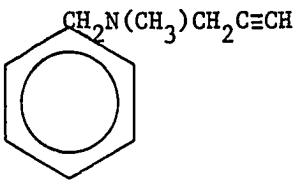
Figure 3-2B. The Effect of Clorgyline Inhibition on the Oxidation of Biogenic Amines by MAO. Substrates: 5-HT (o), DA(*), and beta-phenethylamine (+). Data from reference 33.



Deprenyl



Clorgyline



Pargyline

and type B (Fig. 3-2B) inhibition of serotonin, a type A substrate, phenethylamine, a type B substrate, and dopamine, a substrate for both types A and B. Substrates for both types A and B are typically recognized by a biphasic titration curve as seen for dopamine. Although most experts in the field accept the isozyme concept of MAO, many unanswered questions concerning the multiple forms of MAO still remain. Reference 33 is recommended as an excellent introduction to the large amount of work being done on MAO characterization.

Purification of the individual isozymes has not been accomplished; however, multiple attempts have been reported.[46] Recently, kinetic studies using several different substrates and inhibitors have additionally indicated that type B may be heterogeneous in rat liver, human blood platelets and pig heart.[47-49] Investigators studying oxygen, a natural MAO cosubstrate, have also concluded that a simple binary

classification of MAO is an oversimplification.[50] These results muddle an already confusing picture concerning the existence of multiple forms of MAO.

For the most part, the subcellular localization of MAO in brain is in the outer membrane of mitochondria. The MAO which is primarily concerned with catecholamine and indoleamine degradation is presumed to be located in mitochondria found in the presynaptic nerve ending. In normally functioning neurons, the transmitter is released by electrical stimulation of the neuron. Subsequent diffusion of the transmitter and interaction with the postsynaptic membrane effects communication or transmission of information. This transmission process, for catecholamines, is predominately inactivated by reuptake of the discharged amine into the presynaptic nerve cell. However, MAO provides ultimate inactivation of individual transmitter molecules by converting them to their corresponding carboxylic acids or alcohols. Virtually all the transmitter molecules which are intracellular, but extravesicular, are directly accessible to metabolism by MAO. And, the products of MAO conversion are incapable of eliciting a postsynaptic response.

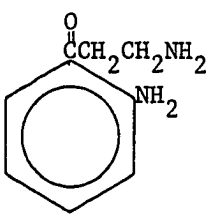
III. ANALYSIS OF MAO

Most of the published assays for MAO use purified mitochondrial preparations as the enzymatic source. Isotonic solutions, commonly containing 0.25 M sucrose in a weak phosphate buffer, are utilized when preparing the various mitochondrial fractions. No apparent reason was given for the majority of the assays as to why these rather laborious sample preparations were performed. Regardless of the sample preparation,

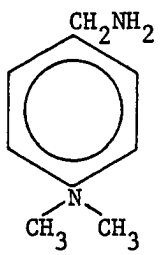
however, all reported MAO assays are conducted in hypotonic buffers, normally 0.050 M phosphate, in an effort to maximize the accessibility to MAO for substrates and cofactors by rupturing the subcellular components.

Analytical evaluation of the various reactants or products from the in vitro incubation of MAO has employed the following techniques: radiometry,[12,14,31] spectrophotometry,[10,20,24,26] fluorometry,[9,13,19] potentiometry,[15,36] polarography,[57] gas chromatography,[11] and manometry.[56] These techniques are employed to measure one of the following: (1) disappearance of the substrate,[10] (2) consumption of oxygen,[36,56] (3) production of peroxide,[13] (4) production of ammonia,[15] (5) formation of the aldehyde,[11,25] or (6) formation of a carboxylic acid following enzymatic conversion of the initial aldehyde product.[9,19]

Many of these techniques have inherent drawbacks. Manometric and polarographic techniques,[56] which measure oxygen uptake, lack sufficient detection limits and selectivity. Colorimetric assays commonly employ substrates which are not physiological in nature, e.g., kynuramine(I) or p-dimethylaminebenzylamine(II):



Kynuramine
(I)



p-Dimethylamine-
benzylamine
(II)

A gas chromatographic assay[11] with electron capture detection also suffers from this drawback by using m-iodobenzylamine(III). Potentiometric assays, which measure the generated ammonia or peroxide, are relatively rapid and exhibit moderate detection limits. However, they lack selectivity and are useless for studies involving mixtures of substrates.

An improved colorimetric procedure has been described. It is based on a peroxidase-catalyzed reaction with a colorless fluoroscein dye.[26] However, the biologically important catecholamines, dopamine and norepinephrine, cannot be used as they interfere with the oxidative conversion of the dye into the chromophore.

The measurement of generated peroxide in MAO reactions has also employed a fluorometer.[13] The procedure is based on the reaction of peroxide with homovanillic acid (HVA). In the presence of peroxidase, these two species combine to form a highly fluorescent compound. This assay also suffers from interference by catecholamines and serotonin, since these amines block formation of the fluorophore. Fluorometric techniques are additionally subject to error due to potential variation of background levels of endogeneous, fluorescent materials.

Gas chromatographic and radiometric procedures have been developed which measure aldehyde formation.[11,12,14,31] These assays require an extraction of the aldehyde prior to quantitation. Not only are extractions time consuming and tedious, but studies using these procedures have shown incomplete recovery of indole-3-acetaldehyde and indole-3-acetic acid from solutions containing tissue residues.[12,59] The investigators attribute the low recoveries to nonspecific binding

of the radioactive metabolites to denatured protein.[59] Based on studies in our laboratory, we also concur with this explanation.[1]

Additional difficulties can be encountered in measuring only the generated aldehyde. First, conversion of the aldehyde metabolite to its corresponding acid must be prevented. Secondly, aldehydes have been shown to be highly reactive; the carbonyl group of the aldehyde readily reacts with the free amino groups in proteins and other compounds to form a Schiff's base.[60] In order to circumvent these difficulties, subsequent procedures have attempted to reduce the time spent by the aldehydes in the incubation mixture. Alternatively, total conversion of the amines into their corresponding carboxylic acids by the addition of aldehyde dehydrogenase (AD) may be employed, followed by quantitation of the acids.[9,19,20]

AD, like MAO, is also found in brain mitochondria. Early information leading to the discovery of AD in brain tissue included: 1) the presence of only small amounts of aldehydes in the brain as products of MAO[18] and 2) the facile production of acids from aldehydes by isolated brain tissues.[61] These two pieces of data implicated an enzyme that readily oxidized aldehydes into their corresponding acids.

For a coupled MAO-AD assay to be quantitative, two stringent requirements must be satisfied. Since the AD-formed carboxylic acid is to be measured, it is imperative that the MAO-formed aldehyde be converted both immediately and stoichiometrically. In other words, the production of the aldehyde by MAO must be the rate limiting step in the overall scheme. This also implies that sufficient AD be present to instantaneously transform the intermediate aldehyde into the acid.

Previous work with AD[28] and MAO[36] indicates that an MAO-AD coupled reaction is a completely reasonable approach to measuring MAO activity. Michaelis constants and maximal velocities were reported in these investigations for dopamine, serotonin, and their corresponding aldehydes. The data is summarized in Table 3-2, where it is easily seen that AD exhibits much higher velocities and requires substantially less substrate to achieve saturation kinetics.

Based on these reports and the preceding information, an attempt was made to establish a procedure for the routine analysis of MAO using a combined MAO-AD system and quantitation by LCEC. The following chapter describes the results of this work which was completed during the winter of 1980 and the spring of 1981.

Table 3-2. Kinetic Constants for Pertinent MAO and AD Substrates[a]

Substrate	Enzyme	V_{max} (micromoles/min/mg protein)
5-hydroxytryptamine (5-HT)	MAO	0.0047
5-hydroxyindoleacetaldehyde	AD	84
dopamine (DA)	MAO	0.0065
3,4-dihydroxyphenylacetaldehyde	AD	156

[a]Data from references 28 (AD) and 36 (MAO).

CHAPTER 4

THE DETERMINATION OF MONOAMINE OXIDASE ACTIVITY IN BRAIN TISSUES

I. INTRODUCTION

In developing a procedure for monoamine oxidase (MAO), we attempted to devise a method which would be: 1) simple to perform and involve minimal sample manipulation, 2) inexpensive, requiring a limited amount of equipment and reagents, 3) rapid and capable of incorporating a large number of samples at one time, 4) capable of employing substrates which are biologically important, 5) capable of analyzing mixtures of substrates, 6) sensitive with reasonably low limits of detection, and 7) stoichiometrically correct, or produce exactly one mole of product for each mole of substrate consumed. By utilizing LCEC many of these requirements were inherently satisfied. The liquid chromatograph easily and quickly separates and quantitates a relatively large number of substrates and products, allowing the employment of multiple substrates. The LCEC system is relatively inexpensive, and has previously exhibited low limits of detection. Additionally, the instrumentation existing in our laboratory at the time could be used without any major modifications. Therefore, the primary challenge was to develop a stoichiometrically correct procedure that was simple, rapid, and involved a minimal amount of sample manipulation. Optimization of the various

parameters would also ensure the maximum sensitivity, or tissue/ blank ratio, for the assay.

II. DEVELOPMENT OF THE PROCEDURE

The analysis of MAO in brain tissue may be viewed as a three step process. Step one involves preincubation of the enzyme with appropriate cofactors, aldehyde dehydrogenase (AD), and other essential species under a specific set of conditions. The second step involves addition of substrate to the mixture with subsequent incubation. During incubation, MAO and AD transform the amine substrate into a carboxylic acid. Finally, the enzymatic reaction is terminated and the product quantitated by LCEC.

The development of this assay began with an examination of the chromatographic conditions for separating the electrochemically active components in the incubation mixture. Once an adequate separation was achieved, we proceeded to optimize the preincubation, incubation, and related parameters utilized in the assay.

The following report begins with a description of the materials used and a short outline of the procedure. This is followed by the optimization of individual parameters and an application of the procedure to the determination of MAO activity in brain parts. The concluding remarks cover areas which may prove fruitful for future investigations.

III. EQUIPMENT AND REAGENTS

A. Apparatus.

1. Liquid Chromatograph. The liquid chromatograph with an

electrochemical detector (LCEC) has been previously described by a number of investigators (1-3; also see Chap. 1). The system used in the current investigation was identical to that described by Hsi[80] in many aspects. It contained the following components.

2. Pump. Solvent delivery was accomplished with a Milton Roy Minipump, model no. 13906-1. This unit has a maximum pressure capability of 5000 p.s.i. and a maximum flow rate of 160 ml/hr.

3. Damping Coil. A Li-Chroma-Damp II from Handy & Harmon Tube Company, Norristown, PA, was employed to minimize the pressure fluctuations arising from the reciprocating pump. It was rated at a maximum operating pressure of 3000 p.s.i. The empirical lifetime of this unit was only ca. 2 months during continuous operation. The dampener failed to perform adequately due to excessive formation of small pinhole leaks in the collapsed portion of the coil. Consequently, the dampener was discarded and replaced with a comparable device from Waters Associates (Low Pressure Filter Assembly, part number 98060). The Waters unit provided completely acceptable noise reduction for this assay with a considerably longer lifetime.

4. Connecting Tubing. Stainless steel tubing (1/16" o.d. x 0.030" i.d.) was used to connect the various parts of the chromatograph. Connectors were 1/16" stainless steel Swagelok fittings.

5. Detector. The carbon paste working electrode as well as the reference and counter electrodes have been described in Chapter 1.

6. Injector. Initially, a Rheodyne Model no. 7120 Sample Injector fitted with a 20 microliter fixed volume loop was used. Later

a Waters WISP, model no. 710A, Intelligent Sample Processor was used to automate the injection process.

7. Electronics. The combined potential controller and current monitoring instrument was identical to that previously described.[4]

8. Column. The column was a 4.6 x 150 mm Ultrasphere ODS packed with 5 micron reverse phase particles and obtained from Beckman, Berkley, CA (part no. 256-06). Essential precautions required for proper maintenance of the chromatographic column are described in Appendix A2.

9. Pressure Limiter. A pressure gauge (3000 p.s.i. max.) was electronically modified by P. Lin to limit the operating pressure of the entire system.[59] It automatically shut off the pump when a predetermined value of pressure, typically 3000 p.s.i., was reached or surpassed.

10. Tissue Homogenizer. For sonicated samples, a Sonifier Cell Disruptor, model W200P from Heat Systems Ultrasonics, Inc., Plainfield, NY, was used. The sonicator was fitted with a 3 mm probe. For ground glass samples, a Fisher Dynamix stirrer (Model 43, Fisher Scientific, Dallas, TX) was fitted with a ground glass tissue grinder (part no. K-885450-0023, size C) from Kontes Glass Co., Vineland, NJ.

11. Incubator. A Sherer Controlled Environmental Chamber, model CEL 37-14, was used directly as an air incubator.

12. Shaker. An Eberbach model 6010 shaker, operated at 4 cycles per second (high setting), was placed in the incubator for utilization.

13. Centrifuge. A Sorvall RC2-B refrigerated centrifuge, fitted with an SM-24 rotor, was used for all centrifugal operations.

14. Strip Chart Recorder. An Omniscribe recorder, obtained from Houston Instruments, Austin, TX, was used to record all chromatograms. The recorder was typically operated at a setting corresponding to 20 namp of detector current for a full scale deflection.

B. Animals.

Adult male and female mice were bred in-house from the Sprague-Dawley ARS HA/ICR albino strain. These were maintained on a 12 hour light/12 hour dark cycle and allowed access to food and water ad libitum. They were sacrificed by cervical dislocation, usually between the hours of 9 and 11 a.m. They were typically 2-3 months old, corresponding to body weights of 25-30 g, at the time of death.

C. Chromatographic Conditions.

1. Detector. The detector was packed with carbon paste (CP-W) from Bioanalytical Systems, West Lafayette, IN. The electrode was operated at a potential of +0.70 volts vs. a saturated calomel electrode (SCE). The SCE was similar in construction to that described in Meites.[5] It contacted the chromatographic stream through a short piece of 3 mm Vycor rod which was held onto its tip by a piece of teflon tubing.

2. Mobile Phase. A buffer solution was prepared by dissolving 29.41 grams of trisodium citrate dihydrate (0.050 M) and 21.01 grams of citric acid monohydrate (0.050 M) in 2.0 liters of water. The pH of this buffer was brought to 4.3 by dropwise addition of 1 M NaOH. Prior to use, the solution was passed through a 0.45 micron Millipore filter to remove particulate matter.

3. System Operating Conditions. The flow rate was typically 1.3 ml/min. This corresponded to a pump setting of 50.0 and resulted in an operating pressure of 2500 p.s.i. To minimize solvent preparation, the eluent from the detector was returned to the solvent reservoir to be recycled through the chromatographic system. The recycling of the mobile phase presented no apparent problems even after 1 month of continued use. To prevent dust and other impurities from entering the system, the reservoir was tightly capped with a one hole stopper. The inlet and outlet lines passed through the single hole. The inlet line was also fitted with a metal-sintered filter (50 μ) to prevent large particles from entering the pump.

D. Reagent Sources.

The following chemicals were obtained from the sources noted in the highest available purity.

From Sigma Chemical Co., St. Louis, MO:

β-nicotinamide adenine dinucleotide (NAD^+)

3,4-dihydroxybenzoic acid (protocatechuic acid, DB)

From Aldrich Chemical Co., Milwaukee, WI:

5-hydroxyindole-3-acetic acid (5-HIAA)

3,4-dihydroxyphenylacetic acid (DOPAC)

dopamine hydrochloride (DA)

5-hydroxytryptamine(serotonin)creatine sulfate monohydrate (5-HT)

pargyline (N-methyl-N-propargylbenzylamine hydrochloride)

citric acid, trisodium salt (sodium citrate dihydrate)

From Mallinkrodt, Paris, KY:

citric acid monohydrate

From Pfaltz & Bauer, Flushing, NY:

2-mercaptoethanol (EtSH)

From in-house synthesis:

5-hydroxyindole-3-carboxylic acid (5-HICA). (Synthesis described in Appendix A1).

All other chemicals were purchased in the highest possible purity from readily available commercial sources.

E. Solutions.

1. Stock Solutions. These solutions were prepared on the morning of analysis (See Table 4-1). The solutions were considered unstable and were discarded immediately after use. The values for NAD^+, 5-HT, and DA shown in the table are the derived optimum values (vide infra).

2. 2-Mercaptoethanol. This solution was prepared by dissolving 100 µl or 350 µl of the mercaptan in 10.00 mls of distilled water. Different solutions were required for each of the two substrates investigated. The final stock concentrations were 0.143 M and 0.499 M for DA and 5-HT, respectively.

3. Phosphate Buffer. A 0.50 M solution of phosphate having a pH of 9.6 was prepared in the following manner. A 0.50 M solution of Na_2HPO_4 was made by dissolving 35.5 grams of the anhydrous compound in 500 mls of water. A second solution, containing 0.50 M Na_3HPO_4, was made by dissolving 95.0 grams of the dodecahydrate salt in 500 mls of water. The first solution was then titrated with the second until

Table 4-1. Preparation of the Stock Standard Solutions

Compound	Weight of Compound (mg)	Final Volume (ml)	Final Concentration (moles/l)
NAD^+	65.20	5.00	2.0×10^{-2}
Internal Standard Stock Solutions			
5-HICA[a]	1.18	5.00	1.33×10^{-3}
DB[b]	1.20	10.00	7.79×10^{-4}
Working Standard Stock Solutions			
5-HIAA[a]	1.14	10.00	5.96×10^{-4}
DOPAC[b]	.94	10.00	5.59×10^{-4}
Substrate Stock Standard Solutions			
5-HT[a]	34.47	5.00	1.70×10^{-2}
DA[b]	10.2	5.00	1.08×10^{-2}

[a]Used when 5-HT is substrate.

[b]Used when DA is substrate.

a pH of 9.6 was obtained.

4. Homogenization Solution. A 0.050 M hypotonic phosphate buffer having a pH of 10.0 was prepared by first diluting each of the 0.50 M phosphate buffers described above by a factor of 10. The solution containing Na_3PO_4 was then titrated with the solution containing Na_2HPO_4 until a pH of 10.0 was reached.

5. Isotonic Sucrose. An isotonic solution of sucrose was prepared by dissolving 58.2 grams of this compound in 500 ml of water. The final concentration was 0.35 M.

6. Postincubation Solution. The solution added to the incubation mixture immediately after incubation contained acetic acid and $NaClO_4$. It is described as the 'homogenization solution' in Chapter 2.

7. Kidney Homogenate. Kidneys were removed from female laboratory mice and weighed in a ground glass tissue grinder. An appropriate volume of isotonic sucrose was added to produce a solution containing 0.10 grams tissue/ml. The mixture was homogenized at a setting of 4 using multiple up/down strokes, transferred to a 15 ml polypropylene centrifuge tube and centrifuged at 4°C and 39,900 x g (18,000 rpm) for 20 min to remove unwanted particles. The supernate was stored on crushed ice for use in the analysis.

