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# SIMULTANEOUS MEASUREMENT OF TYROSINE, TRYPTOPHAN AND RELATED MONOAMINES FOR DETERMINATION OF NEUROTRANSMITTER TURNOVER IN DISCRETE RAT BRAIN REGIONS BY LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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## SUMMARY

Concomitant measurement of monoamine neurotransmitter turnover in discrete rat brain areas with the use of radiolabeled amino acid precursors permits simultaneous evaluation of interacting transmitter systems. [$^3$H]Tyrosine and [$^3$H]tryptophan were administered via indwelling catheters to unrestrained rats. Content and specific activity of norepinephrine, dopamine, 5-hydroxytryptamine, and the metabolites dihydroxyphenylacetic acid, homovanillic acid, and 5-hydroxyindoleacetic acid in addition to tyrosine and tryptophan were quantified by liquid chromatography with electrochemical detection and liquid scintillation counting. The method employs a simple extraction procedure without prior cleanup for chromatography. Neurotransmitter turnover rates that incorporated tyrosine- or tryptophan-specific activities were found to be two to four times greater than those that did not include them.

## INTRODUCTION

Recent efforts to correlate behavioral observations in rodents with neurochemical findings have been most successful when data have been evaluated in terms of central nervous system (CNS) neurotransmitter interactions and relatively simple neuronal circuits [1—5]. In many instances these interactions have involved the monoamine neurotransmitters norepinephrine (NE), dopamine (DA), and 5-hydroxytryptamine (5-HT). Behavioral, neurochemical,

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and anatomical evidence support the view of the existence of functional interactions between neuronal elements of the monoamines. For example, considerable evidence has accumulated that 5-HT neurons provide inhibitory influences to dopaminergic neuronal activity [1, 6, 7]. The function of these biogenic amines depends closely on their distribution in particular brain areas. Therefore, evaluation of specific neurotransmitter roles requires their assessment in discrete neuroanatomical regions or specific nuclei.

It is not surprising that methods for the determination of monoamines in brain have been the focus of many investigators for a number of years. Several analytical methods for their separation, detection, and quantitation have been employed. Spectrophotofluorometric procedures have been widely used to assay the biogenic amines [8, 9], but require either the isolation of each individual compound or the use of compound-specific methods of fluorescence development and in some instances lack adequate sensitivity. For these reasons fluorometry has not proven conducive to the simultaneous estimate of a large number of compounds. Gas chromatography–mass spectrometry while offering identification of compounds with excellent selectivity and limits of detection requires equipment that is not routinely available and utilizes relatively complex derivatization techniques [10, 11]. Radioenzymatic methods rely on enzymatic labeling of the catechol or indole nucleus with subsequent isolation followed by counting of radioactivity [12, 13]. This methodology, although highly sensitive, requires multiple-sample handling steps, considerable technical skill, and does not permit the simultaneous measurement of the precursors and metabolites of the biogenic amines under study.

TABLE I

MAJOR PUBLISHED REPORTS OF METHODS MEASURING BIOGENIC AMINE NEUROTRANSMITTERS, PRECURSORS, AND METABOLITES IN BRAIN

| Compounds analyzed | References |
|---|---|
| Single compounds | |
| NE | 16 |
| 5-HT | 17, 18 |
| MHPG | 19 |
| Catecholamines/metabolites | |
| EPI, NE, DA | 20 |
| NE, DA | 21–25 |
| DA and metabolites | 26–28 |
| NE, DA and metabolites | 29, 30 |
| Indoleamine/metabolites | |
| 5-HT and metabolites | 31, 32 |
| 5-HT and metabolites + TRP | 33, 34 |
| Multiple neurotransmitters/metabolites/precursors | |
| DOPA, 5-HTP | 35 |
| DA, 5-HT | 36, 37 |
| NE, DA, 5-HT | 38 |
| MHPG, DOPAC, HVA, 5-HIAA | 39 |
| DA, 5-HT and metabolites | 40, 41 |
| NE, DA, 5-HT and metabolites | 42–45 |
| NE, DA, 5-HT and metabolites + TRP | 46 |
| NE, DA, 5-HT and metabolites + TYR, TRP | 47, 48 |

The utility of determining picomole quantities of the neurotransmitters, their precursors, and metabolites by the use of reversed-phase liquid chromatography with electrochemical detection has been clearly established within a relatively short time since its potential and specificity were first recognized by Adams [14] and applied by Kissinger et al. [15] as a rapid, inexpensive and highly sensitive procedure for the assay of these compounds. Table I lists the most important published reports using this mode of detection in brain tissue and the compounds quantified [16—48]. As can be seen, numerous investigators have described procedures to measure various combinations of the catecholamines, indoleamines, and their respective metabolites. However, few papers [47, 48] have described the simultaneous determination of the two amino acid precursors, tyrosine (TYR) and tryptophan (TRP), concomitantly with these other compounds.

