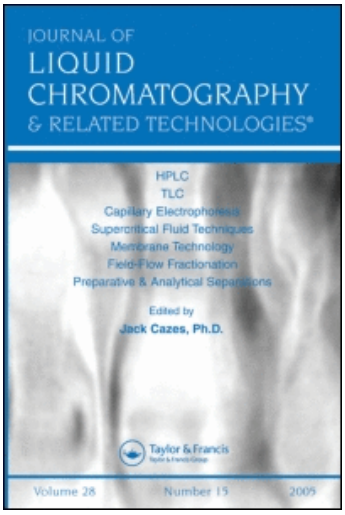

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The Determination of Catecholamines, Indoleamines, Metabolites, and Related Enzymatic Activities Using Three Micron Liquid Chromatography Columns

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THE DETERMINATION OF CATECHOLAMINES, INDOLEAMINES, METABOLITES, AND RELATED ENZYMATIC ACTIVITIES USING THREE MICRON LIQUID CHROMATOGRAPHY COLUMNS

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ABSTRACT

Liquid chromatography with electrochemical detection has become an established technique for the determination of catecholamines, indoleamines, precursors, metabolites, and related enzymatic activities in tissues and fluids. Previously available instrumentation, however, has limited the number of individual species readily and simultaneously accessible with reasonable throughput to only a few. Determinations of other species required either extended amounts of time per individual chromatogram or the use of an entirely separate chromatographic setup employing different columns and eluting solvents. Using reversed-phase columns packed with 3 micron particles, we have been able to produce the separation of 16 different catecholamine and indoleamine related species and two different internal standard compounds in 5 or 7 minutes. Samples may be analyzed directly after only homogenization, centrifugation, and clarification by filtration. No further purification steps are required. The enzymatic activities of 6 separate enzymes may be determined using the same chromatographic apparatus and simply monitoring selected metabolites following appropriate incubation of pretreatment. The metabolites and transmitters currently accessible with this apparatus include norepinephrine, dopamine, epinephrine, serotonin, 3,4-dihydroxyphenylacetic acid, 3,4-dihydroxyphenylalanine, normetanephrine, metanephrine, 3-methoxytyramine, 3,4-dihydroxyphenylethyleneglycol, vanillylmandelic acid, homovanillic acid, 5-hydroxytryptophan, 5-hydroxyindoleacetic acid, 5-hydroxytryptophol, and N-acetyl-5-hydroxytryptamine. The enzymatic activities include tyrosine hydroxylase, tryptophan hydroxylase, dopa decarboxylase, 5-hydroxytryptophan decarboxylase, monoamine oxidase, and catechol-O-methyltransferase.

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INTRODUCTION

The initial utilization of liquid chromatography combined with electrochemical detection for the determination of catecholamines by Refshauge et al. (1) offered a unique and highly selective technique for this purpose. Likewise, the same basic technique was found applicable to the determination of serotonin (2). This was quickly followed by announcements of procedures to determine catecholamine (3-8) and indoleamine (9-11) metabolites. Other workers (2, 12-19) reported the determination of enzymatic activities by coupling liquid chromatography with electrochemical detection to previously established pretreatments or incubation procedures.

The desire for greater amounts of information per sample motivated some to attempt to increase the number of individually determined species in single chromatographic runs. Such efforts (2, 3, 20-27) have been shown capable of separating as many as 10-12 endogenous species, although individual chromatograms may have required as much as 30-60 minutes to accomplish this task. This is clearly too long for most routine applications. On the other hand, the determination of only 3 or 4 major species, which may be obtained on a more rapid basis, leaves the investigator with the feeling that s/he would like to know a little more about the sample.

The recent advent of reversed-phase columns containing 3 μ packing materials has offered a substantial improvement in this situation along with some notable difficulties (28-32). The difficulties primarily arise from two major factors. First, the extremely small peak widths of the early eluting components require much greater attention to dead volumes and detector response times. Secondly, the large number of conceivably desirable tyrosine and tryptophan metabolites, which may be as great as 50, means we still cannot measure all the metabolites in a reasonable period of time. Nonetheless, the judicious choice of some 10-14 commonly measured metabolites will allow their rapid and routine determination in 4-7 minutes. This is exactly

what we have done in the current work. While our selection of the particular species to be included is unquestionably a biased one, we feel that the individual components and enzymatic activities to which the separation applies will be generally useful to a number of other investigations.

MATERIALS

Liquid Chromatograph

The apparatus employed for this report was constructed from readily available components. Two separate systems are described, since both have been shown independently capable of performing all of the described determinations. System A employed a Beckman Ultrasphere ODS reversed-phase (75 x 4.6 mm) column, while system B employed a Perkin-Elmer HC-18 reversed-phase (100 x 4.6 mm) column. The average particle diameter for both columns was 3 microns. Besides the obvious differences in columns and eluting solvents (vide infra), the two systems further differed only in respect to flow rates (system A - 2.2 ml/min; system B - 1.85 ml/min) and mean operational back pressures (system A - 3350 p.s.i.; system B - 3525 p.s.i.). The pump was a Milton Roy reciprocating Minipump. This was connected to a Mark III pulse dampener from Alltech Associates for mechanical dampening and a 5000 p.s.i. pressure gauge. In succession, this was connected to an injection port, the column, the electrochemical flow cell, the reference electrode compartment, and the waste container. The entire system was not thermostatted (room temp. = 21°C), since it was felt that most potential users would not have access to this capability. However, even further improvements in the currently reported separations could feasibly be obtained through judicious use of temperature control (28). The Rheodyne model 7010 injection port was modified to contain a 5 µl injection loop by replacing the standard loop with a short piece of 0.006 inch (i.d.) stainless steel tubing (1/16 inch o.d.). The same, narrow bore tubing was employed to connect the injection port

to the column inlet (6 cm length) and to connect the column outlet to the flow cell (6 cm length). The flow cell was similar to a BioAnalytical Systems model TL-3 with the following exceptions. The tubing forming the inlet to the flow cell was press fitted into the upper Kel-F block such that it penetrated completely to the level of the cell gasket. The carbon paste working electrode was located in the upper Kel-F block between the inlet and outlet openings. The bottom block was constructed entirely of stainless steel and served as the auxiliary electrode. The reference electrode, a Ag/AgCl (3 N NaCl) unit obtained from BioAnalytical Systems, was located downstream in the reference electrode holder unit. A model LC-4B dual electrochemical potential controller, also from BioAnalytical Systems, was employed to maintain a constant potential of 0.85 to 0.90 volts vs. Ag/AgCl. Only one channel of output is displayed in this report. We also would advise using a potential setting of only 0.65 to 0.70 volts for the in vitro tryptophan hydroxylase determination; the large amounts of L-tryptophan used as substrate in this assay will otherwise cause the noticeable appearance of this component at an elution time of approximately 15 min when using the higher potential. The lower potential could also be used to gain a higher signal to noise ratio in many of the other determinations described. However, it does provide a considerable lowering of signal for the methoxylated catecholamines and, thus, should not be employed when these are of primary concern.

The time constant afforded by the RC damping of the LC-4B detector was modified to a value of 0.25 sec. This was accomplished by altering the capacitor in the primary amplification stage. While such modification is essential to avoid instrumental broadening of the early eluting components, it also allows considerable amounts of pump pulsation, with a period of ca. 0.5 sec, to feed through to the detector as noise. The use of more extensive mechanical damping, a 6-or 8-pole Butterworth filter, a higher frequency pump, curve fitting by subtracting the underlying pump 'signal,' or all of these may, thus, be very appro-

priate to obtain the maximum signal to noise ratio for systems employing 3 μ columns.