8. Brain Homogenate. To each whole brain, 4.00 ml of homogenization solution was added, and the mixture was homogenized on crushed ice by sonication (power setting 5; 50% duty cycle). For brain parts, only 1.00 mls of homogenization solution was added. This volume had to be adjusted to 2.00 mls for cortex samples. A 500 µl

aliquot of the homogenate, whether from whole brain or parts, was then used directly in the analysis of each sample.

IV. THE STANDARD MAO PROCEDURE

A brief diagrammatic outline of the MAO procedure is presented in Fig. 4-1. The brains were rapidly removed after sacrifice, placed in 15 ml centrifuge tubes, weighed and stored on crushed ice. Whole brains were used directly or dissected into seven regions (cerebellum, medulla-pons, midbrain, diencephalon, hippocampus, striatum, and cortex) by the method of Wassil.[8]

Kidneys were dissected by first removing the skin from the back of the animal. A small incision was made on each side of the exposed backbone, approximately one inch below the neck. The kidneys, easily seen as small, brownish-red bean shaped objects, were removed with the aid of a scalpel. These were homogenized as described above. During the twenty minutes required for centrifugation of the kidney homogenate, power was applied to the temperature controlling unit of the incubator, stock solutions were prepared, the centrifuge tubes to be used for incubation were numbered, and the reagents were dispensed into the labeled tubes. The volume of each reagent as well as the order in which it was added are presented in Table 4-2. The brain tissues were homogenized immediately before addition in this sequence. For all experiments, the total incubation volume was kept at 1.750 mls.

After solutions 1 through 10 (Table 4-2) were added and mixed, the tubes were capped and the samples preincubated at 44°C, without

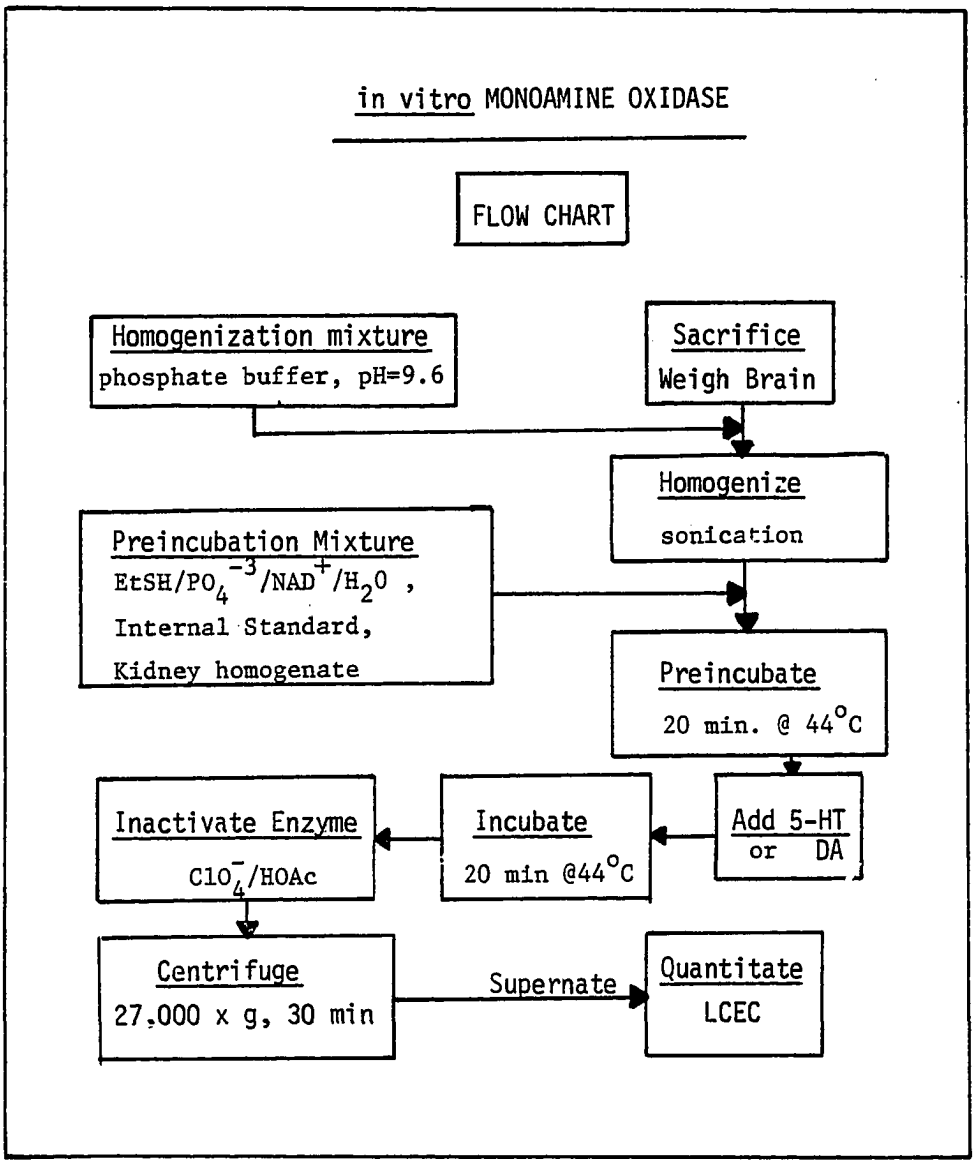


Figure 4-1. Flow Chart for Monoamine Oxidase Procedure.

Table 4-2. Reagent Volumes of Stock Solutions Used in MAO Assay

Reagent and Order of Addition	Quantity (microliters)	Final Concentration in Tissue Samples (M)	Final Concentration in Standard Samples (M)	Final Concentration in Blank Samples (M)
1. Phosphate buffer	500	0.14	0.14	0.14
2. Homogenization solution	500	--	0.014[a]	0.014[a]
3. 0.34 M Sucrose	250	--	0.048[b]	--
4. NAD^+	100	0.00113	0.00113	0.00113
5. Water	100	added[c]	--	added[c]
5-HT as Substrate				
6. EtSH	100	0.0330	0.0330	0.0330
7. 5-HICA	100	7.6×10^{-5}	7.61×10^{-5}	7.61×10^{-5}
8. 5-HIAA	100	--	3.19×10^{-5}	--
11. 5-HT	100	9.67×10^{-4}	9.67×10^{-4}	9.67×10^{-4}
DA as Substrate				
6. EtSH	100	0.00813	0.00813	0.00813
7. DB	100	4.42×10^{-5}	4.42×10^{-5}	4.42×10^{-5}
8. DOPAC	100	--	3.19×10^{-5}	--
11. DA[d]	100	6.16×10^{-4}	6.17×10^{-4}	6.17×10^{-4}
9. Kidney homogenate	250	36 mg/ml	--	36 mg/ml
10. Brain homogenate[e]	500	36 mg/ml	--	--
Total Volume	1750			

[a]substitutes for brain homogenate [b]substitutes for kidney homogenate [c]substitutes for 100 microliters of working standard [d]added after 20 min preincubation [e]assuming an ave. brain wt. of 0.5 gm

shaking, for 20 minutes. After preincubation, the substrate (either 5-HT or DA) was added, a stopwatch started, and the samples incubated for a further 20 minutes. During incubation, the samples were continuously subjected to shaking at 4 cycles/sec with the tubes in a horizontal position having the long axis of the tubes parallel to the direction of shaking. The measured pH of the incubation mixture was 8.1. After 19.0 minutes of incubation, the tubes were removed from the incubator. The reaction was terminated at 20.0 minutes by placing a 250 µl aliquot of the reaction mixture into 750 µl of the postincubation solution. The tubes were capped, mixed, and centrifuged for 1 hr. at 39,900 x g (18,000 rpm) and 4°C. The supernatant fractions were decanted into clean, labeled 1.5 ml polypropylene centrifuge tubes and stored on dry ice until analysis by LCEC. Between 5 and 20 µl of supernate were typically required for separation and quantitation of the reaction product(s).

V. CALCULATIONS

Calculations were virtually identical to those described by Wong.[1] The amount of acid formed by the deamination and dehydrogenation of the amines is a direct measurement of the MAO activity. The results, expressed as nanomoles product/milligram wet tissue/minute (nmol/mg/min) were calculated as follows:

$$\text{activity(nmol/mg/min)} = \left(\frac{R_{sample} - R_{blank}}{R_{std}}\right)\frac{(\text{nmol acid in working std.})}{(\text{milligrams tissue})(\text{incubation time, min})}$$

where

$$R = \left(\frac{\text{peak height, acid}}{\text{peak height, int. std.}}\right)$$

The R_{std} and R_{blank} values represent the average ratios for all standard samples and blank samples, respectively. The acid refers to 5-HIAA or DOPAC and the internal standard refers to 5-HICA or DB; the choice as to which is appropriate depends on whether 5-HT or DA, respectively, is used as the substrate. The weight of tissue added to each incubation tube was calculated as follows:

wt. tissue incubated (mg) =

$$\frac{(\text{mls tissue homogenate taken})(\text{wt. tissue, g})}{(\text{mls homog. solution taken} + (\text{wt. tissue, g})/(1.2\ \text{g/ml}))}$$

This assumes a density of 1.2 g/ml for the brain tissue.

Unless otherwise specified, each result represents the average of at least three separate determinations. All uncertainties are expressed as the standard deviation (S.D.). Statistical significance was examined by use of Student's t-test.[7]

VI. INVESTIGATION OF CHROMATOGRAPHIC CONDITIONS

Utilizing the chromatographic principles and theories presented in Chapter 1, a separation was achieved for MAO determinations which provided adequate resolution between neighboring chromatographic peaks. The total time requires was less than 12 minutes for a single chromatogram. The conditions employed also permit the simultaneous quantitation of 5-HIAA and DOPAC as well as their associated substrates and internal standards. A sample chromatogram, shown in Fig. 4-2, illustrates the separation achieved for 5-HIAA, DOPAC, 5-HT, and DA. The related chromatographic data is presented in Table 4-3, while the

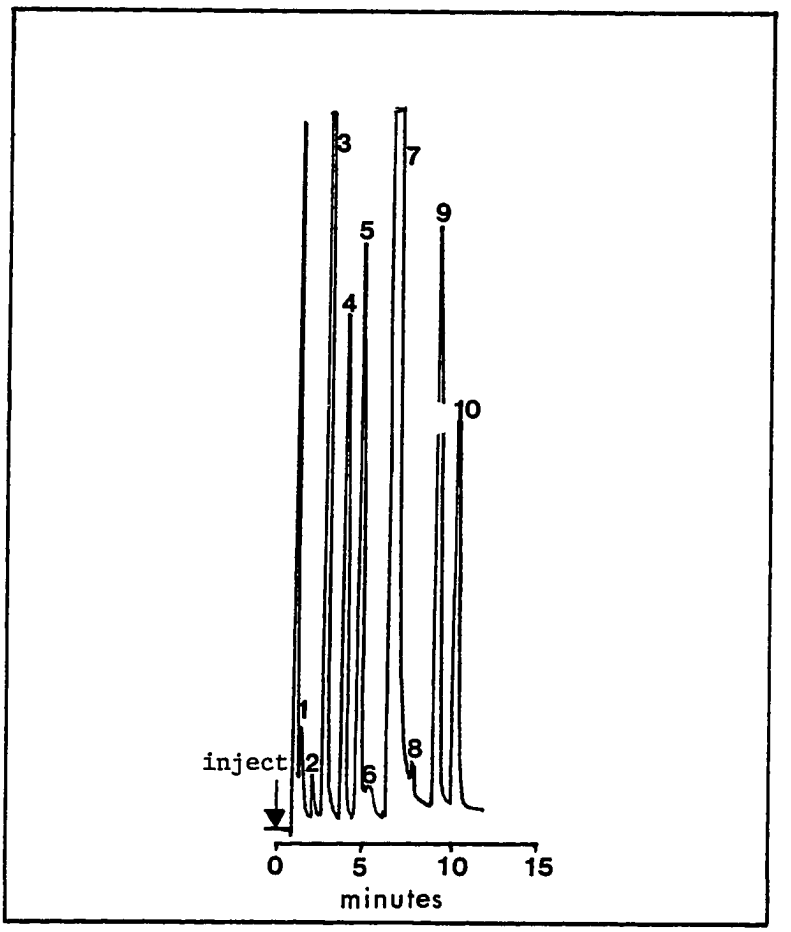


Figure 4-2. Typical Chromatogram from an MAO Analysis Employing Both Dopamine and Serotonin as Substrates. Numbers used for peak identification are defined in Table 4-3.

Table 4-3. Chromatographic Data for Compounds Associated with MAO Determinations

Compound No.	Compound Identification	Retention Time (min:sec)	Capacity Factor (k')
0	Solvent	----	0.52
1	NADH	0:51	1.23
2	Unknown	1:36	2.10
3	Dopamine (DA)	2:22	2.57
4	3,4-Dihydroxybenzoic Acid (DB)	3:29	3.56
5	3,4-Dihydroxyphenyl-acetic Acid (DOPAC)	4:36	4.55
6	Unknown	5:04	5.32
7	Serotonin (5-HT)	6:51	6.53
8	Impurity from 5-HIAA	7:58	7.52
9	5-Hydroxyindole-3-acetic acid (5-HIAA)	9:09	9.15
10	5-Hydroxyindole-3-carboxylic Acid (5-HICA)	10:38	10.26

eluting solvent is described in Section III.C.2 (vide supra). These same chromatographic conditions are also suitable for the analysis of other electrochemically active substrates. For example, 3-MT (k'= 6.5) can be studied through its product HVA (k'=10.0) either alone or in combination with DA and/or 5-HT. Due to their extremely small capacity factors, the β-hydroxylated catechols, NE and NORMET, cannot be determined using these conditions. A solvent having a low pH (less than 4), some sample clean-up, and the use of quaternary ammonium salts as ion-interaction agents are suggested as possible modifications to make these species accessible to the interested reader.

Optimization of the applied potential for the electrochemical detector focused on providing the maximum sensitivity with a minimum amount of baseline noise. For quantitative work, it is also preferred to have a signal which is relatively independent of the applied working potential. This is helpful in avoiding major signal fluctuations as a result of inevitable, but minor, fluctuations in applied potential. To minimize this problem, the potential of the working electrode was successively varied while a constant amount of the investigated compounds were injected into the chromatograph. The observed peak heights at each potential were recorded. The results are shown in Fig. 4-3 for 5-HIAA and 5-HICA. The ratio of the peak heights is also presented in this figure. The background current or noise, although not shown, increases in a fairly linear fashion over the range shown. For maximum sensitivity with minimum noise, a potential was selected on the portion of the graph where the peak height ratio is relatively independent of the applied potential, i.e., +0.700V vs. SCE. This value was used

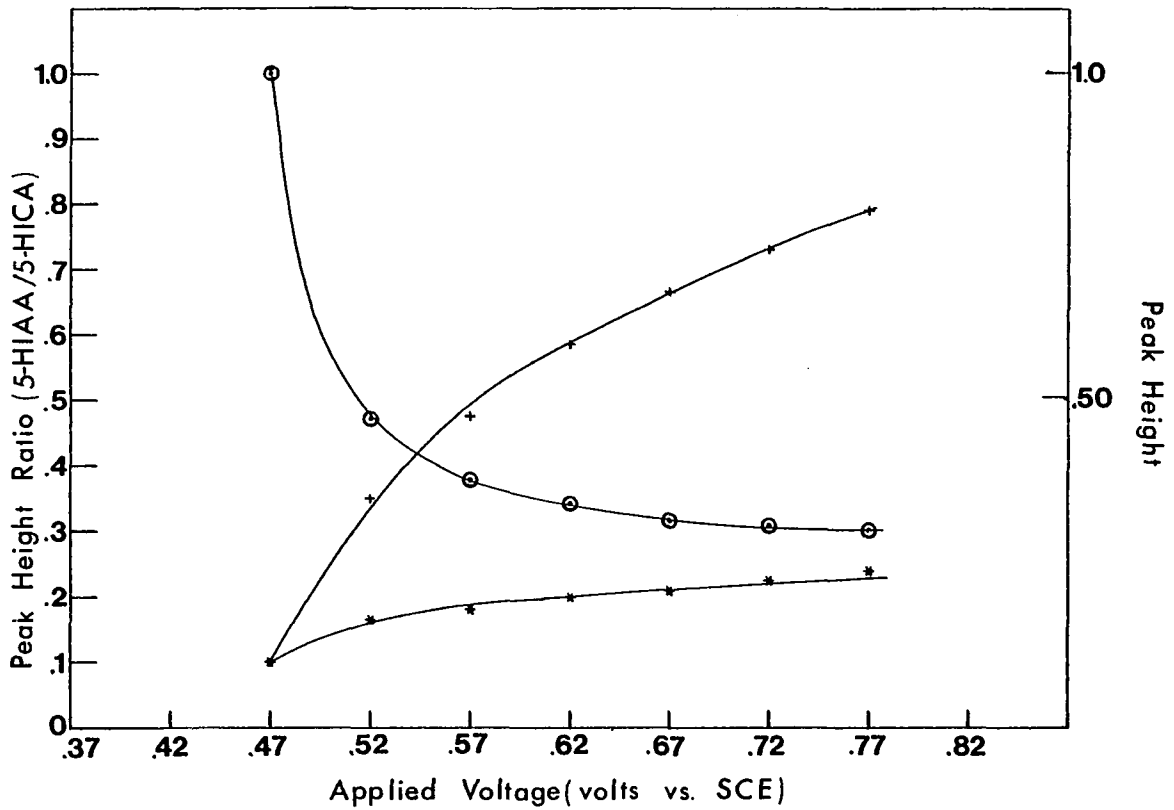


Figure 4-3. Effect of Applied Working Potential on the Peak Height Ratio of 5-HIAA to 5-HICA. Symbols: peak height ratio (o); peak height 5-HICA (+); peak height 5-HIAA (*).

in all subsequent work. Similar studies of DOPAC and DB were not undertaken. These compounds had been previously shown to be easier to oxidize than the indolic species by cyclic voltammetry; thus, the value of +0.700V is also completely applicable to their quantitation.

The linearity of the peak height ratio [(peak height of acid)/(peak height of internal standard)] as a function of the amount of acid was also studied for both DOPAC and 5-HIAA. The results are plotted in Fig. 4-4. The concentrations were derived from stock standard solutions by appropriate dilution. No tissue was present in this study. As can be seen, excellent linearity was obtained for a range of values which encompasses those typically encountered for 5-HIAA and DOPAC in an MAO assay.