Measurements of steady-state tissue content of NE, DA, 5-HT and their metabolites in specific brain areas provide limited insight into changes occurring within these neuronal systems. The determination of neurotransmitter turnover has proven to be a more meaningful and useful estimate of neuronal activity. Various methods of assessing monoamine turnover have been extensively employed in recent years. These non-radiometric techniques have included measurement of: (1) the rate of accumulation of L-dihydroxyphenylalanine (DOPA) or 5-hydroxytryptophan (5-HTP) after decarboxylase inhibition [49, 50]; (2) the rate of decrease in DA or NE concentration following tyrosine hydroxylase inhibition with $\alpha$-methylparatyrosine [51]; (3) the rate of 5-HT accumulation following inhibition of monoamine oxidase [52, 53]; (4) the concentration of dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), or 5-hydroxyindoleacetic acid (5-HIAA) at steady state or following inhibition of formation (by monoamine oxidase inhibitors) or transport (by probenecid) [52, 54—58]. Nevertheless, these methods have considerable drawbacks. First, techniques inducing neurotransmitter changes beyond the range of normal concentrations produce abnormal behavioral states in the animal. This eliminates any possibility of relating neurochemical findings in this situation to ongoing behavior observed in a test paradigm. Second, methods resulting in the accumulation or decline of neurotransmitters, precursors, or metabolites produce an abnormal neurochemical environment by altering normal biochemical feedback and regulatory processes. This seriously hinders attempts to investigate neurotransmitter interactions. Third, changes observed in metabolite concentrations can result from more than one synaptic mechanism, and consequently they provide information of limited value. The radiolabeled turnover procedure described in this report is not susceptible to these criticisms, and provides data that can be evaluated in terms of behavioral correlates and the interactions of different neuronal systems. Radiolabeled turnover can be determined by applying steady-state kinetics to the decline of transmitter specific activity over time utilizing a single-compartment model [59]. Moreover, accuracy requires that the specific activity of the precursor amino acids be included in the determinations also [59].

This report describes a procedure by which content and radioactivity of the monoamine neurotransmitters and metabolites can be measured simultaneously with the precursors TYR and TRP within small tissue samples ($< 10$ mg) for

## EXPERIMENTAL

### *Animals*

The male Long—Evans hooded rats (450—500 g) used in this work were progeny of rats originally obtained from Charles River (Wilmington, MA, U.S.A.) and bred in-house. The animals were housed in stainless-steel wire cages within a temperature (22 ± 1°C), light (12:12 cycle commencing at 07.00 hours), and humidity (50%) controlled environment ventilated with filtered air. NIH-07 laboratory chow (Ziegler Bros., Gardners, PA, U.S.A.) and distilled water were provided ad libitum.

### *Surgical preparation of animals*

Administration of the radiolabeled precursor amino acids TYR and TRP was via a chronic indwelling jugular catheter. Application of the tracer dose in this manner requires no anesthesia and avoids stress, both of which influence neuronal activity, while enhancing the precision of the administered doses as compared to manual restraint and intravenous injection. Animals were surgically implanted with catheters as described originally by Weeks [60] and modified by Lane et al. [61]. Briefly, the animals were anesthetized with pentobarbital, an opening made on the ventral neck, and a small polyvinyl chloride catheter (Tygon®, 0.01 in. I.D.) inserted into the external facial vein and run approximately 25 mm toward the heart. The catheter was anchored with surgical silk and run under the skin to a point above the scapula where it exited through a small polyethylene harness. The catheter was filled with heparinized saline and enclosed in a small brass shimstock box attached to the harness. At least ten days were allowed for the animals to recover from the surgery and become accustomed to the cannula harness attached to their backs. Function of the catheter was checked by injection of thiopental 2—3 days before sacrifice.

### *Precursor injection*

On the day of sacrifice L[$^3$H] 2,6-TYR (specific activity, 30 Ci/mmol) and L[$^3$H(G)] tryptophan (specific activity, 4.5 Ci/mmol) (New England Nuclear, Boston, MA, U.S.A.) were dried under a stream of nitrogen and redissolved in saline so that 100 μl of the mixture contained 1.0 mCi of [$^3$H] TYR and 0.5 mCi of [$^3$H] TRP. Animals were injected with 100 μl of the mixture (33 and 110 nmol of amino acid, respectively) between 09.00 and 11.00 hours and were decapitated either 60, 90, 120, or 180 min later ($n$ = 3 to 4 animals per time point).