Eluting Solvents

The eluting solvents for the two systems were quite similar and are presented in Table 1. The mobile phase is typically prepared in 4.0 liter batches, which is described as follows for System B. Approximately 0.5 to 1.0 g of NaOH and 74.45 mg $Na_2EDTA \cdot 2H_2O$ are added with stirring to 3700 ml of distilled/deionized (Milli-Q) water. The dissolution of the EDTA is aided here by the initially basic nature of the solution. After dissolution, citric acid monohydrate (84.04 g), diethylamine (3.4 ml), and sodium octyl sulfate (236.9 mg) are added with stirring. The mixture is filtered through an 0.22 μ Millipore vacuum filtering unit and combined with 300 ml of previously filtered acetonitrile. The final mixture is then titrated to a measured pH value of 2.45; no further filtration is effected in order to preserve the acetonitrile content. The diethylamine, only necessary when using the Perkin-Elmer column, is added to eliminate peak tailing problems which were particularly observed with the amine-containing species.

Fine tuning of the solvent conditions appears to be essential to obtain the desired separation for each new column received as well as, occasionally, throughout the lifetime of individual columns. We are currently preparing a paper which will discuss this problem in more detail. Briefly, however, useful adjustments have been determined to be afforded by the pH (in 0.02 to 0.05 unit increments), the concentration of SOS (in 0.05 to 0.10 mM increments), and the concentration of acetonitrile (in 0.5% increments). The latter parameter simply effects contraction (upon increased CH_3CN concentration) or expansion (upon decreased CH_3CN concentration) of the chromatogram. The former two parameters, however, alter the relative retention times of individual species. Acetonitrile and pH effects typically require only a few hours, at normal flow rates, to become apparent. Effects

TABLE 1

Eluting Solvents

Parameter	Concentration or Value*	
	Beckman Ultrasphere (System A)	Perkin-Elmer HC-18 (System B)
Citric Acid	0.1 M	0.1 M
EDTA	0.05 mM	0.05 mM
CH_3CN (vol:vol)	7.5%	7.5%
pH (final measured value)	2.50	2.45
Sodium Octyl Sulfate (SOS)	0.175 mM	0.255 mM
Diethylamine (wt./vol.)	0	0.06%

*Final concentrations assume the aqueous and nonaqueous portions of the solvent are completely additive.

due to increasing (decreasing) SOS concentrations, represented by stronger (weaker) relative retention of the amines and amino acids, may require as long as 48 hours.

Chemicals

All chemicals used were obtained from commercial sources at the highest available purity.

Animals

All mice used were adult males of the ARS-HA/ICR strain (Sprague-Dawley, Madison, WI) weighing 20-30 g at the time of sacrifice. The rats were of the Sprague-Dawley strain and typically weighed 300-325 g at the time of sacrifice. These animals were maintained on a 12 hr. light/12 hr. dark cycle and allowed access to food and water ad libitum. No animal was used until at

least one week after arrival. Also, the animals were typically sacrificed at 3-4 hr into the light cycle.

METHODS

Tissue and Fluid Determination of Endogenous Components

A sample of tissue (0.5 g) or bodily fluid (0.5 ml) is precisely measured and added to 750 µl of homogenizing solution. Appropriate adjustments are made in these quantities for different weights or volumes, although a lower limit of 250 µl of homogenizing solution is employed for practical manipulative purposes. The homogenizing solution contains 0.5 M acetic acid, 0.5 M sodium acetate, 0.4 M sodium perchlorate, and has a pH of 4.8. It is degassed for ca. 15 min before use with O_2-free N_2 (1). A 100 µl aliquot of internal standard, containing approximately 300 ng 3,4-dihydroxybenzylamine and 250 ng N_ω-methyl-5-hydroxytryptamine (for whole mouse brain) then added to each sample and the resultant mixture is subjected to ground glass homogenization. Standard samples are prepared by replacing the tissue or fluid with a roughly equivalent volume of a stock external standard solution containing the species to be quantitated at levels appropriate to the unknown samples. Thus, for a whole mouse brain (ca. 0.5 g), 0.5 ml of a stock external standard containing ca. 200 ng norepinephrine, 450 ng dopamine, etc. would be employed. In general, we recommend that the concentration of substances to be measured not be lower than 100 ng/ml in the external standard to minimize the attendant problems associated with extracting the signal from the noise. Standard samples also receive a 50 µl aliquot of 1 mg/ml ascorbic acid, prepared fresh on the day of analysis.

Following homogenization, the sample is centrifuged at 50,000 x g for 45 min or 40,000 x g for 60 min to separate the macromolecules and cellular debris from the supernate. An aliquot of the supernate is then transferred to an MF1 Microsample Filtering unit (BioAnalytical Systems) and clarified by centri-

fugation/filtration at 13,000 x g for 15 min. The pore size of the filter is 0.22 μ. The temperature during both centrifugal operations is 4°C. A 5 μl aliquot of the filtrate is then injected into the liquid chromatograph for quantitation.

Two internal standards are employed in this determination; one (3,4-dihydroxybenzylamine) is more suited to the catechols, while the other (N_ω-methyl-5-hydroxytryptamine) is more suited to the indoles. This use, which might be labelled extravagant by some, is really quite appropriate since neither of these substances overlaps any of 26 tested catecholamine and indoleamine derivatives. However, the N_ω-methyl-5-hydroxytryptamine could be eliminated to obtain faster throughput, since it elutes last at the current time.

in vitro Tyrosine Hydroxylase Activity

The in vitro tyrosine hydroxylase determination described is for whole mouse brains, although it has been used for brain parts and should be applicable to other tissues.

The tissue is removed and weighed. A tissue homogenate is prepared by adding ca. 0.5 g tissue to 2.00 ml H_2O and 200 μl of an internal standard solution containing 8×10^{-5} M 3,4-dihydroxybenzylamine in 1×10^{-3} M ascorbic acid. Homogenization is effected with a glass/Teflon mechanical unit. Standard 'homogenates' are prepared by adding 500 μl of a solution containing 9.84 μg L-3,4-dihydroxyphenylalanine (L-DOPA) in 10 ml of 1×10^{-3} M ascorbic acid to 2.00 ml H_2O and 200 μl of the above internal standard solution.

The incubation mixture is prepared by mixing the following components to obtain a final volume of 500 μl: 100 μl of 2.0 M acetate buffer having a pH of 6.40; 50 μl of a solution which contains 20.0 mM 6-methyltetrahydropterin, 0.50 M 2-mercaptoethanol, and 0.4 mM NSD-1015 (3-hydroxybenzylhydrazine); 50 μl of 1.0 mM ferrous sulfate; 50 μl of 2.5 mM L-tyrosine in 0.01 M HCl; and, 250 μl of brain (or standard) homogenate. All the solutions except the L-tyrosine and the homogenate are preincu-

bated for 30 min at 37°C after mixing. The L-tyrosine and homogenate are then added and the incubation is carried out, with shaking, at 37°C for 30 min.

Since tyrosine solutions contain L-DOPA, the reaction product, as an impurity and L-DOPA is also produced by non-enzymatic routes, it is important to run blanks with these determinations. A blank 'homogenate' is prepared by mixing 500 µl of 1 x 10^{-3} M ascorbic acid, 2.00 ml H_2O, and 200 µl of the above described internal standard solution. Blanks then receive the same treatment as the tissue samples except that 250 µl of this 'homogenate' replaces the 250 µl of tissue homogenate in the above sample preparation.