VII. OPTIMIZATION OF INCUBATION CONDITIONS

The conditions used by Lovenberg et al.[9] were initially employed for the MAO assay without any major modifications. However, the currently developed method is unique in a number of respects. Thus, we decided to carefully examine some of the more important parameters. An appropriate range for each parameter was initially selected and examined in a rather cursory manner to determine a value which produced an optimum activity. The values derived in this manner were then combined to form a 'tentative' optimal procedure. These values also were used to indicate appropriate ranges to be further investigated. Using the tentative procedure and the new ranges, each parameter was reexamined in detail in the order presented below. The optimized value for a given individual parameter was always incorporated

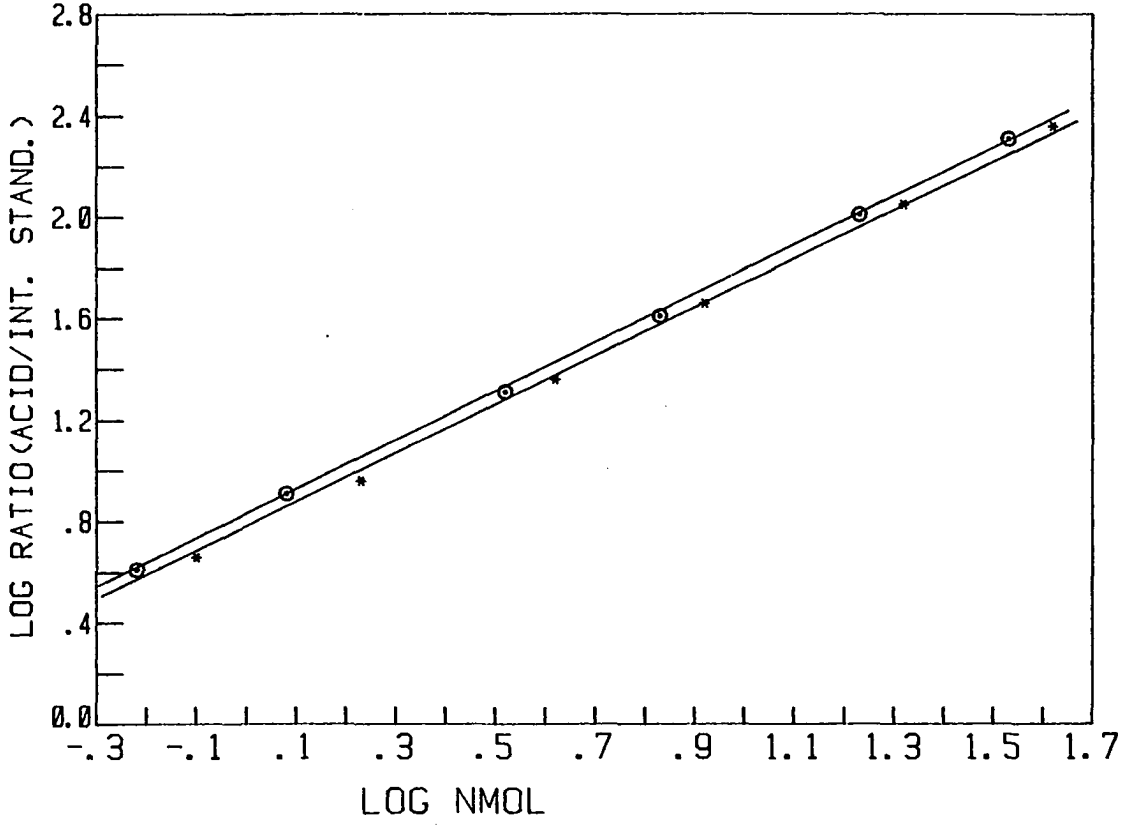


Figure 4-4. Relationship of the Peak Height Ratios DOPAC/DBA (o) and 5-HIAA/5-HICA (*) as a Function of the Amount (nmol) of Injected DOPAC and 5-HIAA, Respectively.

into the tentative procedure immediately after it was determined. Both the initially employed conditions and the ones used in the beginning tentative procedure are presented in Table 4-4.

A. Incubation Temperature

A review of previous procedures for MAO showed no single incubation temperature common to all. Room temperature,[10] 30°C[11,12] and 37°C[13,14,15] had all been employed. The consensus appeared to be

Table 4-4. Procedural Conditions Used At Various Stages in the Development of the MAO Assay.[a]

Condition	Initial Procedure[b]	Intermediate, 'Tentative' Procedure
Temperature	37°C	37°C
Kidney Homogenate	0	36 mg/ml
2-Mercaptoethanol	0	33.0 mM (5-HT) 8.3 mM (DA)
pH	7.4	8.1
Nicotinamide	1 mM	0
NAD^+	1 mM	1 mM
Substrate Concentration	1.18 mM (5-HT) not tested (DA)	1.18 mM (5-HT) 0.50 mM (DA)
Incubation Time	30 min	20 min
Preincubation Time	10 min	20 min
Homogenization	Ground Glass	Ground Glass

[a]All concentrations are referred to the final incubation mixture.
[b]Patterned after Lovenberg et al.[9]

at 37°C, although none of the papers reported optimization of this parameter.

Initially, temperatures of 30, 37 and 44°C were investigated with 37°C yielding the maximum activity. However, once the tentative procedure had been established, the maximum activity was observed to occur at 44°C. The results are presented in Table 4-5. Preincubation was performed at the same temperature as incubations to eliminate time delays that would have been required for stabilizing the air bath.

B. Aldehyde Dehydrogenase.

The conversion of amines into the corresponding acids by MAO relies on a second, NAD^+ dependent enzyme: aldehyde dehydrogenase (AD). Kinetic measurements of MAO determined by the amount of acid formed are reliable only if the MAO activity is rate limiting. The AD must spontaneously convert the aldehyde(s) produced by MAO into the corresponding acid(s).

The need to fortify the brain tissue homogenates with AD originated with the commencement of this work. Incubated tissue samples initially exhibited a broad peak eluting near 5-HIAA when using 5-HT as the substrate (Fig. 4-5). This peak was partially eliminated with the addition of 2-mercaptoethanol (EtSH), a compound known to stimulate AD; simultaneously, an increase was observed in the measured 5-HIAA activity. This led us to believe that the broad, unidentified peak was 5-hydroxyindole-3-acetaldehyde. Additional evidence to support this hypothesis was provided when two broad peaks appeared following incubation with both 5-HT and DA in the absence of NAD^+, a cofactor for AD (Fig. 4-6A). These peaks disappeared when NAD^+ was incorporated

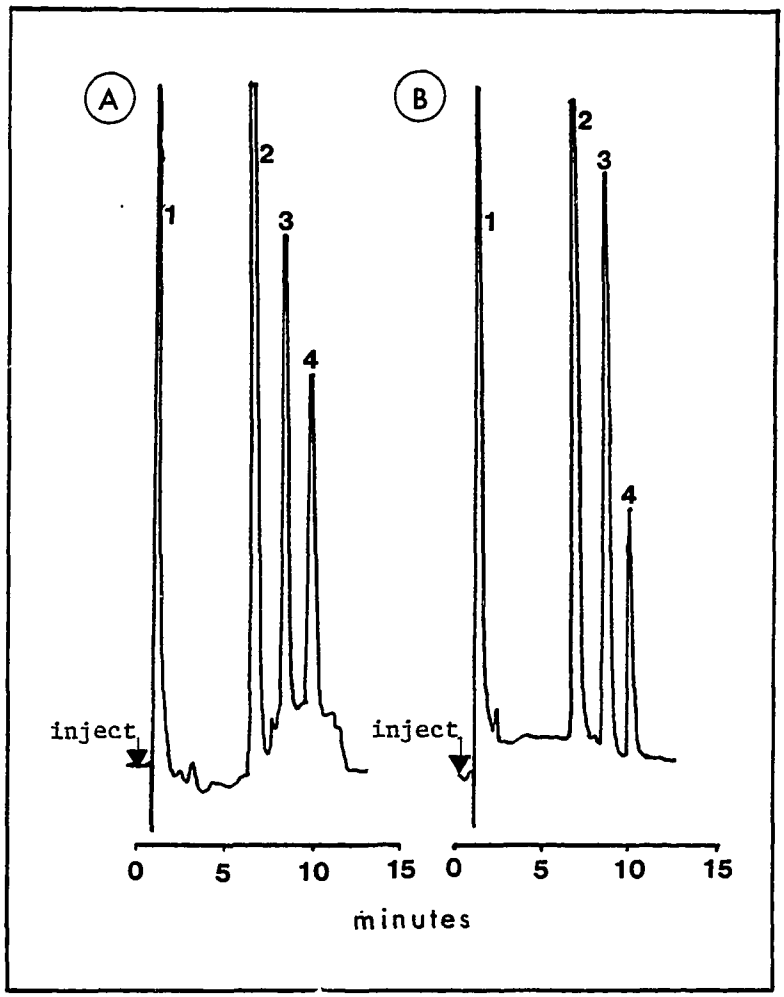


Figure 4-5. Removal of Broad Peak by the Addition of EtSH. Fig. A was obtained with no EtSH. Fig. B was obtained with an EtSH concentration of 0.033 M. Both mixtures contained mouse kidney and NAD^+. (For chromatographic conditions see section III-C). Peaks are: solvent (1), 5-HT (2), 5-HIAA (3), and 5-HICA (5).

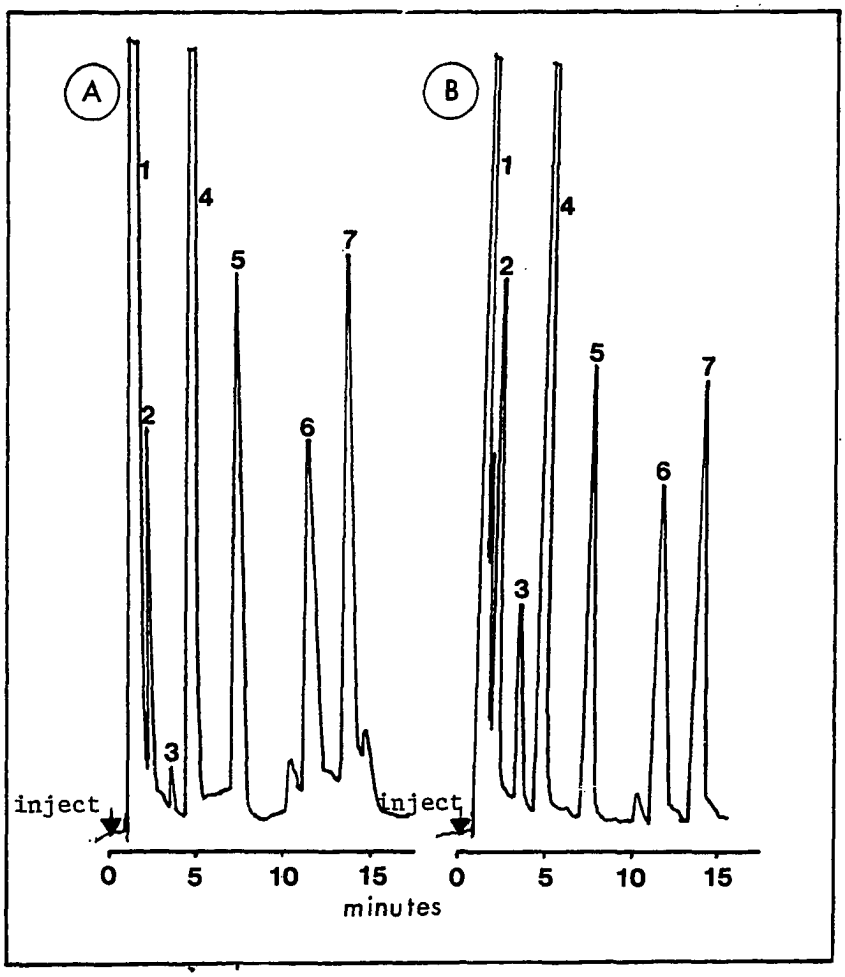


Figure 4-6. Addition of NAD^+ to Incubation Mixtures. Fig. A was obtained with no NAD^+; Fig. B was obtained with NAD^+. Note the absence of the two broad peaks in chromatogram B. (Chromatographic conditions are the same as in section III-C except the pH was 4.6 and 0.080 mM sodium octyl sulfate was added to the mobile phase.) Peaks are: solvent (1), 2,3-dihydroxybenzoic acid (2), DOPAC (3), DA (4), 5-HT (5), 5-HIAA (6), 5-HICA (7).

Table 4-5. MAO Activity vs. Incubation Temperature for Whole Mouse Brain

Incubation Temperature (°C)	Activity DA (nmols DOPAC/mg/min)	Activity 5-HT (nmols 5-HIAA/mg/min)
30	0.0562±.0021	0.0418±.0057
37	0.0883±.0052	0.0606±.0055
44	0.111±.0040	0.0797±.0072
50	0.103±.0110	0.0653±.0046

into the reaction mixture containing added AD (Fig. 4-6B).

The capability to detect these aldehydes in MAO incubation mixtures gives the LCEC assay definite advantages over other MAO-AD coupled procedures. Other assays commonly involve coextraction of the acids and aldehydes into a nonaqueous solvent.[9] Such extractions do not, however, provide any distinction between aldehyde and acid. This causes difficulties in distinguishing between MAO and AD effects. The LCEC assay partially alleviates this problem by allowing any substantial amount of aldehyde to be easily seen in the chromatogram.

It was considered justifiable to add a source of AD to prevent the possibility of this enzyme being a limiting factor in the overall reaction. A commercial preparation of AD was first attempted. One milligram of this preparation reportedly will oxidize 4.5 μmol of acetaldehyde to acetic acid in one minute at pH = 8.0 and 25°C. This

AD was added to the incubation mixture in amounts ranging from 0 to 1.1 mg. Surprisingly, the highest activity occurred when no commercial AD was added at all! This approach was thus abandoned. But, previous investigators had reported enhancement of MAO assays by incorporating additional AD.[9,18-20] Studies with guinea pigs,[16] e.g., had shown that MAO activity increased when kidney was used as a source for AD. In the light of this information, isotonic kidney homogenates were prepared and added to the incubation mixtures. The kidney homogenates did, indeed, produce higher activities (Fig. 4-7). A homogenate containing 50 mg of tissue was subsequently added to all samples.

During the course of this optimization work, notable sex differences were observed. Initially, the mice used were exclusively females. However, as our female population slowly diminished, a switch was made to males. A considerable drop in observed MAO activity was noticed at this time, coupled with the reappearance of the aldehyde peak in the chromatography. An examination of the literature revealed that a sex difference in kidney AD had previously been reported for rats[17] where the velocity for females was over twice that of males. In light of our findings and these reports, only female kidney preparations were used for succeeding investigations.

The possible necessity for blanks was also considered at this point in the initial optimization. Blanks would be required if either the endogenous amount of product(s) in brain homogenates or the amount of product(s) produced by incubation without brain tissues were considerable. A substantial amount of endogenous compound was eliminated due to the relatively low concentration of such species found in the

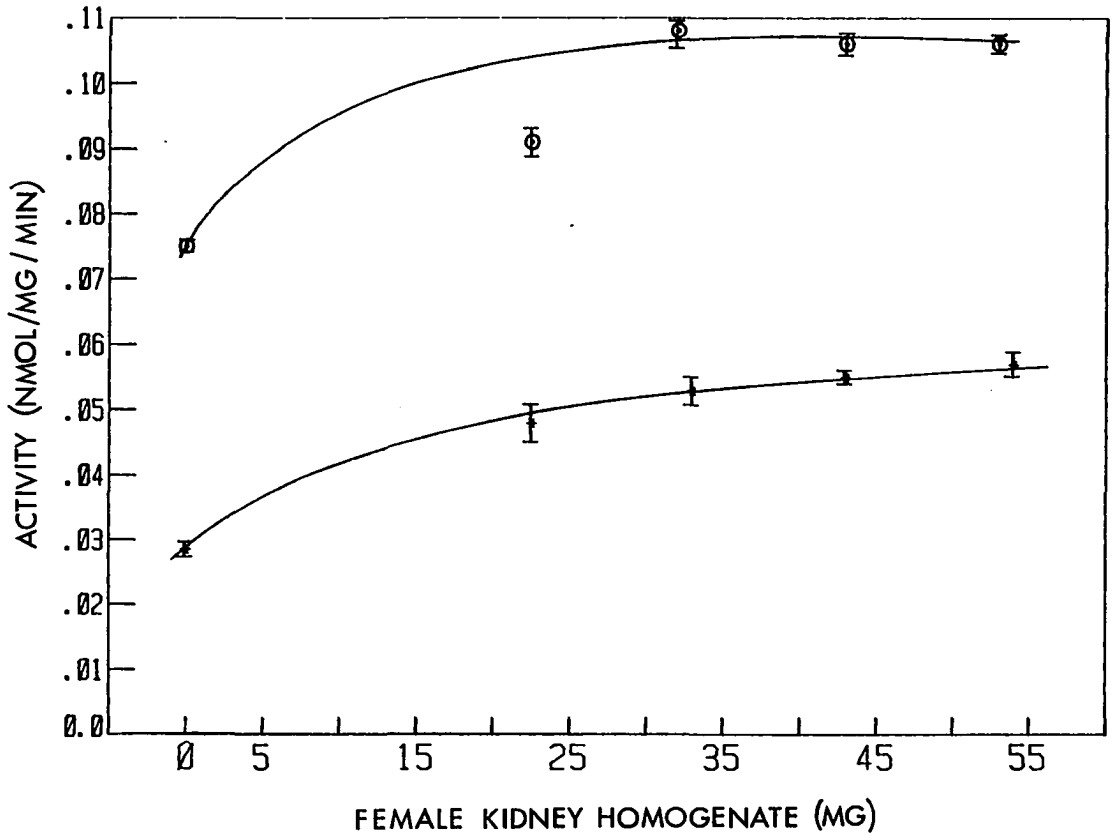


Figure 4-7. Effect of Female Mouse Kidney Homogenate on MAO Activity. Substrates: 5-HT (*), and DA (o). Amount of kidney homogenate indicated were added to each incubation mixture.

brain. For example, a typical whole brain contains 400 ng 5-HIAA/gram wet tissue. A typical MAO assay, employing 63 mg tissue and incubated for 20 min, yields an average activity of 0.0835 nmol/mg/min. Calculating the 5-HIAA produced by the enzyme and that derived from the endogenous content, we find:

endogenous: 0.33 nmol 5-HIAA

MAO produced: 105.0 nmol 5-HIAA

Thus, under the given conditions, the amount of product generated by the enzyme is 300 times greater than that commonly encountered in brain tissues. However, when incubations were performed without any brain homogenate, a substantial amount of product was formed. Representing approximately 5-10% of the usual activity, this material was presumed to result from residual MAO activity in kidney homogenates. This observation simply means that blanks are required and must be included for assays employing this procedure.