### *Tissue dissection and extraction*

Brains were quickly removed, rinsed in isotonic saline (4°C), and dissected on ice essentially according to the method of Heffner et al. [62] using a modified slicing apparatus. Brain regions were quickly frozen on dry ice, weighed, and stored at −70°C until extraction.

Individual brain regions were homogenized in ground-glass tissue grinders (1.0 ml volume; Radnoti Glass Technology, Monrovia, CA, U.S.A.; 10 ml volume; Kontes, Evanston, IL, U.S.A.) and extracted essentially according to

Co et al. [45] with 25 volumes of ice-cold 1.0 *M* formic acid—acetone (15:85, v/v) containing epinine (N-methyldopamine) as internal standard. Aliquots (300 μl) were taken from each homogenate and centrifuged at 1000 *g* for 10 min at 4°C (Sorvall RC2-B, DuPont Instruments, Newtown, CT, U.S.A.). Supernatants were extracted with 1.0 ml cold heptane—chloroform (8:1, v/v), the organic layer discarded, and the aqueous layer dried under nitrogen and stored at −20°C until analysis (< 1 week). Samples were thawed and redissolved in mobile phase (see below) prior to injection of 20 μl into the liquid chromatograph.

*Apparatus*

The chromatographic system consisted of a 25 cm × 4.6 mm I.D. Biophase-ODS 5-μm $C_{18}$ column (Bioanalytical Systems, West Lafayette, IN, U.S.A.), a PM-11 Milton-Roy minipump (State College, PA, U.S.A.), and a Model 7125 syringe-loading rotary injection valve (Rheodyne, Cotati, CA, U.S.A.). A pre-column 2-μm filter (Rheodyne) minimized the accumulation of particulate matter on the analytical column. The signal from a TL-8A glassy carbon detector cell was amplified by a Model LC-3 amperometric controller (Bioanalytical Systems) with output to dual-chart recorders set at 20 and 200 nA full-scale deflections. Two recorders set at different amplification ranges were used since tissue concentrations of the compounds of interest often differ by more than an order of magnitude. The flow-rate was 1.5 ml/min with a back-pressure of 172 bars. Columns were continuously perfused with mobile phase at minimum flow-rates when not employed for actual tissue analyses.

*Chromatography*

The mobile phase consisted of 0.15 *M* monochloroacetic acid in triple glass-distilled water made 0.1 m*M* with disodium-EDTA and adjusted to pH 3.00 with 10 *M* sodium hydroxide. This solution was passed through 0.22-μm pore nitrocellulose filter supported by a coarse-grade fritted-glass funnel (Millipore, Bedford, MA, U.S.A.) and degassed under vacuum. Sodium octyl sulfate was added as the ion-pairing agent at 200 mg/l, and acetonitrile added to a final concentration of 7.3%. The solution was pumped at ambient temperature during chromatography.

Detector potential was maintained at +950 mV vs. Ag/AgCl reference electrode. These chromatographic conditions permitted the routine quantitation of TYR, TRP, NE, DA, and 5-HT as well as DOPAC, HVA, and 5-HIAA (Fig. 1).

*Scintillation counting*

All samples were spiked with $^{14}$C-tracer amounts of NE, DA, 5-HT, TYR, and TRP (ca. 500 dpm each) to provide a measure of the accuracy of peak collection for these compounds for scintillation counting. Eluate corresponding to specific peaks was collected quantitatively from the detector in scintillation vials according to the deflection of the recorder pen. Volumes of 15 ml of Aquasol-2® (New England Nuclear) were added to individual chromatographic fractions, and the vials were placed in a Packard Tri-Carb 460 CD Liquid Scintillation System (Packard Instruments, Downers Grove, IL, U.S.A.) for