Following incubation, the reaction is terminated by the addition of 200 µl of 1.0 M $HClO_4$. 50 µl of a solution containing 0.30 M $NaHSO_3$ and 0.030 M EDTA is also added to prevent oxidation of the catechols. The samples are centrifuged at 16,000 x g and 4°C for 15 min. 500 µl of the supernatant fraction is transferred to a small plastic vial and subjected to the alumina isolation procedure (1). This is the only determination of all being described which requires such a special isolation step. However, it must be used here to prevent substantial interference between the 6-methyltetrahydropterin and L-DOPA in the succeeding chromatography. The final eluent from the Al_2O_3 is subjected to centrifugation/filtration for sample clarification. 5 µl of the filtrate is injected into the liquid chromatograph for quantitation.

in vivo Tyrosine Hydroxylase and Tryptophan Hydroxylase Activities

The simultaneous determination of the in vivo hydroxylase activities are surprisingly simple. The method is patterned after that presented by Carlsson et al. (33, 34). Mice are injected with 200 mg/kg (i.p. or i.v.) NSD-1015 (N-3-hydroxybenzylhydrazine) 2-30 minutes prior to sacrifice. A dose of 150 mg/kg is completely adequate for rats. The longer the time between injection and sacrifice, the greater the resultant signal to noise

ratio obtained. Following sacrifice, the desired tissue is removed, weighed, and subjected to the procedure described above for "Tissue and Fluid Determinations." Of course, the standard 'homogenates' in these determinations should contain appropriate concentrations of L-DOPA and L-5-hydroxytryptophan, the tyrosine and tryptophan hydroxylase products, respectively. The NSD-1015 blocks metabolism of these products by DOPA decarboxylase and 5-hydroxytryptophan decarboxylase. Thus, their buildup following blockade is a direct measure of the in vivo activity of their respective hydroxylase source activities. While endogenous concentrations of these two products are typically very small (ca. 2 ng/g and 15 ng/g), blanks may be appropriate for very short times between treatment and sacrifice, i.e., 2-3 minutes. The blanks would simply be saline injected controls.

in vitro DOPA Decarboxylase Activity

DOPA decarboxylase and 5-hydroxytryptophan decarboxylase may well be a single enzyme, or at least the same entity with two active sites (35). Alternatively, they may represent two distinct, but very similar, entities (36). We have proceeded as if the two substrates act differently toward decarboxylation and, thus, have optimized assays for each. The procedures of Sims et al. (36) were used to obtain workable conditions, and the determinations were then optimized for whole mouse brain tissue. However, we feel the results should be readily applicable to other tissues and fluids as well.

For the assessment of DOPA decarboxylase activity, a 10% tissue homogenate is formed by adding 500 mg of tissue to 500 µl of 2.0×10^{-3} M 3,4-dihydroxybenzylamine or epinine, either being a satisfactory internal standard, and 4.00 ml of H_2O. The water content is actually adjusted for each sample to assure a 10% homogenate. A standard 'homogenate' is prepared by adding 500 µl of 2.0×10^{-3} M dopamine to 500 µl of the same internal standard solution, 50 µl of ascorbic acid (75 mg/ml), and 3.95 ml of an 0.10 M acetate buffer of pH 4.80. Homogenization is effected by

ultrasonication or with a ground glass apparatus. For each sample, the following components are combined in a small incubation tube: 100 l of a 1:1 mixture of 1.0×10^{-3} M pargyline and 1.0×10^{-3} M pyridoxal-5'-phosphate; 100 µl of 0.75 M phosphate buffer of pH 6.60 (this is replaced with 100 µl of the pH 4.80 acetate buffer mentioned above for standards); and 100 µl of 1.0×10^{-2} M L-DOPA as substrate. The samples are briefly mixed by vortexing and then incubated at 37°C for 30 min. Inactivation of the enzyme is accomplished by heating the sample at 100°C, by immersion in boiling water, for 45 seconds. 100 µl of a solution containing 1.5 M HCl and 0.10 M Na_2EDTA is added to each incubation tube. The samples are then clarified by centrifugation/filtration through 0.22 µ MFl Microsample Filters (BioAnalytical Systems) at 13,000 x g and 4°C for 15 min. A 5 µl aliquot of the filtrate is finally injected into the liquid chromatograph for quantitation of the product, dopamine.

in vitro 5-Hydroxytryptophan Decarboxylase Activity

As with dopa decarboxylase, this determination initially employed the procedural conditions outlined by Sims et al. (36). These were then modified in our laboratory to provide optimal results for whole mouse brains.

The tissue homogenate is prepared by adding 500 mg of tissue to 500 µl of 8.0×10^{-4} M N_ω-methyl-5-hydroxytryptamine, the internal standard, and 4.00 ml of water. Again, the actual amount of water is adjusted for each sample according to the weight of tissue to yield a constant 10% homogenate. A standard 'homogenate' employs 500 µl of 8.0×10^{-4} M serotonin, the product of the reaction, 500 µl of the same 8.0×10^{-4} M internal standard solution, 50 µl of 75 mg/ml ascorbic acid, and 3.95 mls of an 0.1 M acetate buffer of pH 4.80. Homogenization is accomplished with either ultrasonication or a ground glass apparatus. For incubation, the following solutions are added to the individual tubes: 100 µl of a 1:1 mixture of 1.6×10^{-3} M pargyline and 5.0×10^{-3} M pyridoxal-5'-phosphate; 100 µl of 0.75 M phosphate buffer of

pH 8.40; 100 µl of 4.0 x 10^{-3} M L-5-hydroxytryptophan as substrate; and 100 µl of the homogenate. For standards, the phosphate buffer is replaced with 100 µl of 0.1 M acetate buffer of pH 4.80. After incubation at 37°C for 30 min, the reaction is quenched by immersion of the tube in boiling water for 45 sec. A 100 µl aliquot containing 1.5 M HCl and 0.1 M Na_2EDTA is added to prevent subsequent loss of the product, 5-hydroxytryptamine, through oxidation. The samples are then clarified by centrifugation/filtration, as described above for DOPA decarboxylase, and the activity quantitated by injection of a 5 µl aliquot into the liquid chromatograph.

in vitro Tryptophan Hydroxylase

This procedure was also optimized in our laboratory for whole mouse brains, although it has been shown applicable to other species, other tissues, and brain regions. Tissue homogenates are prepared by homogenizing the following mixture with a ground glass apparatus: 500 mg of tissue; 50 µl of a solution containing 12.2 µg of N_ω-methyl-5-hydroxytryptamine (internal standard) in 1 x 10^{-3} M HCl; and 3.00 mls of H_2O. The volumes, of course, should be adjusted appropriately for other tissue sizes. The incubation mixture is prepared by adding the following components to each sample: 100 µl of a 2.0 M acetate buffer of pH 8.0; 50 µl of a solution containing 5.0 mM 6-methyltetrahydropterin, 0.20 M 2-mercaptoethanol, 0.10 mM NSD-1015, and 52.5 units of catalase per µl; 50 µl of 100 mM Ca^{2+}; 50 µl of 4.0 mM L-tryptophan, the substrate; 200 µl of homogenate; and, 50 µl of 1.0 mM ascorbic acid. For standards, 50 µl of a working standard solution containing 3 µg/ml of L-5-hydroxytryptophan in 1 x 10^{-3} M ascorbic acid replaces the 50 µl of ascorbic acid in the above incubation mixture. Blanks are prepared exactly as described above for tissue samples. Both blanks and standards, however, are additionally treated with 50 µl of 60% $HClO_4$ prior to incubation. For all samples, incubation is carried out at 37°C for 25 min. For tissue samples, the reaction is terminated by the addi-

tion of 50 µl of 60% $HClO_4$. After an initial centrifugation at 27,750 x g and 4°C for 20 min, the sample is clarified by centrifugation/filtration at 13,000 x g and 4°C for 15 min. The quantitative results are obtained by injection of 5 µl aliquots into the liquid chromatograph.