C. Interference by Aldehyde Reductase.

The possible interference by aldehyde reductase, which would convert the intermediate aldehyde into an alcohol, was considered but eliminated for a number of reasons. First, NADH, the primary cofactor for aldehyde reductase, is only present in very low concentration in the incubation mixture. Secondly, aldehyde reductase is notably inhibited at alkaline pH values.[37] Finally, there are only low concentrations of this enzyme in brain tissues.[37]

D. 2-Mercaptoethanol (EtSH).

The addition of EtSH to the incubation mixture noticeably increased the activity. This compound is frequently employed in assays

as a general enzyme activator.[21] Houslay et al.,[20] for example, observed activation upon treatment of tissue preparations with EtSH. Lundquist[22] included it in his assay for AD. Sigma Chemical also suggests addition of thiols to their purified AD preparations. These reports as well as our preliminary observations clearly indicated the inclusion of EtSH. When EtSH was added to incubation mixtures containing either DA or 5-HT as substrate, we obtained the results shown in Fig. 4-8. It is not currently known why the two substrates require unique EtSH concentrations. One possibility might be the involvement of multiple forms of AD; however, isozymes of AD have not yet been reported.[27] Another possibility is that the EtSH is having a differential effect on the two isozymes of MAO in addition to its effect on AD.

E. pH Optimization.

The control of pH was accomplished by the addition of a 0.50 M solution of a phosphate buffer. During optimization, the buffers were prepared by mixing together various amounts of 0.50 M solutions of mono-, di-, and tri-sodium phosphate. This molarity did not provide the desired buffer capacity, but higher concentrations were not practically obtainable due to dissolution problems. The resulting pH of the incubation mixture was, thus, measured as a function of the buffer pH (Table 4-6) using the standard amount of brain tissue (60 mg/sample). The resulting pH was also measured as a function of the amount of brain tissue (Table 4-7) using the standard buffer pH of 9.6. The results were not surprising, since a simple titration of phosphoric acid with 0.10 M NaOH clearly shows a lack of buffering capacity in this pH range.[30] The final pH of the incubation mixture was, thus, measured for each test in the optimization

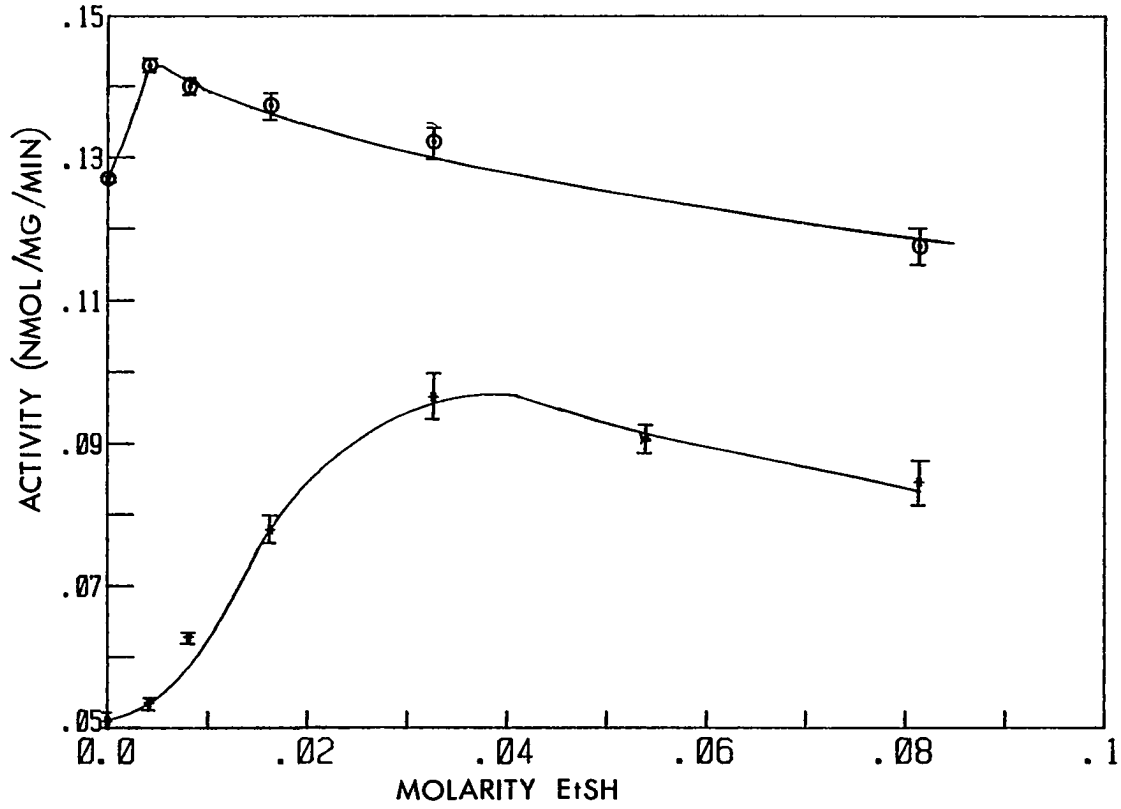


Figure 4-8. Effect of Mercaptoethanol on MAO activity. Substrates: 5-HT (*), and DA (o).

procedure. The resulting activities for the two different substrates were plotted as a function of the measured pH (Fig. 4-9). The pH which produced the maximum activities for both substrates fell in the range of 8.0-8.4. This is in agreement with others who have measured MAO activities through the acid product in preparations incorporating AD.[16,18,20] However, the majority of assays which measure MAO do not incorporate AD and, thus, focus on the aldehyde product. Such assays have reported pH optima in the range of 7.2-7.8.[10-14,29,23-26]

The discrepancy between the optimum pH for the single enzymatic reaction (aldehyde measurement) as opposed to the coupled reaction (acid measurement) may possibly be due to some unknown cooperativity between MAO and AD. Alternatively, the aldehyde product of the single enzyme assays may experience more extensive interaction with macromolecular (and other) amines at elevated pH values. Such reactions would lead to an excessively and incorrectly low pH optimum.

The use of other buffering agents was not investigated in the current study. The obvious alternatives would be TRIS (tris-hydroxymethylaminomethane) and borate. However, Tabakoff[31] has already reported that TRIS causes a 30% reduction in MAO activity.

Table 4-6. pH of Incubation Mixtures as a Function of the Phosphate Buffer pH.

pH of Phosphate Buffer	7.51	8.42	9.57	10.16	10.51	11.24
pH of Incubation Mixture	7.51	7.98	8.23	8.68	9.36	11.09

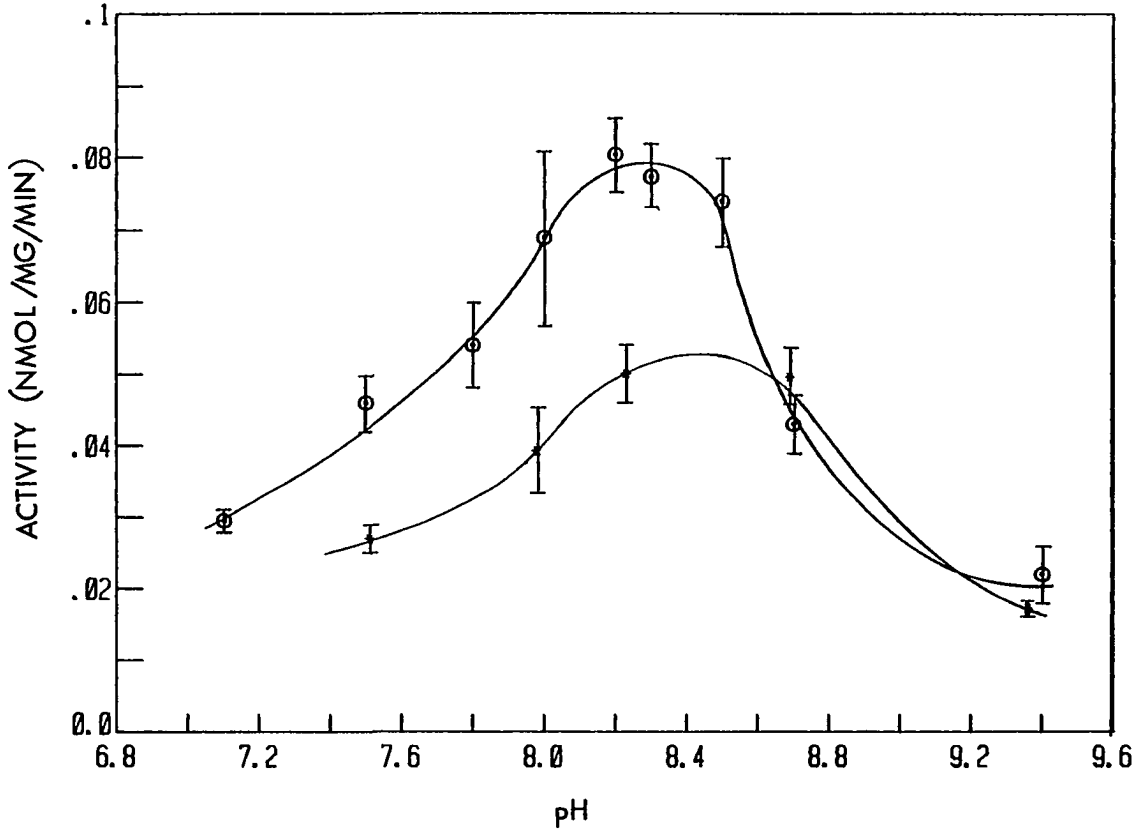


Figure 4-9. Effect of pH on MAO Activity. Substrates: 5-HT (*) and DA (o).

Table 4-7. pH of Incubation Mixture as a Function of the Amount of Added Brain Tissue.[a]

Weight of Tissue (mg)	5.89	11.8	23.6	35.3	58.9
pH of Incubation Mixture	8.26	8.22	8.18	8.15	8.13

[a]All other components of samples were the same as those described for the standard MAO assay. The amount of tissue was adjusted by varying the concentration of the tissue homogenate as expressed in mg/ml.

And, Snyder[13] has observed a decrease in MAO activity when using either borate or TRIS.

The possible effects of ionic strength were also considered but not investigated. Tabakoff[31] has previously found no difference in MAO activity with a threefold change in ionic strength, and Houslay et al. have reported virtually identical results.[20]

F. Nicotinamide.

Some researchers have included nicotinamide in incubation mixtures of coupled MAO assays.[9,19] However, no effect on MAO activity was observed in the current investigation for nicotinamide concentrations varied between 1×10^{-7} and 1×10^{-3} M. Thus, nicotinamide was eliminated from the procedure.

G. NAD^+.

NAD^+ was added to incubation mixtures containing either DA or 5-HT. Plots of MAO activity as a function of the concentrations of

this component are shown in Fig. 4-10. While the observed activity is virtually constant for either substrate with concentrations above 5.0×10^{-4} M, a value of 1.0×10^{-3} M NAD^+ was selected as optimal to provide a comfortable margin of safety. This value is in strong agreement with the work of Dietrich et al., who consistently used the same concentration in their studies with aldehyde dehydrogenase,[17,28,32] and Houslay et al., who used 7.5×10^{-4} M in their work.[20]

H. Substrate.

The effects of the substrate concentration in the incubation mixture are presented in Fig. 4-11. The 1/V vs. 1/S plots were used to determine the K_m and V_{max} values. The K_m results are compared to other reported values for this constant in Table 4-8. The V_{max} for 5-HT and DA, respectively, were 0.0910 nmol/mg/min and 0.164 nmol/mg/min. Since most of the previously reported values for V_{max} are for purified mitochondrial preparations and are expressed for mg protein rather than per mg tissue, no direct comparisons can be made at this time. Based on the K_m values and the ease of preparation, optimal substrate concentrations of 9.67×10^{-4} M for 5-HT and 6.17×10^{-4} M for DA were selected.

I. Incubation Time.

The linear accumulation of product with incubation time is shown in Fig. 4-13. From this figure, it is apparent that the reagents described in Table 4-2 are present in sufficient quantity to sustain a constant production of acid for 20 minutes.

J. Preincubation Time.

Preincubation of some enzymes with their cofactors has been shown to occasionally provide greater activities.[1] The results of

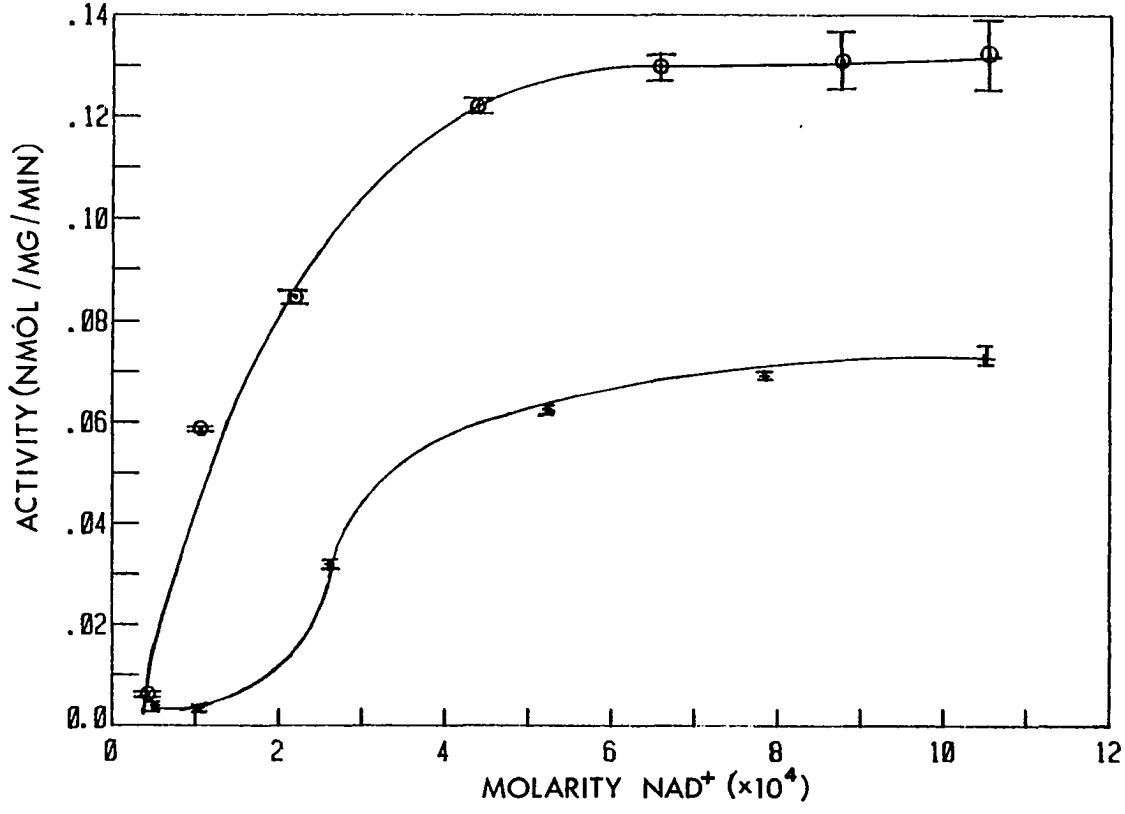


Figure 4-10. Effect of NAD^+ on MAO Activity. Substrates: 5-HT (*) and DA (o).

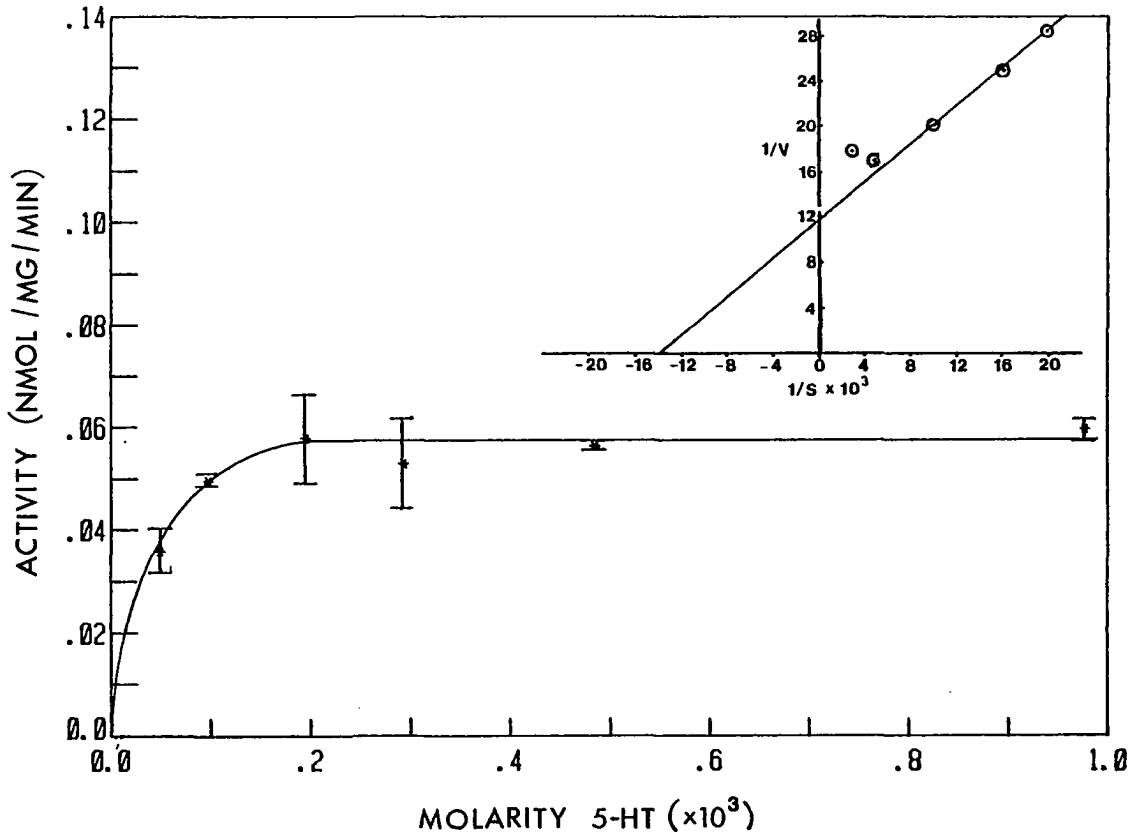


Figure 4-11. Effect of Serotonin (5-HT) Concentration on MAO Activity.

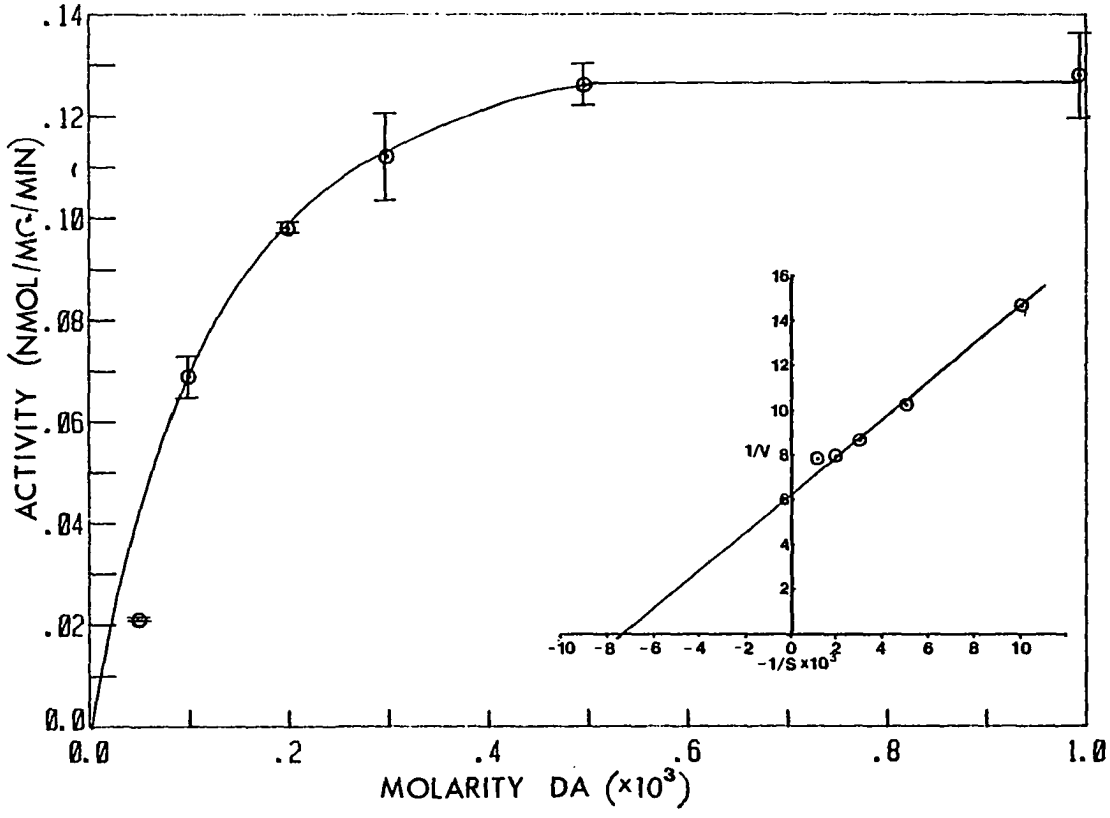


Figure 4-12. Effect of Dopamine Concentration on MAO Activity.

Table 4-8. Comparison of K_m Values for DA and 5-HT as Substrates in MAO Analyses[a]

Substrate	Technique	K_m (μM)	MAO Source	Reference
5-HT	LCEC	71	mouse brain homogenate	this work
5-HT	Radiometric	130	rabbit brain purified mitochondria	12
5-HT	Potentiometric	80	rat brain purified mitochondria	15
5-HT	Potentiometric	57	rat liver purified mitochondria	36
DA	LCEC	167	mouse brain homogenate	this work
DA	Radiometric	210	rabbit brain purified mitochondria	12
DA	Spectrophotometric	210	rat liver purified mitochondria	20
DA	Potentiometric	171	rat liver purified mitochondria	36

[a]The literature values for techniques other than LCEC refer to samples which are not fortified with AD.

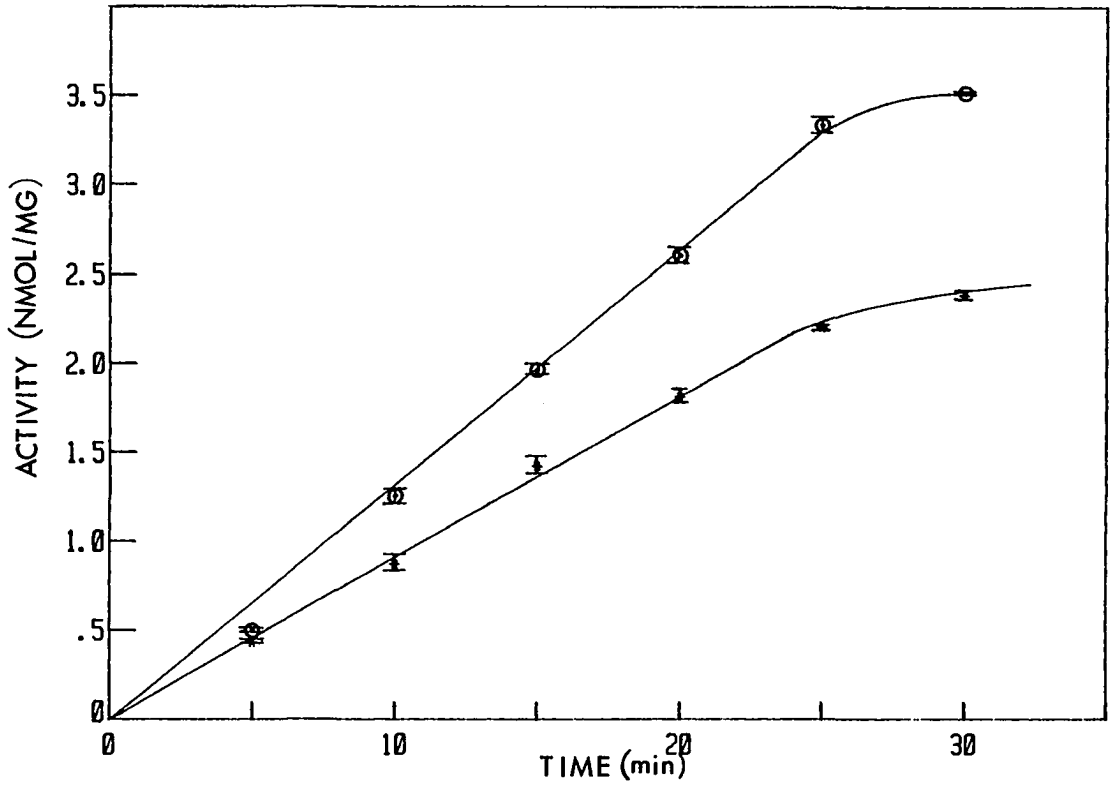


Figure 4-13. Effect of Incubation Time on MAO Activity. Substrates: 5-HT (*) and DA (o).

preincubating samples for MAO determinations are shown in Fig. 4-14. As can be seen, activities are 20 to 50% higher after about 20 minutes of preincubation. It is believed that this time allows the enzyme, in the presence of the added substances, to assume an appropriate structural configuration. An increase in the solubility of the enzyme with increased temperature may also occur. Another possible advantage of preincubation is obtained by raising the temperature of the mixture to the optimal value prior to addition of substrate. This helps to eliminate any non-linearity of activity with incubation time.

K. Sonication and Ground Glass Homogenization.

It is much more convenient, from a procedural aspect, to homogenize small tissue samples like brain parts with ultrasonics. The small tip of the sonicator fits very nicely into the constricted bottom of a 1.5 ml centrifuge tube. In the previous experiments, however, whole brains were homogenized with ground glass due to anticipated destruction of MAO by ultrasonication. But, to our surprise, a comparison of sonication versus ground glass homogenization, given in Table 4-9, showed activities to be significantly higher in the sonicated samples. We feel that sonication might provide better disruption of particulate matter, thus increasing exposure of the mitochondrial bound MAO and, possibly, increasing the solubility of the enzyme. It should be noted that these studies employed brain homogenates derived from males instead of the usual female mice, although we do not believe this would have any effect on the outcome.

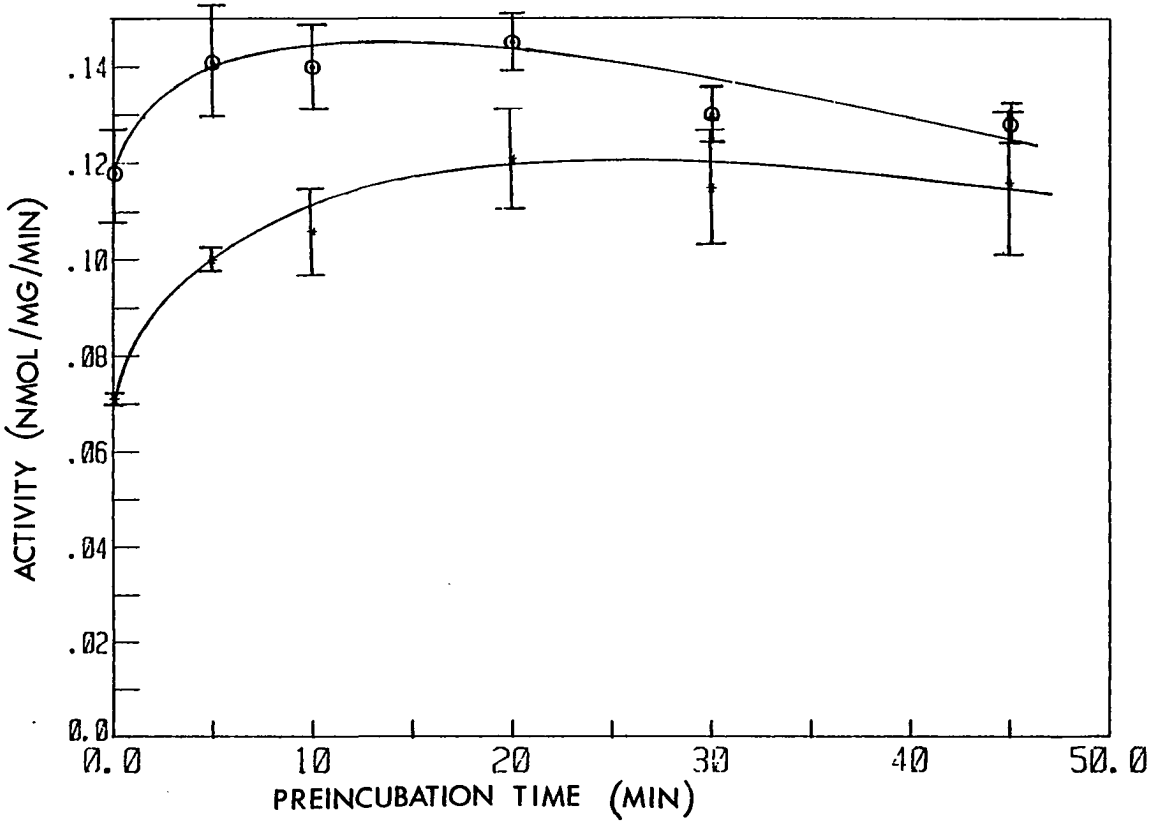


Figure 4-14. Effect of Preincubation on MAO Activity. Substrates: 5-HT (*) and DA (o).

Table 4-9. Comparison of Homogenization Methods for the Determination of MAO Activity

Substrate	Activity (nmol/mg/min)	
	Ground Glass	Sonication
DA	0.0677±.0047	0.0738±.0041
5-HT	0.0360±.0032	0.0434±.0039

L. Shaking.

The effect of shaking the reaction tubes during incubation was studied using DA as the substrate. The values obtained were 0.0148± .0007 nmol/mg/min for still samples and 0.133±.004 for samples which were shook. Needless to say, shaking was continued for all further experiments.

M. Oxygen.

Our laboratory is currently not equipped to measure oxygen concentrations in gas or liquid samples. Nonetheless, a simple experiment was conducted to test the effect of high oxygen levels on MAO activity. A series of tubes containing male mouse brain tissue were filled with pure oxygen, DA was added as the substrate, the tubes were tightly capped, and the samples were incubated along with similar samples containing an air atmosphere. The results were 0.1105+.0107 and 0.0652+ .008 nmol/mg/min for oxygen-enriched and regular samples, respectively.

As expected, the oxygen samples provided much greater activities. A problem occurred, however, with the oxygen samples. After incubation at 44°C, the samples containing oxygen were very dark purple, indicating the chemical destruction of DA by oxidation. The effect of these degraded compounds on MAO is unknown, but it should be investigated if further work is to be done using oxygen sensitive compounds as substrates.

N. Stability of Samples Prior to Injection.

After the MAO activity was halted by addition of the post-incubation solution, the samples were centrifuged and stored on dry ice until immediately before injection into the LCEC system. Studies were, thus, conducted on both working standards and brain samples to examine possible sample degradation with storage. Samples were prepared using two different postincubation media, 1.0 M HCl and 1.0 M HOAc/0.40 M $NaClO_4$. These were kept at two different temperatures, dry ice and room temperature, and analyzed at various times following preparation. The results, shown in Table 4-10 for standard samples, indicate the instability of the 5-HIAA in 1 M HCl at room temperature. The corresponding results for tissue-containing samples exhibited less sensitivity to storage. Thus, the HOAc/$NaClO_4$ mixture was selected as the postincubation medium and all samples were stored on dry ice.

O. Enzyme Concentration.

The relationships between the enzyme concentration and the rate of acid production for the two substrates are shown in Fig. 4-15. The enzyme concentration was varied by modifying the concentration of the tissue homogenate (mg/ml). The resulting range of tissue weights incorporated into individual samples, 5-60 mg, is appropriate for either

Table 4-10. Stability of Standard Samples in Different Media Following Storage at Various Times and Temperatures.

Temperature	Postincubation Medium	Compounds Investigated	Peak Height Ratio[a]			% change after 6 hrs
			Start	6 hrs	3 days	
23°C	HCl	DOPAC/DB	0.57	0.56	0.56	2%
23°C	HOAc/$NaClO_4$	DOPAC/DB	0.57	0.57	0.56	0%
dry ice	HOAc/$NaClO_4$	DOPAC/DB	0.64	0.63	0.63	2%
23°C	HCl	5-HIAA/5-HICA	2.08	9.29	--	347%
23°C	HOAc/$NaClO_4$	5-HIAA/5-HICA	2.60	2.69	--	3%
dry ice	HOAc/$NaClO_4$	5-HIAA/5-HICA	2.23	2.32	3.46	4%

[a]Ratio of acid to internal standard.

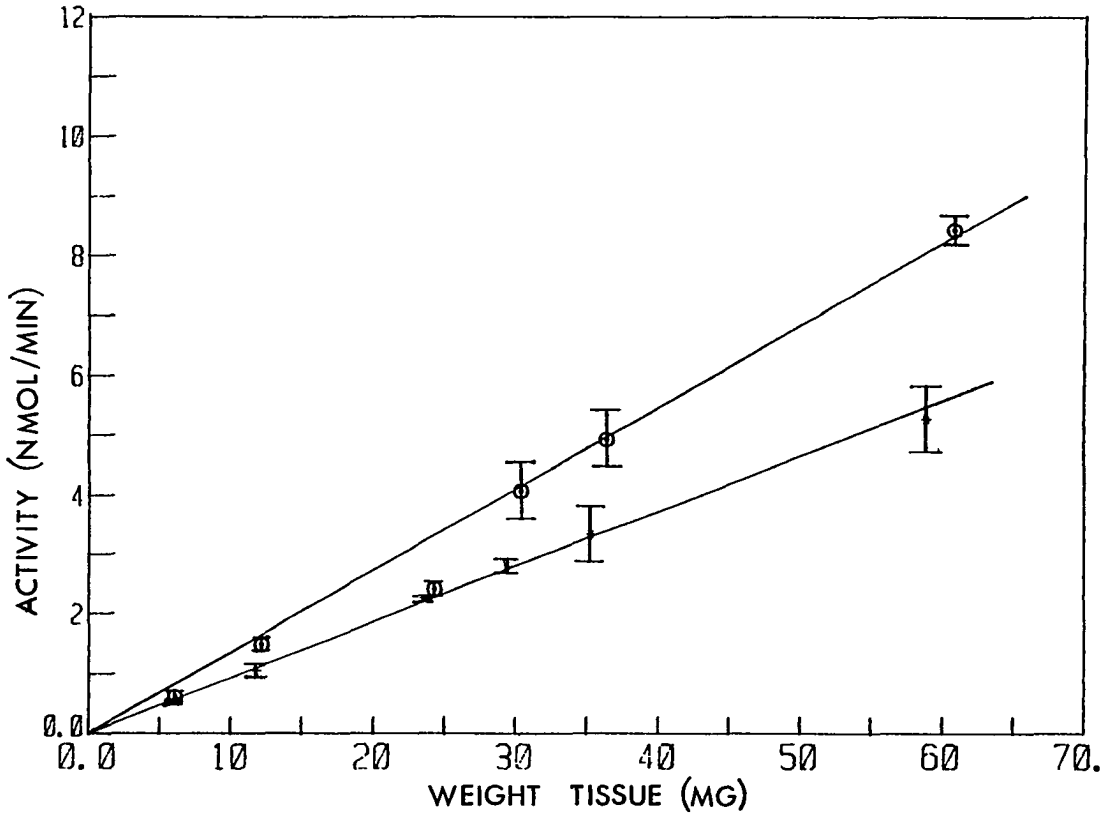


Figure 4-15. Relationship Between MAO Activity and the Amount of Brain Tissue Added to Incubation Mixtures. Substrates: 5-HT (*) and DA (o).

mouse brain parts or whole brain.

P. Stoichiometry of the Method

Once all the various parameters had been optimized, a stoichiometric analysis of the coupled MAO-AD determination was conducted. The relationship between the amount of substrate consumed and product formed is given in Table 4-11. Since the MAO activities, as calculated from both substrate disappearance and product formation show no statistical difference, the enzymatic procedure is stoichiometric. One notable exception for this test is that the substrate added to individual samples was reduced by a factor of three from the optimal value.

Q. Summary of Optimization.

The various parameters investigated during the optimization of the current procedure are presented in Table 4-12. The initial conditions, ranges examined, and final values are also presented in this table.

Table 4-11. Stoichiometric Relationship of Enzymatic Reaction

Substrate	Compound Measured	Observed Activity (nmol/mg/min)
5-HT	5-HIAA	0.0560±.0035
	5-HT	0.0565±.0047
DA	DOPAC	0.149±.0030
	DA	0.142±.0063

Table 4-12. Summary of the MAO Procedure Optimization

Parameter	Initial Value	Range	Optimal Value
Incubation Temperature (°C)	37	30-50	44
Kidney Homogenate AD (mg tissue)	none	0-55	50
Mercaptoethanol (mM)	0	0-83	8.3 (DA), 33.0 (5-HT)
pH	7.4	7.1-9.5	8.1
Nicotinamide (M)	none	10^{-7}-10^{-3}	0
NAD^+ (mM)	none	0-54	1.0
Substrate Concentration (mM)	1.18 (5-HT) 0.50 (DA)	0.05-0.97 0.05-0.99	0.97 0.62
Incubation Time (min)	30	0-30	20
Preincubation Time (min)	10	0-30	20
Homogenization	Ground Glass	Ground Glass/ Ultrasonics	Ultrasonics
Shaking	No	Yes/No	Yes
Sample Storage Prior to Injection			
Temperature	dry ice	room temp./ dry ice	dry ice
Time (hr.)	<24	0-72	≤72

VIII. APPLICATION OF FINAL PROCEDURE.

The results of typical male mouse whole brain and brain parts MAO determinations are given in Table 4-13. For the determination of MAO activity in brain parts, the samples were split into two groups. Cerebellum, midbrain, and medulla-pons were run the first day, while the diencephalon, cortex, striatum, and hippocampus were stored on dry ice for analysis on the second day.