in vitro Monoamine Oxidase Activity

Based upon previous work by others (37, 38), we decided to design a determination of monoamine oxidase which coupled this enzyme to aldehyde dehydrogenase. Thus, the final product would be the corresponding acid rather than the more difficult to analyze aldehyde. However, due to the existence of isozymes of monoamine oxidase (39), we also decided to optimize the determination utilizing two different substrates: dopamine and serotonin. The conditions used by Lovenberg et al. (40) were initially employed, although the final procedure obtained was optimized for whole mouse brains in our laboratory. The following description applies only to the determination employing 5-hydroxytryptamine as substrate.

Tissue homogenates are prepared by adding 500 mg of tissue to 4.00 mls of an isotonic, 0.050 M phosphate buffer of pH 10.0 and sonicating until homogeneous. Kidney homogenates, used as a source of aldehyde dehydrogenase, are prepared by adding an appropriate amount of isotonic, 0.35 M sucrose to whole female mouse kidneys to obtain 0.20 g/ml; the mixture is homogenized in a ground glass apparatus, centrifuged at 39,900 x g and 4°C for 20 min, and the resultant supernatant fraction used as the kidney homogenate.

The preincubation mixture is composed of the following components: 500 µl of 0.50 M phosphate buffer of pH 8.10; 100 µl of 17.5 mM NAD^+; 100 µl of H_2O; 100 µl of 0.578 M 2-mercaptoethanol; 100 µl of 1.33 mM 5-hydroxyindole-3-carboxylic acid, the internal standard; 250 µl of the kidney homogenate; and, 500 µl of the tissue homogenate. Following preincubation at 44°C for 20 min, the substrate is added as a 100 µl aliquot

containing 17.0 mM serotonin and the resultant mixture incubated at 44°C for 20 min. The reaction is terminated by placing a 250 µl aliquot of the incubation mixture into 750 µl of a solution containing 0.5 M acetic acid, 0.5 M sodium acetate, and 0.4 M $NaClO_4$. The resultant solution is centrifuged at 40,000 x g and 4°C for 1 hour. Sample clarification is obtained by centrifugation/filtration at 13,000 x g and 4°C for 15 min. Quantitation is afforded by injection of 5 µl aliquots of the filtrates into the liquid chromatograph. Standard samples are prepared by replacing the 500 µl of brain homogenate with 500 µl of isotonic, 0.050 M phosphate buffer (pH 10.0), replacing the 100 µl of H_2O with 100 µl of 5.58×10^{-4} M serotonin, and replacing 250 µl of the kidney homogenate with 250 µl of isotonic sucrose. Blanks are prepared by only replacing the 500 µl of brain homogenate with 500 µl of isotonic, 0.050 M phosphate buffer (pH 10.0).

in vitro Catechol-O-Methyltransferase Activity

The determination of catechol-O-methyltransferase activity is virtually identical to that described by Shoup *et al.* (41). Dopamine is used as the substrate, while 3,4-dihydroxybenzylamine serves as the internal standard. After terminating the incubation, the solution is clarified by centrifugation/filtration at 13,000 x g and 4°C for 15 min. 5 µl aliquots of the filtrate are used for quantitation by liquid chromatography.

Calculations

The calculation of a single endogenous species in a routine determination of tissue or fluid components is given as:

$$\text{nmol/g} = \frac{R_{sample}}{R_{std}} \times \frac{\text{nmol in std}}{\text{g tissue}}$$

where

R = ratio of peak height (area) of desired component to that of internal standard. The subscripts 'sample' and

'std' refer to the final, injected sample or standard mixtures, respectively.

nmol in std = the number of nmol of the desired substance contained in the final standard mixture, an aliquot of which was injected to obtain the above R_{std}.

g tissue = number of grams of tissue of the sample initially employed.

Of course the final result could be expressed in pmol/ml, or any other suitable unit, by altering the appropriate expressions in this formulation. Expressions of desired components in weight (e.g., ng/g), however, should be careful to use the amount in the standard as the free base.

The calculation of an enzymatic activity is given as:

$$\text{nmol/g/hr} = \frac{R_{sample} - R_{blank}}{R_{std}} \times \frac{\text{nmol in std}}{(\text{g tissue})(\text{time,hr.})}$$

The same definitions and comments apply here as to the simpler calculation above. Additionally,

R_{blank} = ratio of peak height (or area) of desired component to that of internal standard for a blank.

time,hr. = the time of incubation, for an *in vitro* determination, or the time between pretreatment and sacrifice, for an *in vivo* determinations.

The R_{std} expression in the denominator of this calculation should be replaced with $R_{std} - R_{blank}$ for the *in vitro* tryptophan hydroxylase determination.

All results in the current report are expressed as mean $\pm$ S.E.M. for at least 4 separate determinations.

RESULTS AND DISCUSSION

The determinations described may all be performed with a single liquid chromatographic apparatus, as represented by either System A or System B. The individual components, along with their common abbreviations are presented in Table 2 to aid the reader in deciphering the figures.

TABLE 2

Catecholamine and Indoleamine Related Species and Their Abbreviations

Compound (In order of elution)	Abbreviation
3,4-Dihydroxyphenylglycol	DOPEG
3-Hydroxy-4-methoxyphenylglycol	MHPG
Vanillylmandelic acid (coelutes with MHPG)	VMA
Norepinephrine	NE
3,4-Dihydroxyphenylalanine	DOPA
Epinephrine	EPI
3,4-Dihydroxybenzylamine	DHBA
3,4-Dihydroxyphenylacetic acid	DOPAC
Normetanephrine	NM
Dopamine	DA
5-Hydroxytryptophol	5-HTOL
Metanaphrine (coelutes with EPIN)	MET
Epinine	EPIN
5-Hydroxyindole-3-acetic acid	5-HIAA
5-Hydroxytryptophan	5-HTP
N-Acetyl-5-hydroxytryptamine	N-Ac-5-HT
Homovanillic acid	HVA
3-Methoxytyramine	3-MT
5-Hydroxytryptamine	5-HT
N_{ω}-Methyl-5-hydroxytryptamine	N-MET

A computer drawn chromatogram of a synthetic mixture of 18 catecholamine and indoleamine related compounds is shown in Fig. 1. As can be seen, all components are readily resolved in 4.3 and 7.0 minutes on Systems A and B, respectively.