The whole brain activity, as calculated by summing the contribution from individual parts, was found to be 0.0895 and 0.0839 nmol/mg/min for DA and 5-HT respectively. These values are comparable to the values obtained by assessing whole brain directly, i.e., 0.0982 and 0.0797, respectively. Female mice subjected to the same procedure showed a somewhat higher whole brain activity by summing the contribution from individual parts: 0.119±.009 and 0.0896±.0108 nmol/mg/min for DA and 5-HT. The blank values, or MAO activity contributed by the kidney homogenate, were typically 0.0078 and 0.0102 nmol/mg/min (S.D. = 0.001) for DA and 5-HT, respectively. Thus, the kidney preparations contribute ca. 10% of the total measured MAO activity. Forty to fifty samples could conveniently be run with the current procedure on a single day.

IX. PHARMACOLOGICAL EFFECTS

As further evidence for the general applicability of the developed MAO procedure, a brief pharmacological study was undertaken. Previous reports have clearly indicated the existence of two forms of MAO, A and B. These two forms can be differentially inhibited and have different substrate selectivities. The classification of MAO

Table 4-13. MAO Activity in Selected Regions of Male Mouse Brain Tissue

	Whole Brain	Cerebellum	Midbrain	Medulla-Pons	Diencephalon	Hippocampus	Striatum	Cortex
Tissue Weight (mg)	475±20	48.0±4.0	32.8±8.5	31.0±7.1	58.4±14.3	30.9±3.6	38.6±3.1	180±10.5
Activity (DA) (nmol/mg/min)	0.0982±.016	0.112±.009	0.129±.007	0.115±.016	0.0966±.016	0.0897±.009	0.0908±.005	0.0707±.001
Activity (5-HT) (nmol/mg/min)	0.0797±.007	0.124±.003	0.145±.017	0.135±.100	0.0784±.020	0.0795±.014	0.0967±.009	0.0542±.005
Activity Calculated from Parts								
DA	0.0895							
5-HT	0.0839							

isozymes is based on the highly selective inhibition of each form by clorgyline and deprenyl.[33] However, these compounds were not available at the time this study was being conducted. Instead, pargyline, a partially selective inhibitor of MAO-B, was added to samples to examine its effect on 5-HT (a type A substrate) and DA (a type A+B substrate). The results are shown in Fig. 4-16. As expected, the results for DA do not exhibit the double sigmoidal curve which would have been seen with a highly selective inhibitor like deprenyl. However, a slightly increased susceptibility to lower pargyline concentrations is shown by DA than 5-HT.

X. CONCLUDING REMARKS AND AREAS FOR FURTHER INVESTIGATION.

A. Areas for Further Investigation.

Comparison of MAO activities obtained with the current procedure to those obtained with other procedures proved to be fruitless. Most of the published reports describe activities in units of nmol/min/mg protein from purified mitochondria preparations or plasma samples. The advent of modern analytical techniques with their associated low detection limits would appear to have removed the necessity of purified preparations. Thus, we would suggest that the current procedure be compared to, e.g., the radiochemical procedure using the same, wet tissue or mg protein,[35] basis. Similarly, the application of this assay should also be extended to include other tissues and biological fluids.

Three potentially significant advantages of using an LCEC assay for MAO determinations have yet to be fully investigated. First, the high resolution afforded by the chromatography provides the researcher

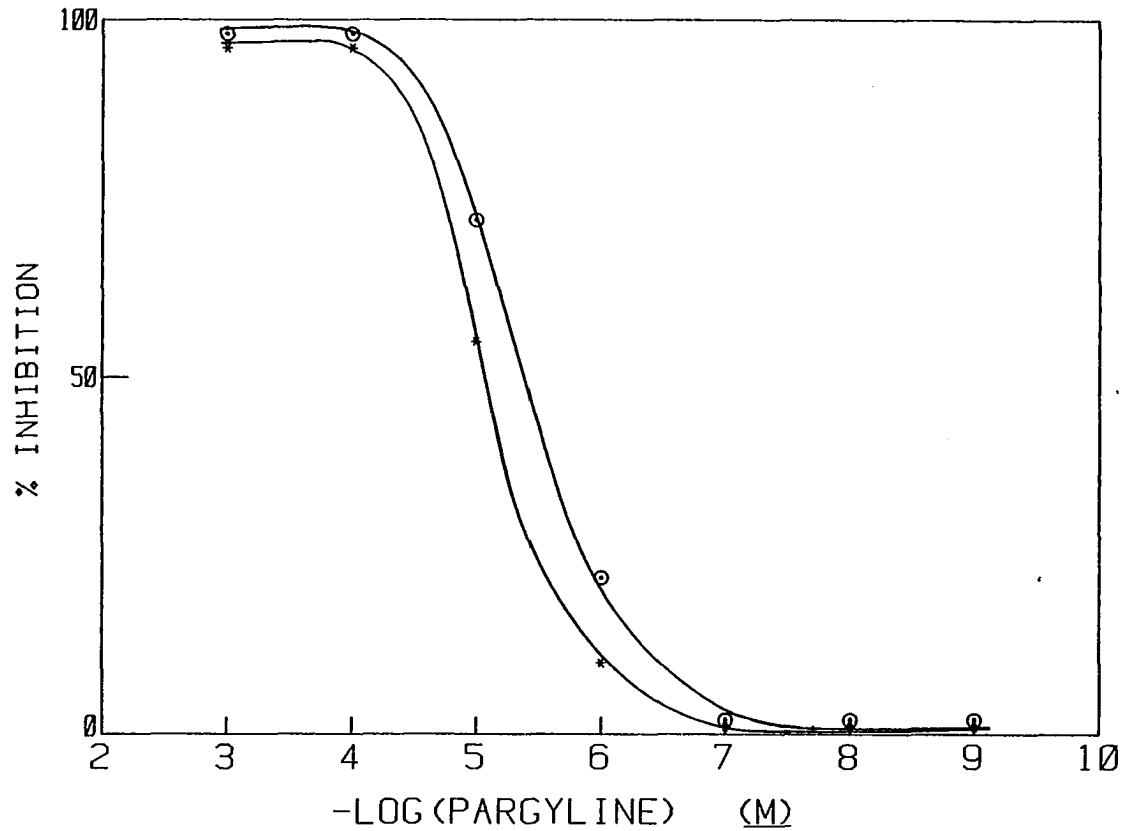


Figure 4-16. Pharmacological Effect of Pargyline on the in vitro MAO Activity of Whole Mouse Brain Tissue. Substrates: 5-HT (*) and DA (o).

with the capability to simultaneously analyze the products derived from multiple substrates. Under the currently employed conditions, 3-MT, 5-HT, and DA can all be investigated separately or concurrently. This capability is not provided by any other published assay. A preliminary study with a mixture of 5-HT and DA showed no inhibition of the activity for 5-HT; however, the activity for DA was cut almost in half when compared to determinations using only a single substrate.

A second area which was not thoroughly investigated was the limit of detection for this assay or the minimum amount of tissue needed to perform the experiment. However, a theoretical calculation was performed to derive this value. Assuming an activity of 0.090 nmol/mg/min, a 20 minute incubation, and a detection limit of 0.15 pmol for 5-HIAA, the amount of tissue required would be 0.00008 mg or 80 ng! This number brings to mind the possibility of determining MAO activities in single cells.

A third possible extension of the current procedure would make it capable of employing even non-electrochemically active substrates. This generalized approach would focus on the measurement of NADH in the incubation mixture. NADH, the reduced form of NAD^+, is produced from the action of AD on the MAO-produced aldehyde intermediate. Preliminary electrochemical studies of NADH have shown an $E_{p/2}$ of 0.65 volts vs. SCE using a carbon paste electrode and run in solvent currently employed in the chromatography (Fig. 4-17). Typical chromatograms from an incubation mixture and a working standard containing NADH are shown in Fig. 4-18. These figures illustrate the potential quantitation of this compound. A comparison of the two chromatograms reveals that

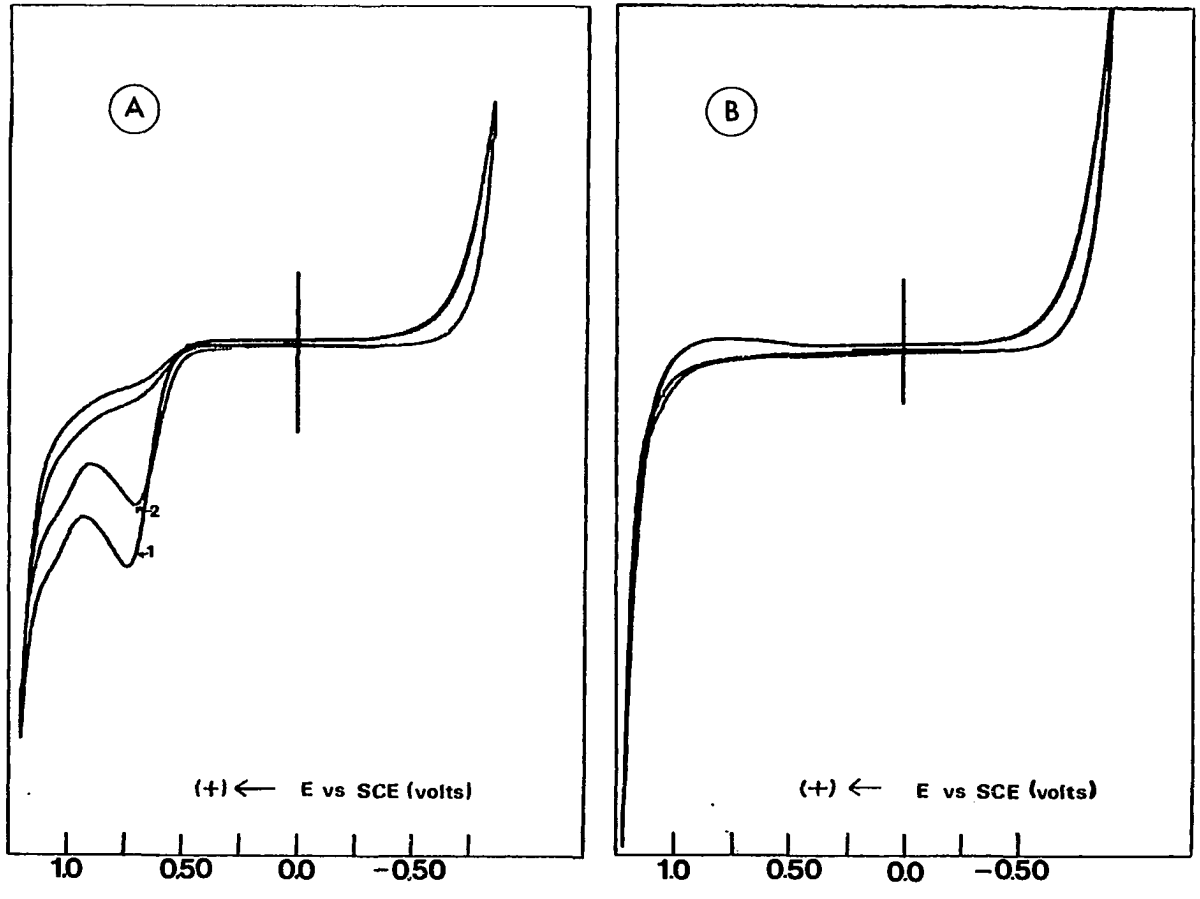


Figure 4-17. Cyclic Voltammograms of NADH (Fig. A) and NAD^+ (Fig. B) in the Current Liquid Chromatographic Solvent at a Carbon Paste Electrode. Fig. B is virtually identical to what is obtained with the solvent alone.

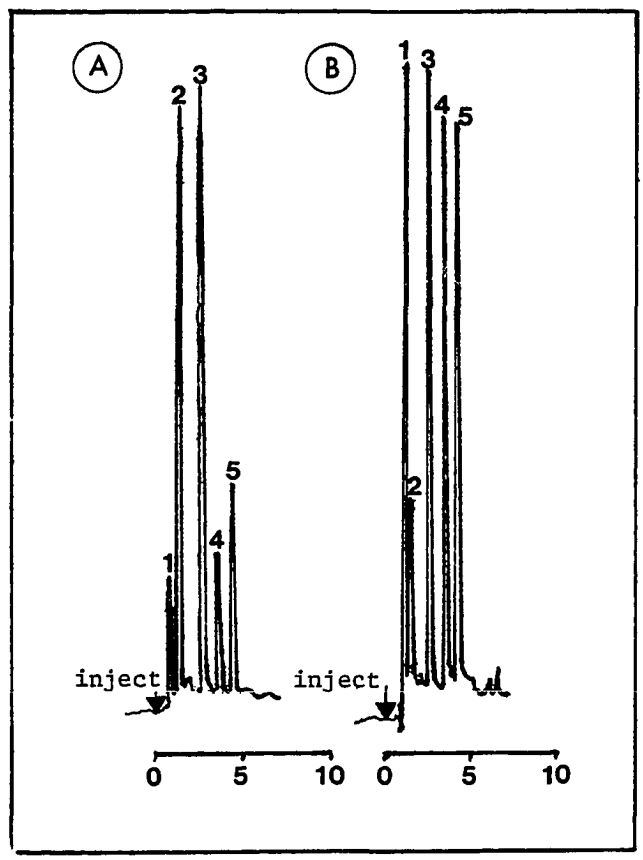


Figure 4-18. Chromatograms Illustrating the Retention of NADH. Fig. A is from a working standard solution with added NADH. Fig. B is from an incubation mixture. Peaks: solvent (1), NADH (2), DA (3), DB (4), DOPAC (5).

the incubation mixture shows a peak with the same capacity factor as authentic NADH. The relatively small size of the NADH peak in such mixtures can be explained by the relatively high peak potential for NADH and the fact that its oxidation only involves a one electron transfer compared to the 2 electron process associated with catechol oxidation.[38] Thus, a loss in sensitivity and detection limits will likely result for MAO determinations employing indirect NADH measurements.

B. Summary

The analysis of MAO activity in tissue samples by LCEC appears to have a number of advantages over existing procedures. The analysis is relatively simple, inexpensive, and involves only a minimum amount of sample manipulation. As many as 40 samples can conveniently be handled by a single operator in one day. As presently configured, this assay can also employ a variety of substrates either individually or simultaneously.

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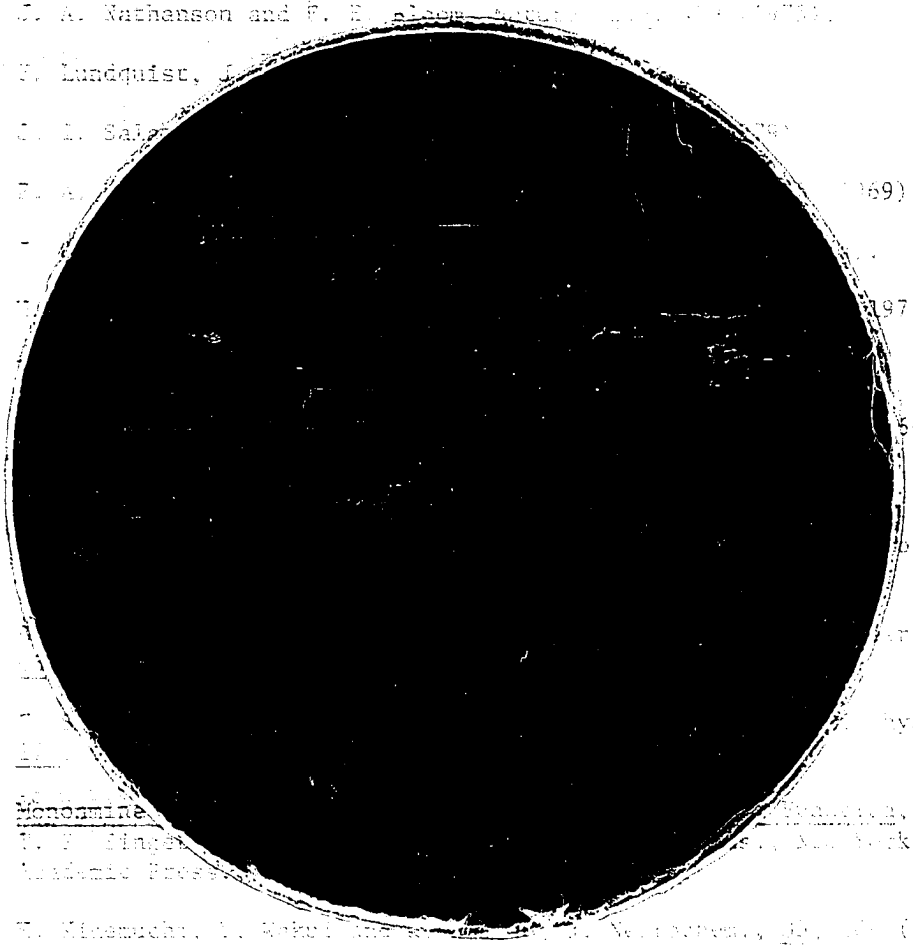
23. Monoamine [illegible]



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APPENDIX A1

SYNTHESIS OF 5-HYDROXYINDOLE-3-CARBOXYLIC ACID

I. INTRODUCTION

The preparation of 5-hydroxyindole-3-carboxylic acid (5-HICA) was adapted from the work of Marchelli et al.[6] who produced the material from 5-benzyloxyindole(I) (Fig. A1-1). The synthesis is based on the formation of 5-benzyloxy-3-carbethoxyindole(III) by reacting the starting material with a Grignard reagent and subsequently esterifying with ethylchloroformate. The mixture, containing esterified indoles (II and III) and starting material, is then subjected to hydrolysis with aqueous alkali, wherein compound III is hydrolyzed into 5-benzyloxyindole-3-carboxylic acid(IV). Indole II is converted into the starting compound (I) during this hydrolysis.[62] Finally, the purified product is debenzylated into 5-HICA(V) by catalytic hydrogenation.

II. REAGENTS

The following chemicals were obtained from Aldrich Chemical Co., Milwaukee, WS in the highest purity available. They were used without further purification:

5-benzyloxyindole

iodoethane

ethylchloroformate

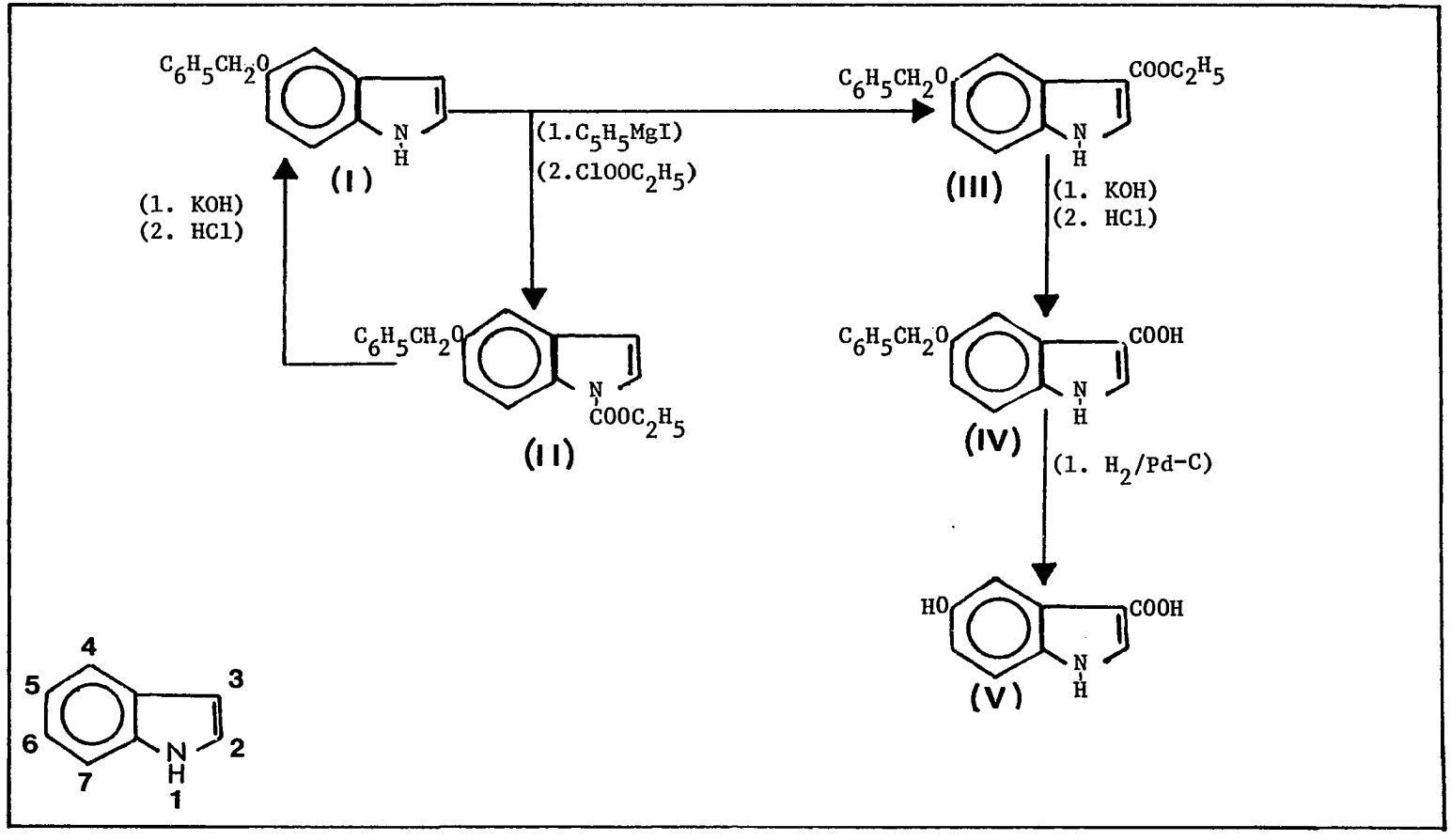

Figure A1-1. Synthesis of 5-hydroxyindole-3-carboxylic Acid.

MCB of Cincinnati, OH, supplied the 10% palladium on charcoal. All other compounds were obtained at the highest available purity from commercial sources. These included iodine, magnesium turnings, ethyl ether, ethyl alcohol, potassium hydroxide, methanol, sodium bicarbonate, anhydrous sodium sulfate, and n-propanol.

III. PROCEDURE

Rxn 1. Formation of Grignard Compounds

The reaction apparatus, similar to that illustrated in Fig. A1-2, was dried at 110°C for 1 hr, assembled while warm, and purged with dry nitrogen. 0.92 grams of magnesium turnings, 30 mls of anhydrous ethyl ether, and 4.8 mls of iodoethane were added to the round bottom flask through the right hand neck. The reaction proceeded very quickly at room temperature, and cooling with crushed ice was necessary to moderate the rate of reaction. After all the magnesium had disappeared, the solution was a pale yellow/black in color.

4.4 grams of the starting material, 5-benzyloxyindole(I), was weighed out and dissolved into 100 mls of anhydrous ethyl ether. The solution was cooled to 5°C, and the indole was added dropwise with stirring through the separatory funnel. After the addition of starting material, the reaction mixture was refluxed carefully for 90 min, after which the heating mantel was removed and the solution cooled to 0°C by immersion in crushed ice. The flask contained two layers: a brown bottom layer under a clear greenish-yellow top layer.

Rxn 2. Formation of Benzyloxycarbethoxyindoles

Into the cold mixture from rxn 1, 2.1 ml of ethyl chloroformate

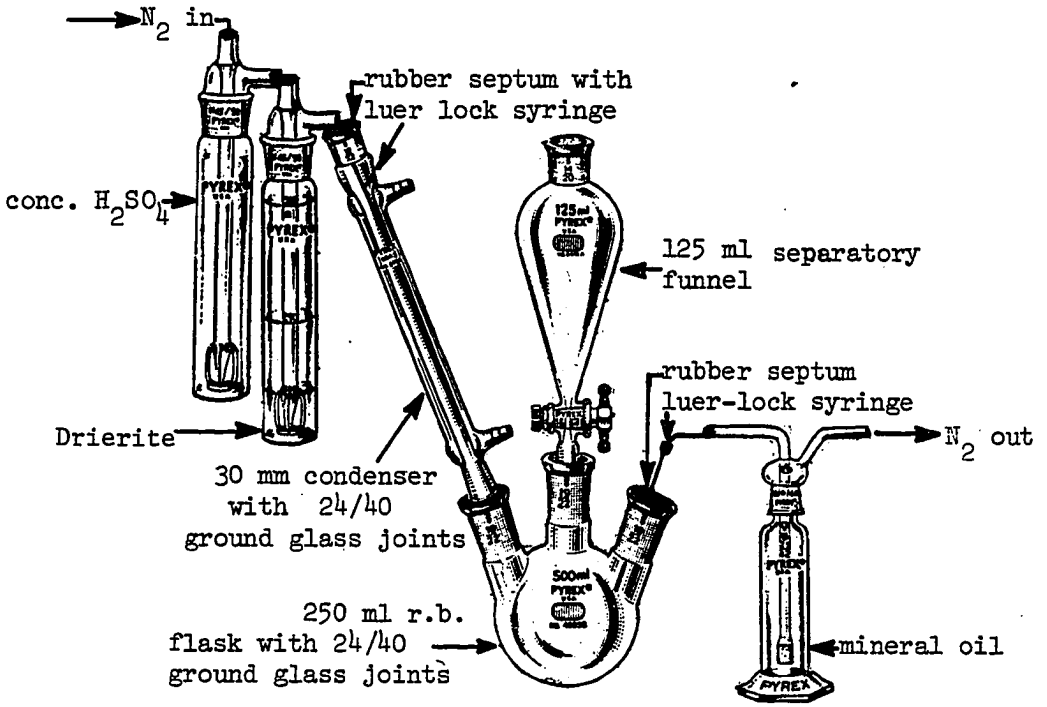

Figure A1-2. Reaction Vessel for Synthesis of 5-benzylcarbethoxyindole.

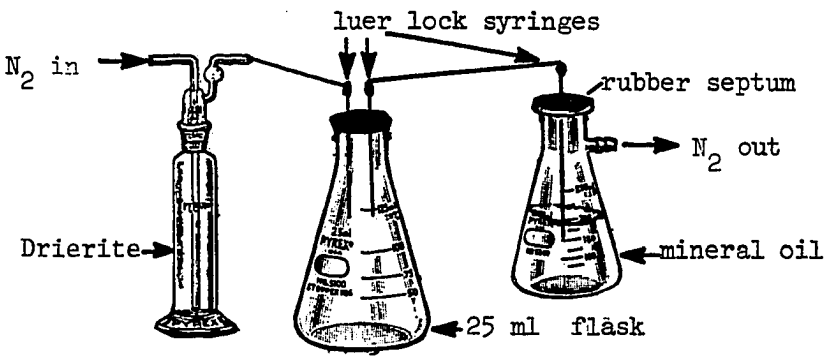

Figure A1-3. Reaction Vessel for Recrystallization of 5-hydroxyindole-3-carboxylic Acid.

in 20 ml of anhydrous ethyl ether was added dropwise while stirring through the separatory funnel. This addition took ca. 30 min. After the addition was complete, the solution was poured into a 1 liter separatory funnel containing ca. 100 of a 50:50 ice and water mixture. The contents were shaken vigorously, and an emulsion appeared. After standing, the ether layer (pale yellow) separated and was saved. The water layer was washed three times with 50 ml portions of fresh ether. The combined ether rinsings were washed three times with a 50 ml portion of a saturated solution of sodium bicarbonate and then dried with anhydrous sodium sulfate. The ether was then removed using a rotary evaporator, leaving a pale yellow solid (5.1 grams, crude product).

Rxn 3. Formation of 5-benzyloxyindole-3-carboxylic acid

2.0 grams of crude product from rxn 2, 150 mls of n-propanol, 15 mls of ethanol, and 70 mls of 2 M KOH were placed into a 500 ml round bottom flask fitted with a reflux condenser and heated for 5 hrs at 75°C in a mineral oil bath. The heat was removed and the esterification process was allowed to continue at room temperature, with stirring, for 24 hrs. The yellow/brown reaction mixture was removed and the volume was reduced to ca. 10 ml using the rotory evaporator. A sandy, yellow precipitate was observed to form at this point. The flask was removed from the rotovap, and 200 ml of ice water and 100 ml of cold ethyl ether were added. Two phases appeared (the top, a dark yellow ether layer, and the bottom, a light yellow aqueous layer). The water layer was separated using a 500 ml separatory funnel; it

was washed with two 50 ml portions of ether to remove the last traces of starting material. Approximately 70 ml of 2 M HCl were added, lowering the pH of the solution to 3 as measured with litmus paper. At this pH, a white precipitate formed, representing the carboxylic acid (IV). The aqueous layer, containing the precipitate, was then extracted 3 times with 50 ml portions of ether to collect the product. The combined ether extracts were placed on the rotary evaporator and the solvent removed, leaving a yellow solid. The solid was dissolved in ca. 50 ml of hot ethanol, filtered while hot, and placed in the refrigerator for crystallization. The melting point of the impure crystals was 190°C. The crystals were redissolved in 4.0 ml of hot ethanol, and the solution was placed back into the refrigerator. Crystals, which again formed within 1 hr, were filtered off using a suction flask and placed in a vacuum desiccator to dry. The final yield was 22% based upon the starting material (M.P. 200°C).

Rxn 4. Formation of 5-hydroxyindole-3-carboxylic acid

This part of the synthesis proved to be the most difficult due to the sensitivity of the debenzylated product to oxygen. Extreme care was taken during the procedure to ensure that all oxygen was excluded from the recrystallization flask. The incorporation of a dry box for future work is strongly suggested.

Into a 50 ml round bottom flask, 15 ml of methanol and a small magnetic stirring bar were added. The flask was then purged with nitrogen. 0.125 grams of 10% palladium on charcoal were added along with 250 mg of the product from rxn 3. Extreme care was taken when adding the catalyst. All oxygen must be removed prior to the

addition of the palladium or a violent reaction will ensue. Once the three ingredients were added, the mixture was placed on the hydrogenator and stirred magnetically at atmospheric pressure for 30 min. After the hydrogenation was complete, the catalyst was removed by filtration with celite and concentrated to dryness on the rotory evaporator. Care was exercised to use as little heat as possible when removing the methanol. After the protecting group is removed from the indole, the compound is very susceptible to air oxidation in solution. And, this oxidation process appears to be highly accelerated at elevated temperatures.

Once the methanol was removed, the flask was tightly stoppered with a rubber septum and the flask fitted with nitrogen (Fig. A1-3). Ethanol (ca. 0.5 ml) was added until the solid just dissolved. Toluene (ca. 4.0 ml) was then added until a cloudiness appeared, and the flask was refrigerated overnight. The next morning an oil was observed in the flask and was discarded. The remaining liquid was placed onto the rotovap and removed, leaving a white solid. The solid was redissolved in ethanol, and toluene was again added. The solution was scratched and placed in the refrigerator for recrystallization. The melting point of the resulting crystals was 180°C, which compares favorably to the literature value of 180°C.[6] A yield of 70% was obtained for this step.

Instrumental observations providing structural verification of the product are presented in Table A1-1. Cyclic voltammetry on the compound exhibited a much more easily oxidized substance when compared to the benzylated precursor run under identical conditions.

Table A1-1. Experimental Data for 5-HICA

Mol. Wt.	Melting Pt.	Infrared (cm^{-1})	Mass Spec[2]	$E_{p/2}$ (Cyclic Voltammetry)
177	180°C	3300(broad); 1630(st); 1655(st)	largest four: 104.1(27.3), 105.1(20.4) 177.0(15.6), 133.0(100)	0.25V[1]

[1]Measured versus SCE at pyrolytic graphite electrode in pH 4.5 acetate buffer

[2]Numbers in parentheses represent % abundance vs. the base peak of 133.

The IR spectrum (KBr pellet) confirmed the presence of a hydroxylated aromatic system and a carbonyl group.

APPENDIX A2

TROUBLESHOOTING THE LCEC

I. INTRODUCTION

Since the initial work of Kissinger et al.[63] the analytical technique of liquid chromatography with electrochemical detection (LCEC) has experienced a steady increase in popularity. A recent review on LCEC describes upwards of 300 papers on the subject.[66] The majority of these publications involve analytical applications of LCEC, but there have also been discussions on the improvement, understanding, and future directions of LCEC.[64,65] However, even with the knowledge we now possess, there are still many questions which have been left unanswered concerning the electrochemical detector. In developing LCEC applications, unusual and paradoxical chromatograms occasionally emerged from the strip chart recorder, leaving us scratching our heads over what appeared as a temporary enigma. When the pen took a turn for the worst, the typical reaction was to wiggle wires and electrodes or tap the side of the electrode's plexiglass body in a rather unsystematic fashion until a normal state of affairs reappeared.

The following report is an attempt to relieve some of the anxiety associated with the use of LCEC by exhibiting the sources of a number of problems that have appeared over the past three years.

These illustrations are meant to be useful in a diagnostic nature, and will be accompanied by a brief discussion of what occurred within the LCEC system, followed by a description of how to restore the chromatograph into a normal state of affairs. The data is very empirical in nature; the actual solution to the problem encountered often appeared only after extensive tapping, wiggling, and reassembling.

II. Experimental

The LCEC system has been previously described in the preceding chapters.

III. Results

The problems which can affect an LCEC can be classified into four general categories according to the particular area of the chromatograph in which they arise. These areas are: 1) the chromatographic solvent and column, 2) the electrochemical detector, 3) the chromatographic pump, and 4) the supporting electronics.

Fig. A2-1 represents a normal state of affairs for an LCEC system operating at a high sensitivity, i.e., 0.1 na/inch on the recorder. The attainment of these conditions is what the chromatographer strives for, and the following results are presented with suggested actions to reestablish this condition when a malfunction does appear.

A. Problems Associated with the Solvent and Column

Fig. A2-2 illustrates three problems normally associated with the liquid chromatography portion of the LCEC. Fig. A2-2A is a chromatogram demonstrating a chromatographic solvent system which has

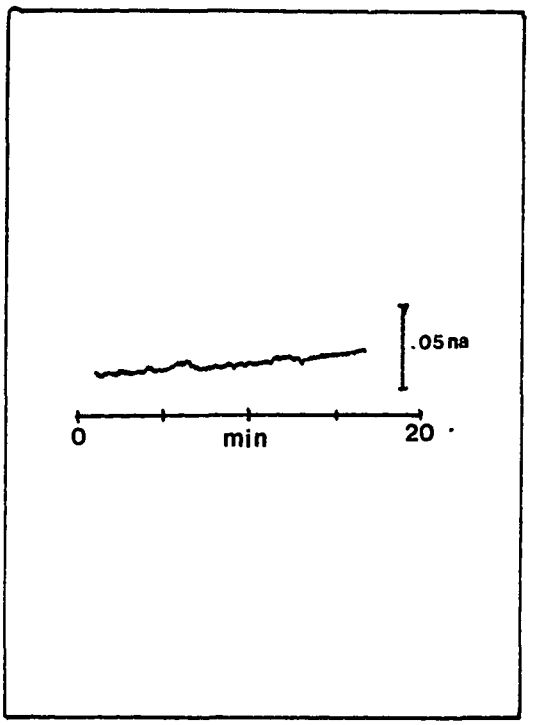

Figure A2-1. Typical Chromatographic Baseline at High Sensitivity.