A typical analysis of a whole rat brain yielded the resultant chromatogram shown in Fig. 2, while the results for a typical analysis of whole mouse brain are presented in Table 3. The values obtained are quite comparable to the many previous reports for such determinations. It is, however, interesting to note the

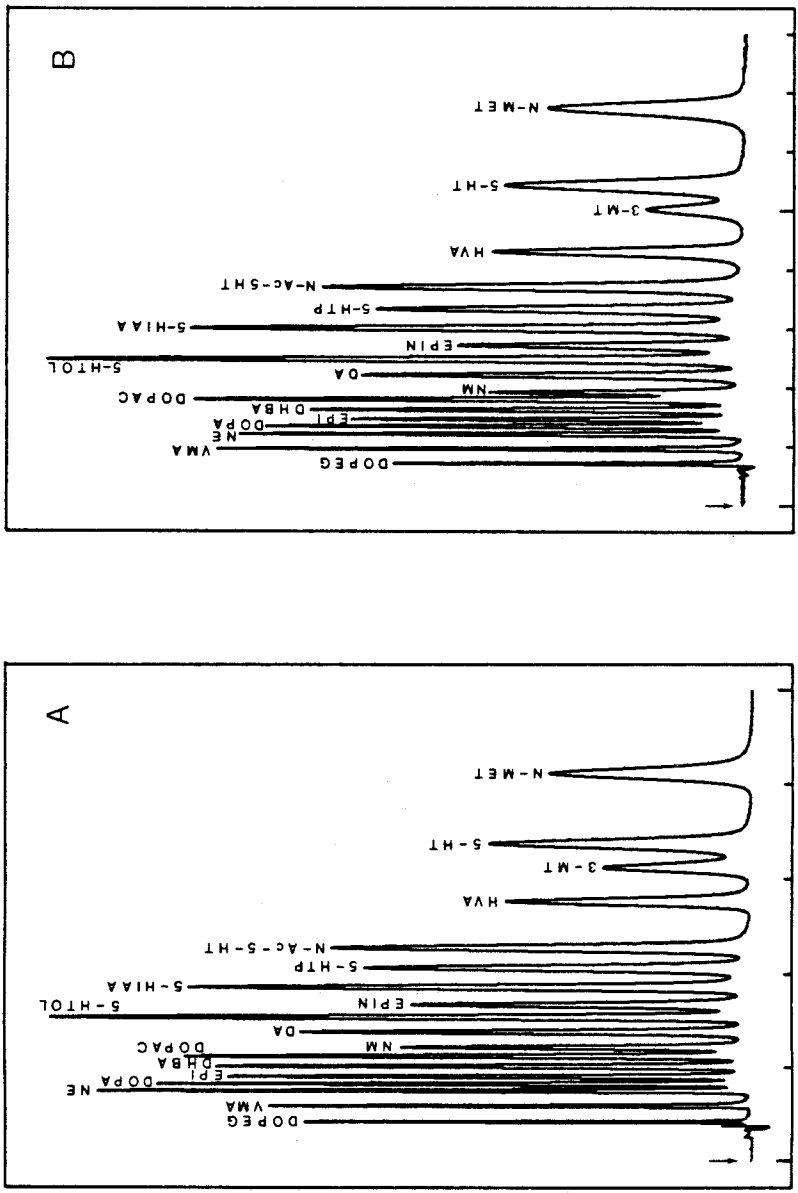

FIGURE 1. Separation of Eighteen Different Catecholamines and Indoleamine Related Compounds. The 5 µl injection contains each compound at a concentration of ca. 1 µM. System used (A or B) is indicated in the upper right of each chromatogram.

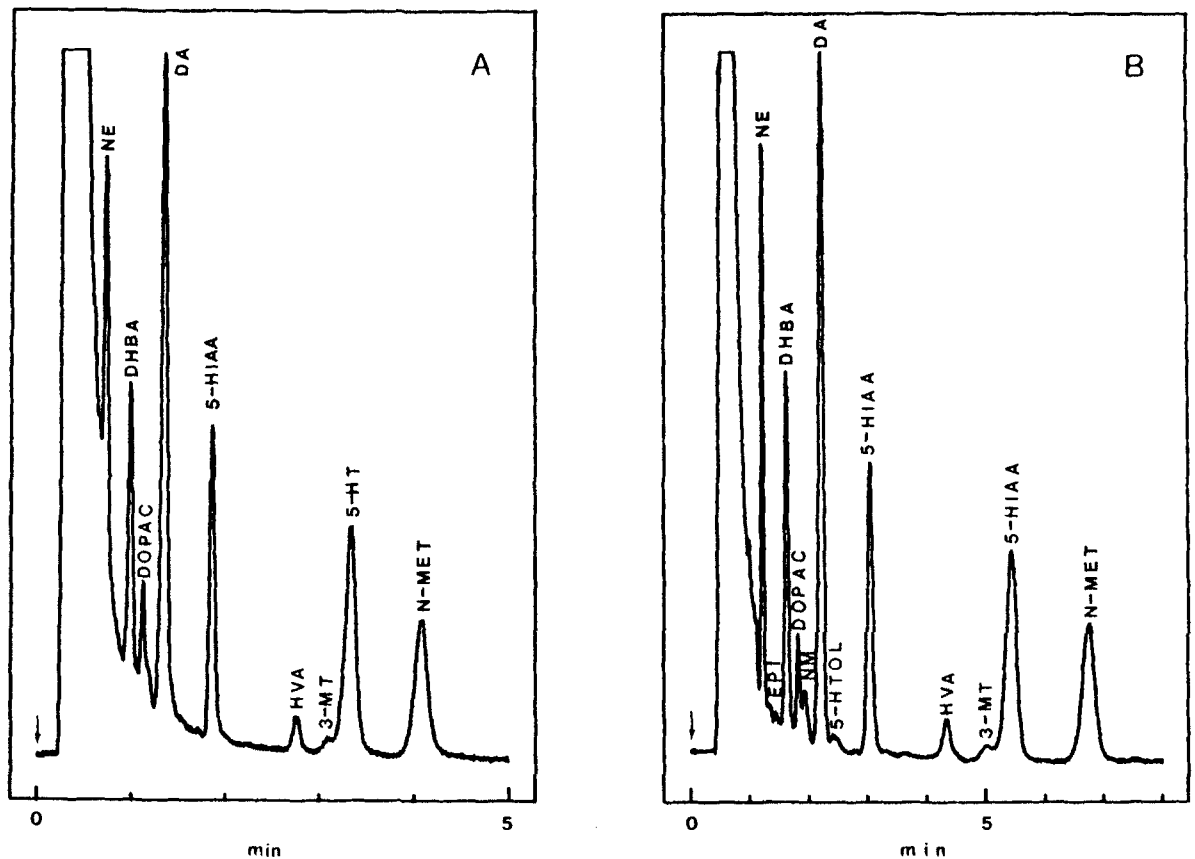

FIGURE 2. Whole Rat Brain Catecholamines, Indoleamines, and Related Metabolites.

TABLE 3

Whole Mouse Brain Content of Various Compounds*

Compound	Content (ng/g)
NE	392 ± 18
EPI	12 ± 14
DOPAC	116 ± 32
DA	1042 ± 83
5-HTOL	28 ± 6
5-HIAA	414 ± 31
HVA	263 ± 46
5-HT	745 ± 57
3-MT	238 ± 64

*Results from system B.

differences observed between the two chromatographic systems. In particular, the early eluting compounds are much more distinctly recognizable in System B. Thus, determinations of these components, especially norepinephrine, epinephrine and DOPA would appear to be better approached with System B. However, the resolution of System A could be enhanced by decreasing the acetonitrile content of its eluting solvent and, simultaneously, increasing its required time per analysis. Alternatively, one could employ an alumina separation prior to injection to greatly decrease the relative size of the solvent front in relationship to these components for either system.

Both systems cannot, unfortunately, be used with the currently described isolation procedure for the determinations of 3,4-dihydroxyphenylglycol, 3-hydroxy-4-methoxyphenylglycol, or vanillylmandelic acid. Further purification of the sample prior to injection would be necessary to avoid their obliteration by the solvent front.