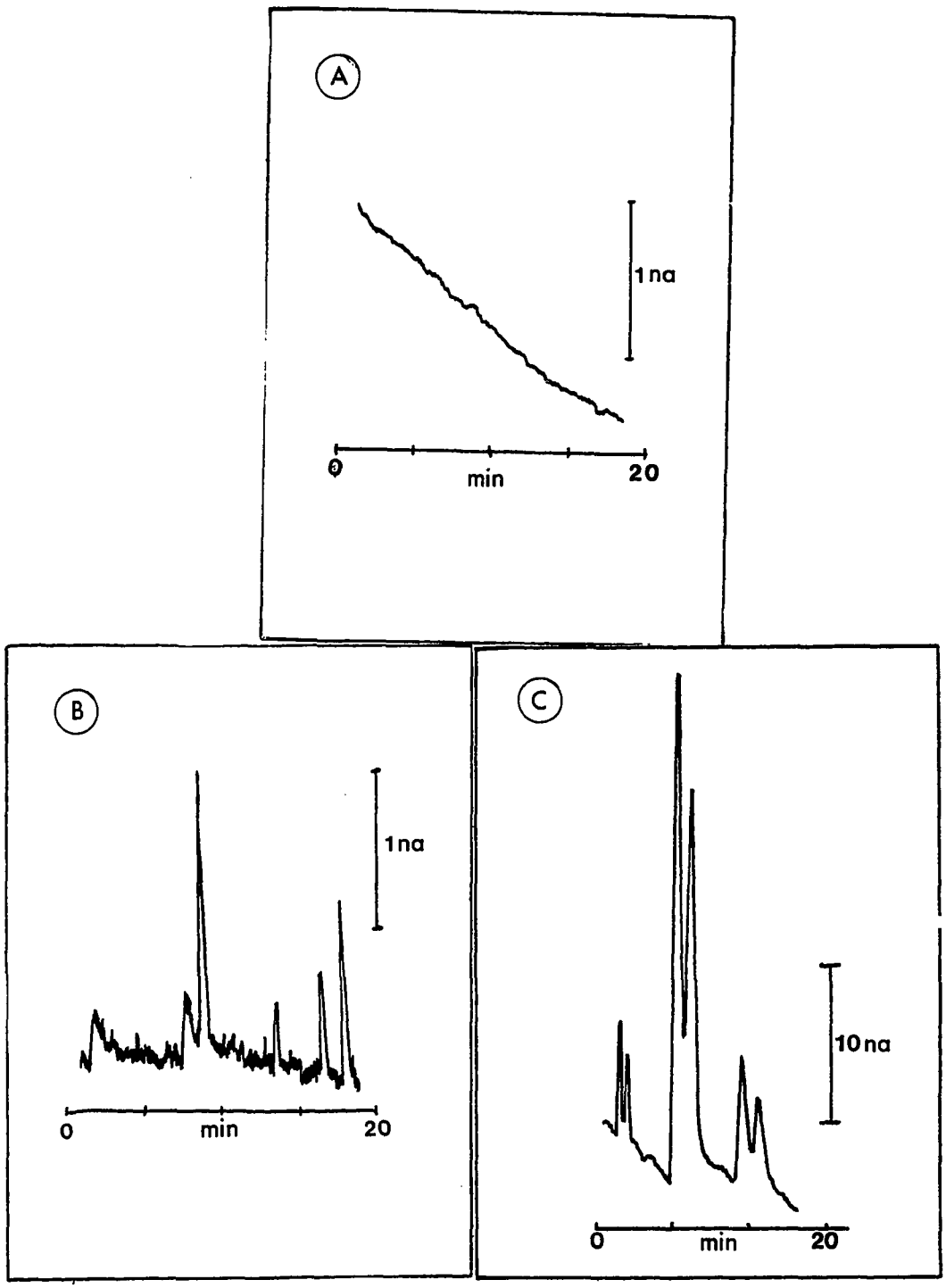

Figure A2-2. Chromatograms Illustrating Problems with Chromatographic Solvents and Columns. Fig. A is baseline from a nonequilibrated LCEC system. Fig. B is the result of oxidizable impurities in the LCEC system. Fig. C is caused by channeling.

not been allowed sufficient time to establish equilibrium with the column or electrochemical detector. The symptoms are either an ascending or descending baseline which is relatively free of "noise." Continued pumping of the solvent with the EC detector in place will eventually correct this situation.

A dirty column or chromatographic system creates a chromatogram similar to that shown in Fig. A2-2B. As impurities bleed from the contaminated area and elute into the detector, spurious peaks appear on the strip chart recorder. The rather obvious solution is to replace the chromatographic solvent and completely flush the system and injection device. Alternatively, the column may be cleaned with methanol/water as described in Table A2-1.

Fig. A2-2C is a chromatogram of three compounds, each resulting in a doublet peak. This condition is believed to be due to channeling within the column. The solute molecules are diverted into two separate paths and emerge from the column at slightly different times, producing two closely spaced peaks. When this situation occurs, the column should be cleaned with a 70% methanol/water solution (see Table A2-1).

B. Problems Associated with the EC Detector

Over a period of time, the polished surface of the carbon paste electrode begins to deteriorate. Small pieces of the electrode surface may be chipped away into the chromatographic stream exposing fresh, electroactive sites on the electrode surface. The result of this deterioration is illustrated in Fig. A2-3A as producing a very jagged and noisy baseline. The electrode should be rebuilt by wiping the

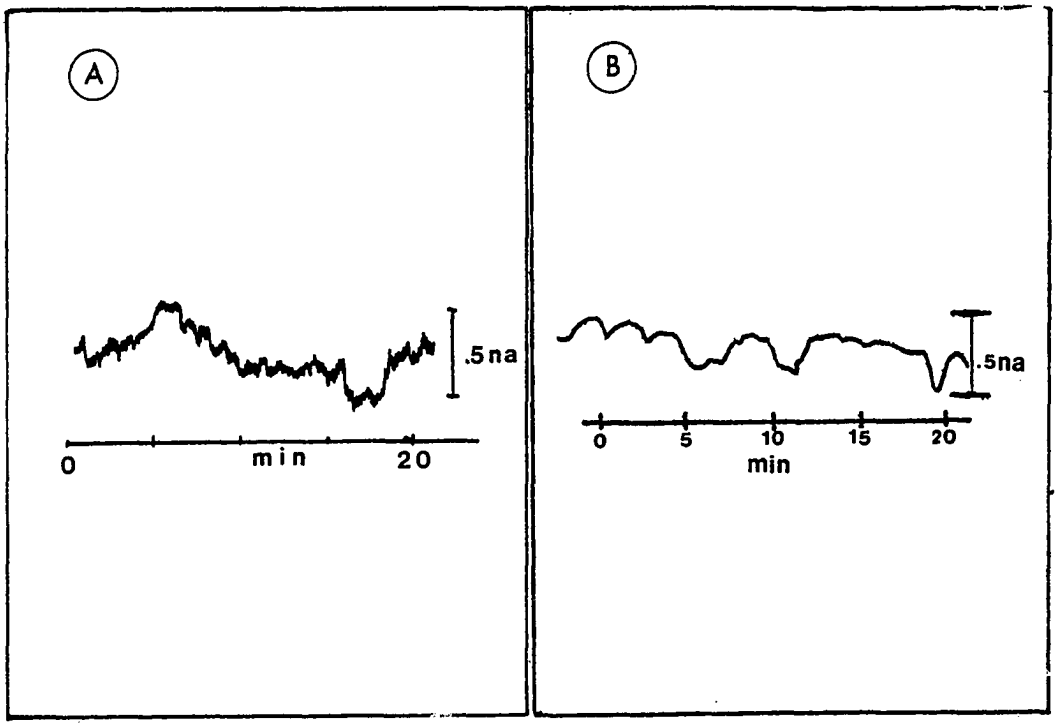

Figure A2-3. Chromatograms Illustrating Malfunctions of the EC Detector. Fig. A was associated with a poorly surfaced working electrode. Fig. B was caused by a bad reference electrode.

Table A2-1. Procedure for Rejuvinating Microparticulate Columns

1. Stop pump and place filtered distilled water into solvent reservoir.
2. Disconnect EC detector and all plastic components at the outlet of the column and start pump.
3. Flush system with distilled water for a minimum of 1 hour to remove last traces of inorganic/organic salts from buffer.
4. Stop pump and fill solvent reservoir with a 70% (v/v) methanol/ water mixture and start pump.
5. Pump the methanol solution through the system at ca. 1 ml/min for a minimum of 4 hours (preferably overnight).
6. Reverse procedure to replace fresh chromatographic solvent.

old surface gently with a paper towel and resurfacing with fresh carbon paste. It should be noted that only the top 0.1 mm of carbon need by removed. This greatly diminishes the amount of time needed for equilibration of the reconstructed electrode with the chromatographic system. It is frequently the case that many resurfacing attempts are required to produce a smooth, noiseless electrode.

Fig. A2-3B represents the baseline obtained from an LCEC system after extensive use with a small Ag/AgCl reference electrode. The fluctuations in the baseline are believed to be due to a nonpolarization of the reference electrode. A flat baseline returned after the reference electrode was replaced with a larger saturated calomel electrode (SCE).

A common nuisance in LCEC is air bubbles forming within the EC detector. Figure A2-4 shows two chromatograms obtained when large air bubbles passed through the detector and either struck the reference

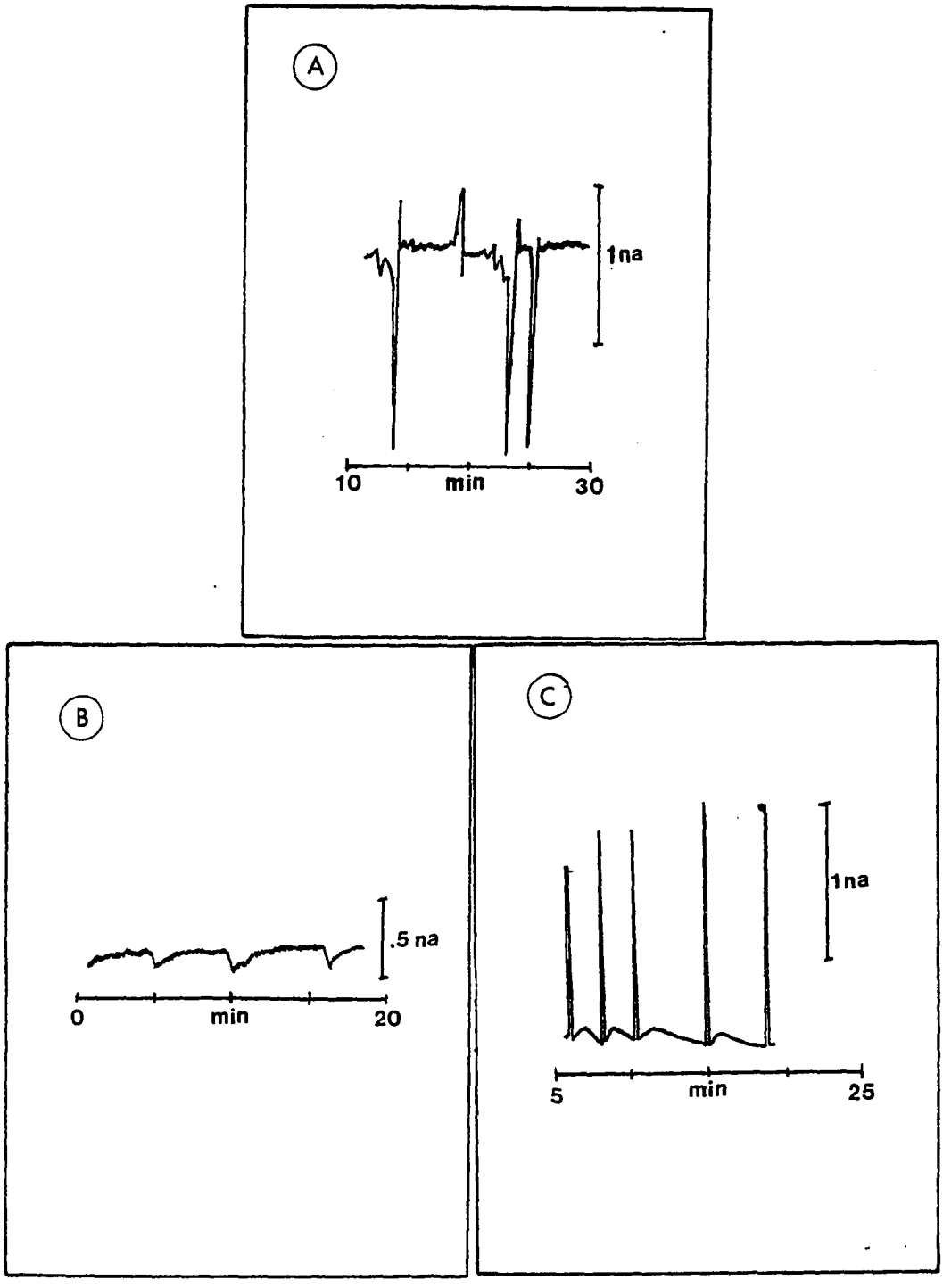

Figure A2-4. Chromatograms Illustrating Air Bubbles Within the EC Detector. Fig. A. Air bubbles striking the reference electrode. Fig. B. Air bubbles collecting on the tip of the reference electrode. Fig. C. Air bubbles streaming across the working electrode surface.

electrode (Fig. A) or collected on the Vycor tip of the reference electrode (Fig. B). Fig. A2-4C is a chromatogram representing a condition in which a regular stream of tiny air bubbles flowed over the working electrode. To help prevent these tiny bubbles from forming in the LCEC system, we recommend that the eluting solvent be deaerated prior to use. This is particularly important for solvents containing methanol. Generally, filtration using an aspirator has been found satisfactory for this purpose. Air bubbles may also be introduced when changing solvents or electrodes. Following these operations, it is very important to check for air pockets trapped within the working electrode or the compartment containing the reference electrode. Any such air can usually be removed by simply gently tapping the electrode with the handle of a screwdriver.

C. Problems with the Pump.

Most problems associated with the hydraulic portions of the LCEC are easily discernible. Leaking fittings, low flow rates, and low operating pressures are easily attributed to a malfunctioning pump or the associated hardware.

A problem that is not so easily recognized and which often occurs with double reciprocating pumps involves trapped air in a pump head (see Fig. A2-5). This situation most frequently develops when changing solvents and sufficient care is not taken to remove air from the solvent inlet lines. In addition to the chromatogram shown in Fig. A2-5, this condition may also be observed as an abnormal drop in operating pressure accompanied by a low flow rate. To remove trapped

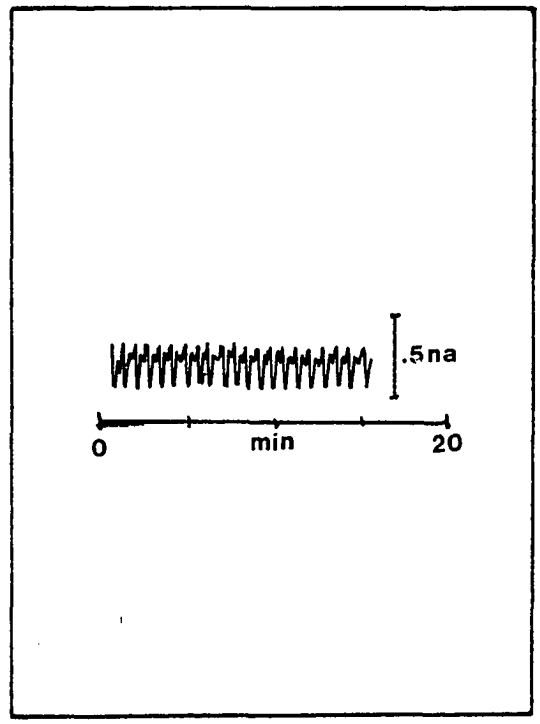

Figure A2-5. Chromatogram Illustrating Trapped Air in Pump Head.

air in the pump, the outlet, or high pressure, side is disconnected and solvent is pumped through the head at a high flow rate for a few minutes.

D. Problems with the Electronics

Our experience with the associated electronics has shown these hardware components to be extremely resilient during extended periods of continuous use. One distinctive problem was easily traced to a failure of the power supply (see Fig. A2-6A). Some minor problems have also been observed, although these can normally be ascribed to operator failure. Forgetting to turn on the power supply or improperly connecting the EC detector to the electronics are represented by an extremely flat, almost noiseless baseline (Fig. A2-6B). A poor connection to the recorder (Fig. A2-6C) also produces flat baselines although in this case they are typically accompanied by large, sudden spikes.

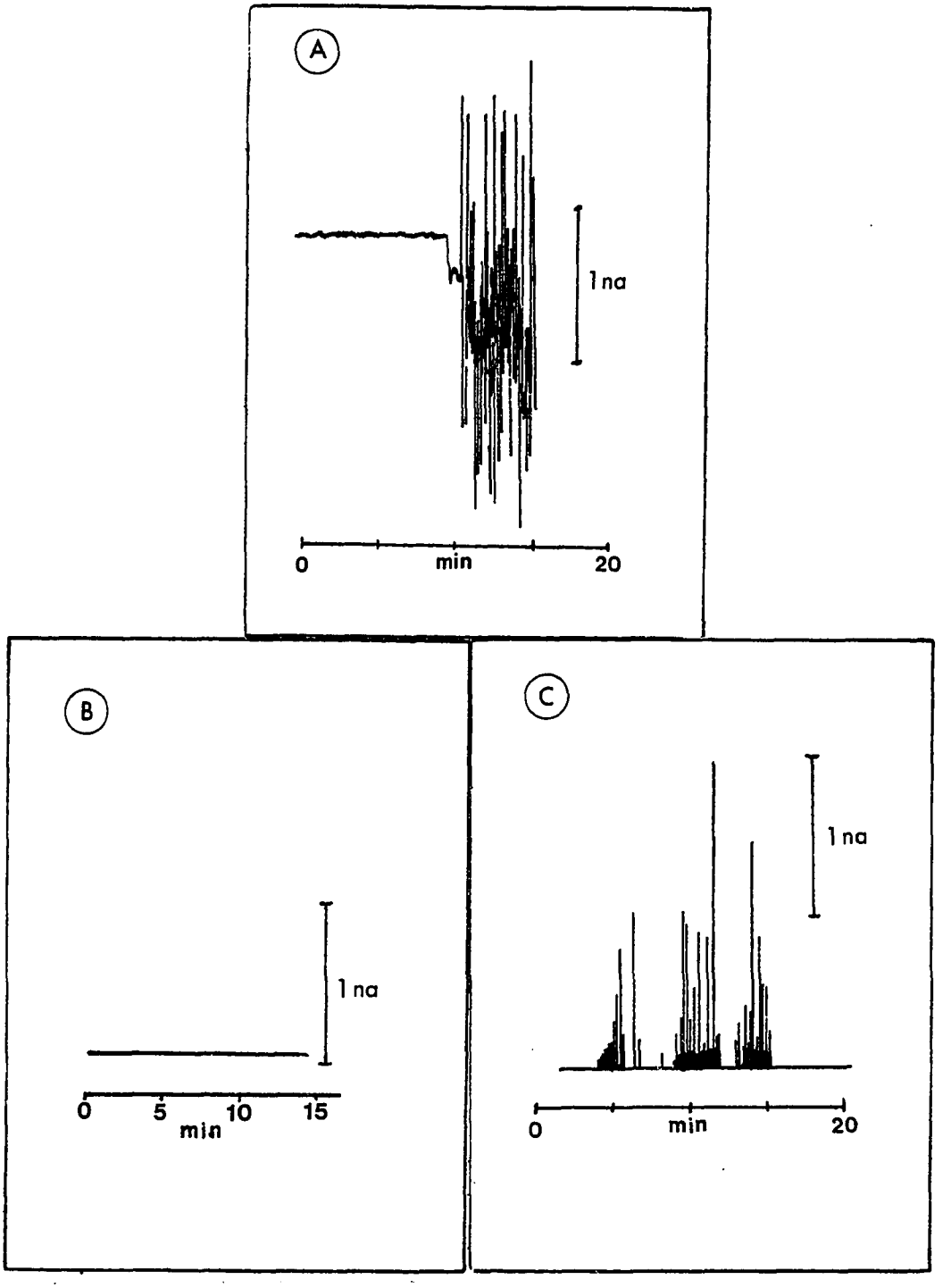

Figure A2-6. Chromatograms Illustrating Electrical Malfunctions. Fig. A was the result of a power supply failure. Fig. B is a baseline from a detector with no applied potential. Fig. C is a baseline obtained from a recorder with poor electrical connections to the associated electronics.