The outlined procedures for the determination of enzymatic activities all rely on the same fundamental bases. The product

of the enzyme of interest is quantitated following blockade of its normal metabolism. For the in vitro determinations, the procedures also require incubation under proper conditions with appropriate cofactors to enhance production.

The simultaneous determination of both tyrosine and tryptophan hydroxylase in vivo incorporates simple blockade of both DOPA decarboxylase and 5-hydroxytryptophan decarboxylase with NSD-1015 at a specified time prior to sacrifice. As seen in Fig. 3, the resultant buildup of L-DOPA and L-5-hydroxytryptophan may then be directly used to assess the in vivo activities of these two enzymes. A typical determination of whole mouse brain yields values of 4.82 ± 0.56 nmol/g/hr and 3.37 ± 0.41 nmol/g/hr for tyrosine and tryptophan hydroxylase, respectively. The pretreatment time for Fig. 3, it should be noted, was only 5 minutes. Thus, the peaks observed are only ca. 1/6 of that which would be obtained at 30 minutes. At this short time, we again observe the difficulty in determining L-DOPA with system A. However, the remedies described above for component analyses of tissue and fluid samples would also apply to this situation.

The in vitro determination of tyrosine hydroxylase, not shown, yields a typical result of 108 ± 4 nmol/g/hr for whole rat brain with a signal to noise (tissue to blank) ratio of 34. This compares favorably to the radiometric results of Waymine et al. (120 ± 4 nmol/g/hr and 36). The in vitro tryptophan hydroxylase assay produced a typical value of 80.4 ± 0.7 nmol/g/hr for whole mouse brain. No comparable report of this tissue's activity by others could be found in the literature. The detection limit for this procedure, assuming a signal to noise ratio of two, is currently 1.0 pmol of 5-HTP. This is compared to the limits of 100 pmol reported for the fluorometric assay of Gal and Patterson (43) and 2.5 pmol for the radiometric assay of Kizer et al. (44).

The chromatographic results of an in vitro DOPA decarboxylase and a 5-hydroxytryptophan decarboxylase determinations are shown, respectively, in Figs. 4 and 5. For DOPA decarboxylase,

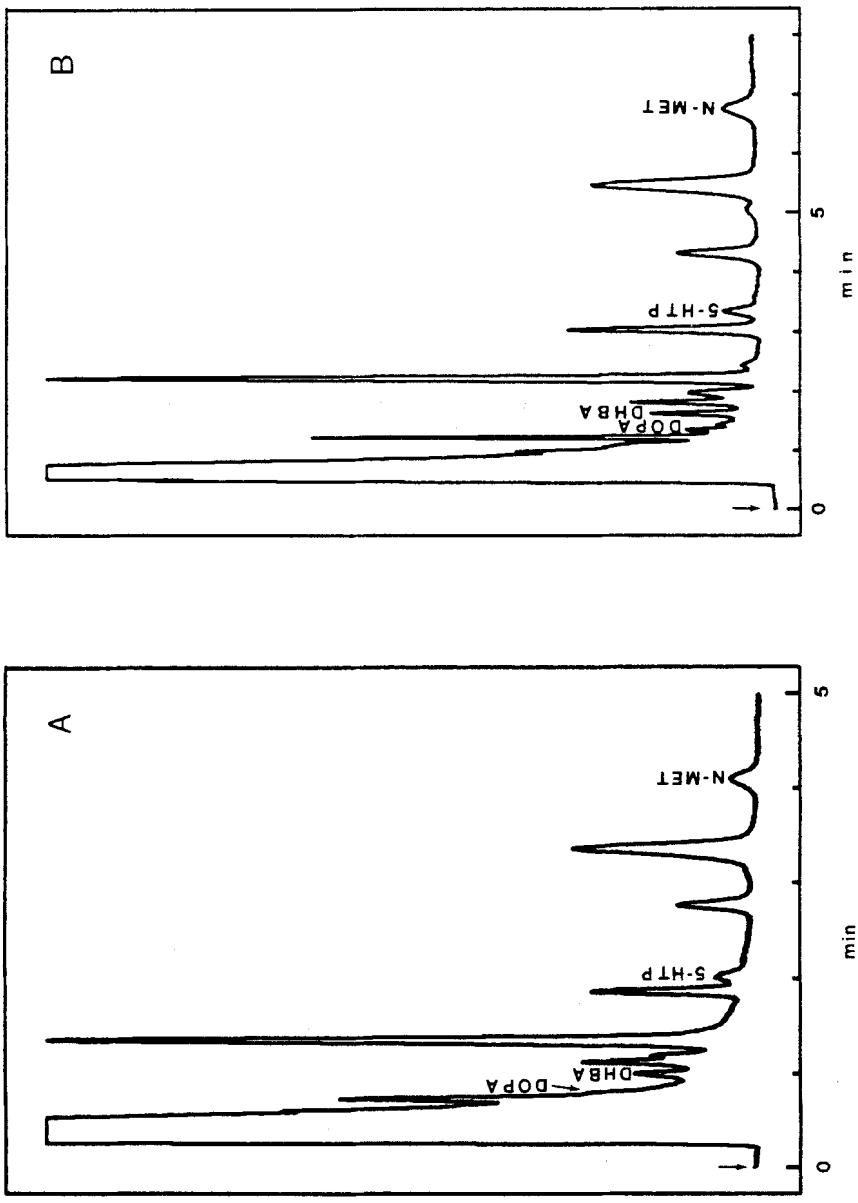

FIGURE 3. Simultaneous Determination of *in vivo* Tyrosine Hydroxylase and *in vivo* Tryptophan Hydroxylase in Whole Mouse Brain.

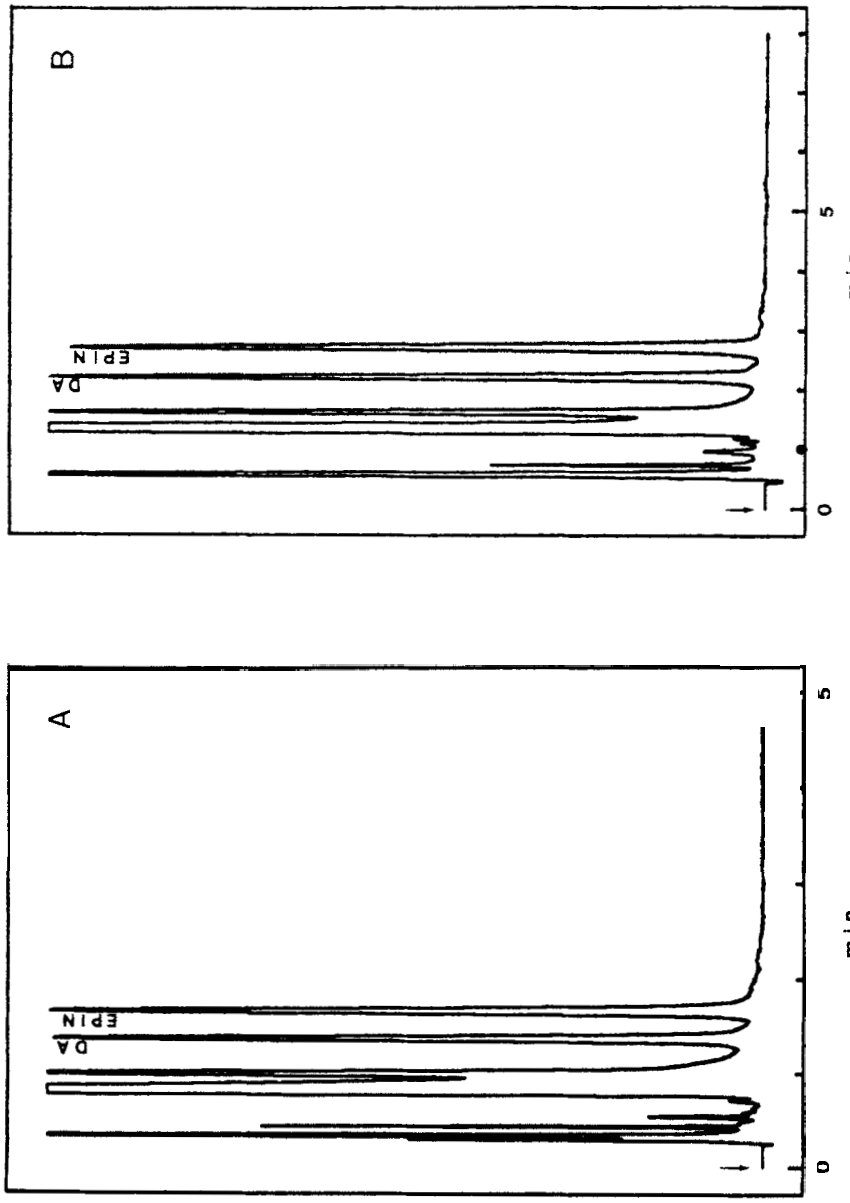

FIGURE 4. Whole Mouse Brain Determination of in vitro DOPA Decarboxylase.

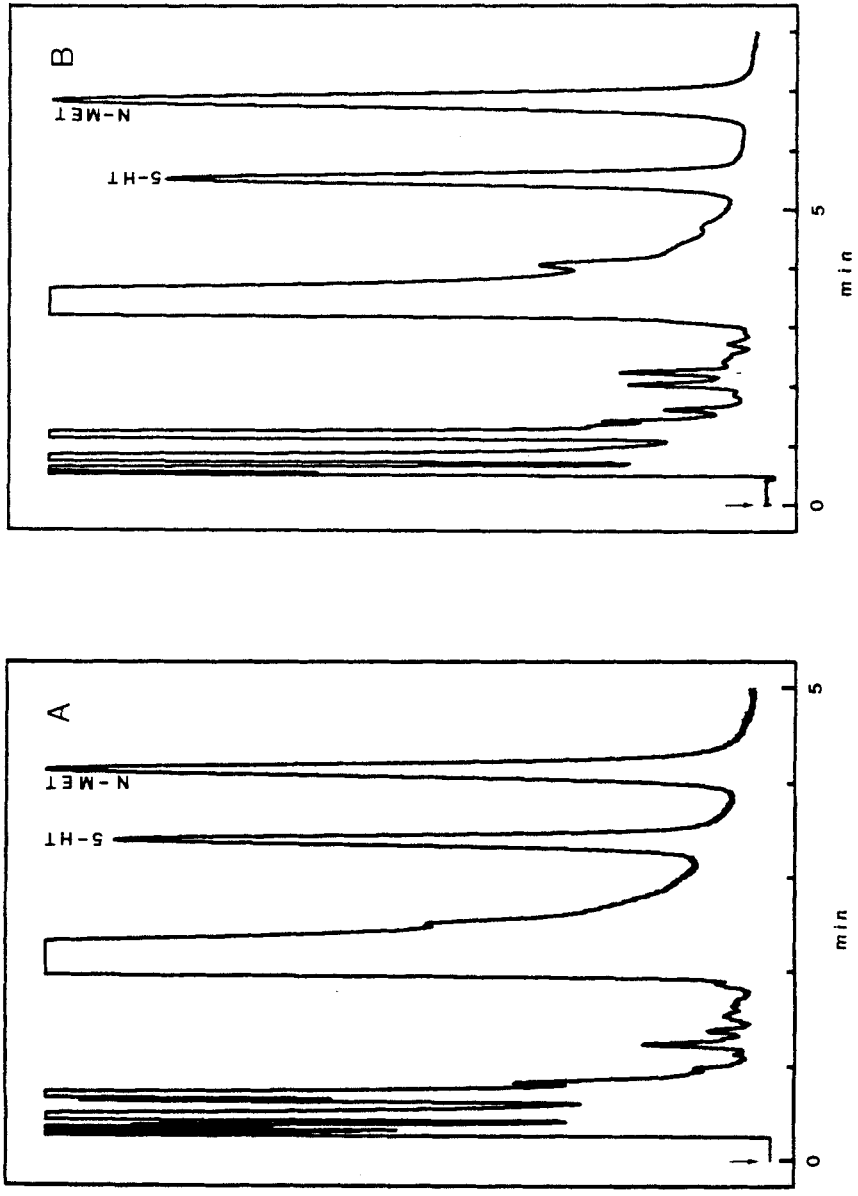

FIGURE 5. Whole Mouse Brain Determination of 5-Hydroxytryptophan Decarboxylase.

epinine has been employed as the internal standard. 3,4-Dihydroxybenzylamine, which can be seen but is not labeled, could also be employed as the internal standard. However, its peak is somewhat crowded by that for L-DOPA, the substrate. Thus, we would recommend the use of epinine. The very large amount of product formed in the DOPA decarboxylase assay, approximately 1000 times the endogenous value for dopamine, provides two advantages. First, blanks are not needed for these determinations. Secondly, the effective time required per individual sample is decreased to 1.8 minutes for System A and 3.0 minutes for System B. This considerably increases the potential throughput for these determinations. Typical results for whole mouse brain are 7.22 $\pm$ 0.58 μmol/g/hr and 0.88 $\pm$ 0.07 μmol/g/hr for DOPA decarboxylase and 5-hydroxytryptophan decarboxylase, respectively.

A typical chromatogram obtained for a sample taken from a determination of mouse whole brain monoamine oxidase activity is shown in Fig. 6. The essentially complete conversion of the intermediate, 5-hydroxyindoleacetaldehyde, to 5-hydroxyindole-3-acetic acid by excess aldehyde dehydrogenase is virtually assured by the lack of any observed broad, underlying background elevation in the chromatography (45). Whole mouse brain values obtained from a typical determination are 76.3 $\pm$ 6.4 pmol/mg/min, while regional values from seven major mouse brain regions varied between 53.8 $\pm$ 4.7 and 151.2 $\pm$ 16.6 pmol/mg/min.

A typical determination of catechol-O-methyltransferase activity in a selected portion of rat liver yielded a value of 462 $\pm$ 7 nmol/g/min for 3-methoxytyramine produced and a value of 135 $\pm$ 2 nmol/g/min for 4-methoxytyramine produced. A representative chromatogram is shown in Fig. 7.

These 3 μ systems do, indeed, exhibit a broad range of applicability to catecholamines, indoleamines, metabolites, and related biosynthetic and degradative enzymes. In addition to the determinations presented here, we feel both of these systems should be readily adaptable to the reported determinations of

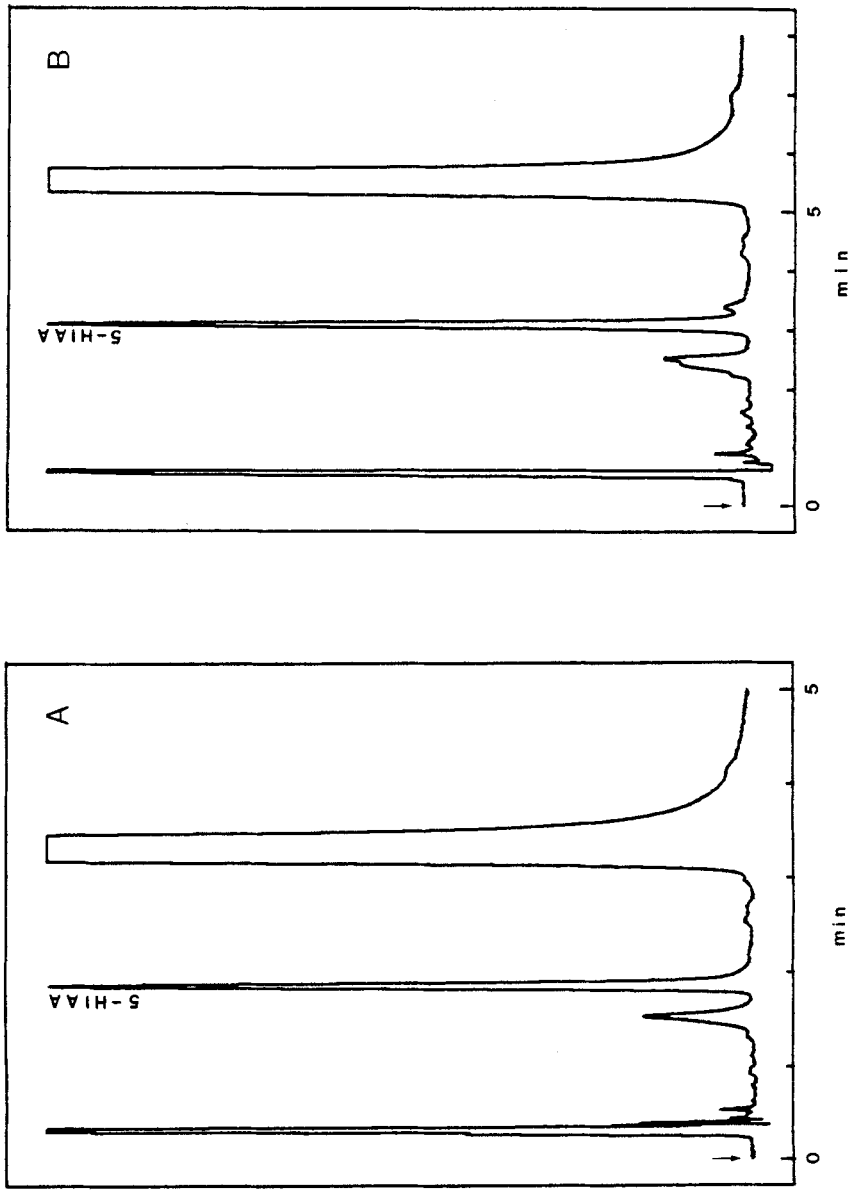

FIGURE 6. Determination of Monoamine Oxidase Activity in Whole Mouse Brain Using 5-Hydroxytryptamine as Substrate.

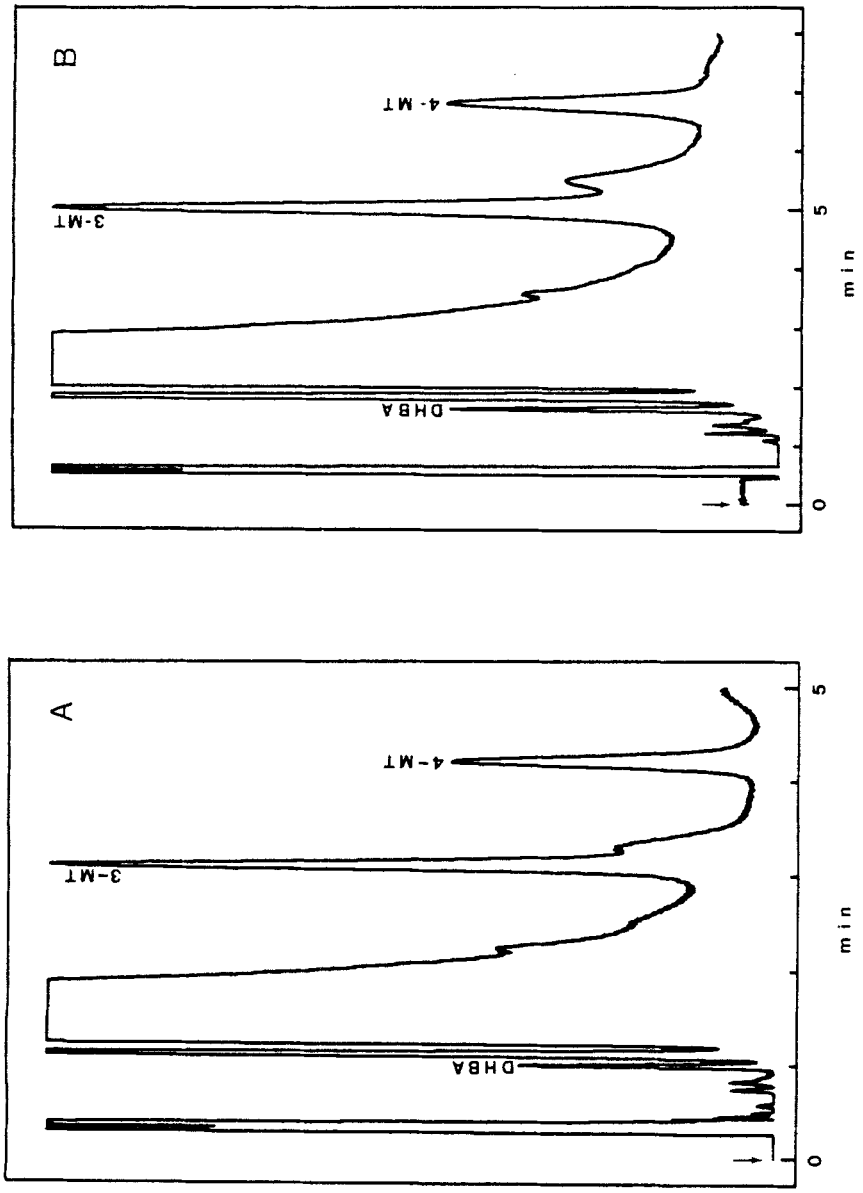

FIGURE 7. Determination of Catechol-O-methyltransferase Activity in a Selected Portion of Rat Liver.

in vitro dopamine-β-hydroxylase (14) and phenethanolamine-N-methyltransferase (15). But, there are cases where the current systems would be advantageously replaced with the older 5μ or 10μ packing materials. In particular, when the concentration of the species to be determined in the final mixture to be injected is extremely low, larger volumes must be injected to allow quantitation. The current systems will allow injection volumes of only 5 μl for early eluting components, while 20 or even 50 μl could be used for the later eluting components. But, injection volumes of 100 or 200 μl would almost certainly require the larger (5 or 10μ) packing materials. This problem could be circumvented by the use of additional purification or preconcentration steps in the procedure; the overall analysis time for the individual determination will need to be examined to obtain optimal throughput.

Three micron systems are also somewhat more 'touchy' than the 5 or 10μ systems. While this problem should diminish as the user's experience increases, more attention to details such as connecting tubing lengths, dead volumes, and damping time constants are essential at the outset.

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