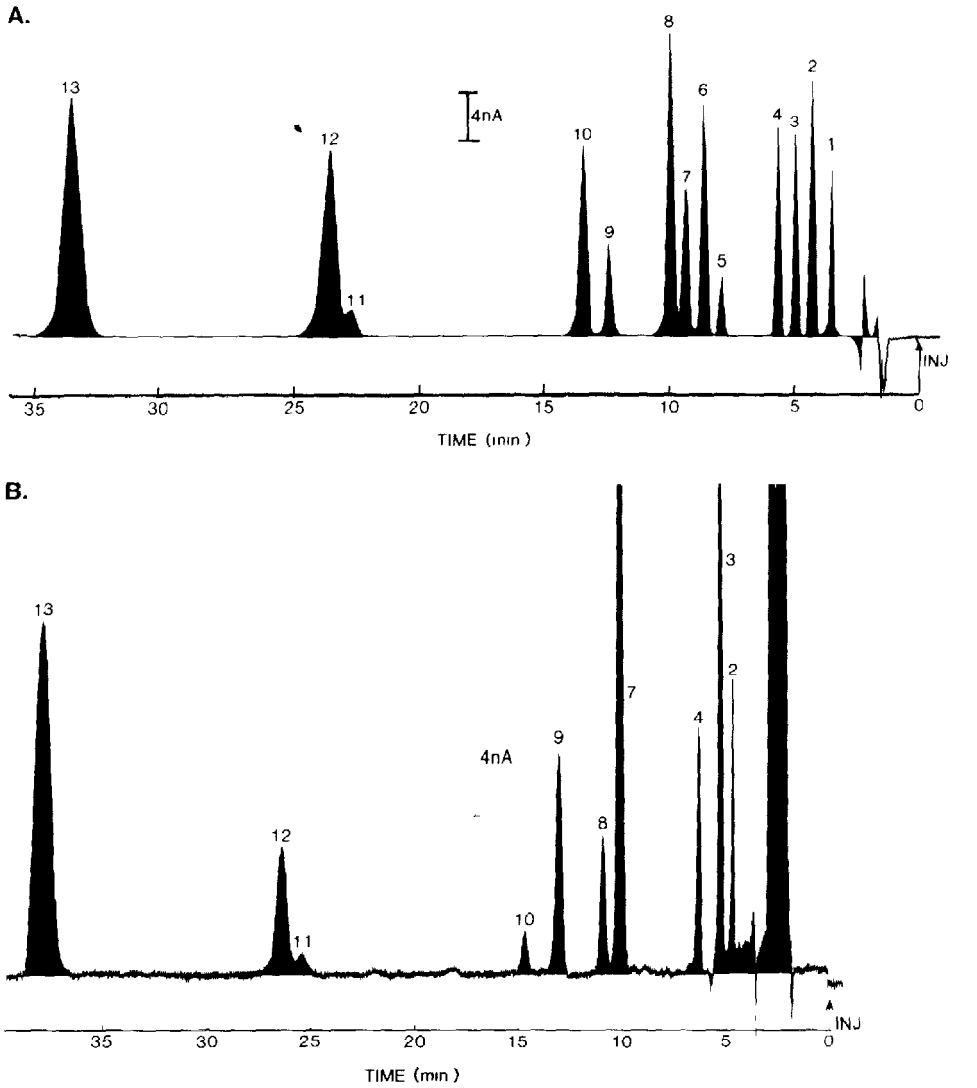


Fig. 1. Chromatograms of (A) 20 $\mu$l of a mixture of working standards containing 1 pmol/$\mu$l of NM and 3-MT, 8 pmol/$\mu$l of TYR and TRP, and 3 pmol/$\mu$l of all the others, and (B) 4.8 mg of tissue from nucleus accumbens showing quantified peaks. Off-scale deflections were measured on a second recorder set at 10% of the above sensitivity. Peaks: 1 = L-DOPA; 2 = NE; 3 = TYR; 4 = DOPAC; 5 = NM; 6 = 5-HTP; 7 = DA; 8 = 5-HIAA; 9 = EPN; 10 = HVA; 11 = 3-MT; 12 = 5-HT; 13 = TRP. See text for chromatographic conditions.

counting of radioactivity. Dual-label ($^3H/^{14}C$) efficiencies were computed by reference to double-quench correction curves developed from a set of external standards.

*Chemicals*

Standard mixtures were prepared from L-norepinephrine bitartrate (NE), L-tyrosine (TYR), dopamine hydrochloride (DA), DL-3,4-dihydroxyphenylalanine (DOPA), 3,4-dihydroxyphenylacetic acid (DOPAC), 4-hydroxy-3-

methoxyphenylacetic acid (HVA), 3-methoxytyramine hydrochloride (3-MT), L-tryptophan (TRP), 5-hydroxytryptamine creatinine sulfate (5-HT), 5-hydroxyindole-3-acetic acid (5-HIAA), 5-hydroxy-L-tryptophan (5-HTP), and deoxyepinephrine hydrochloride (epinine), all obtained from Sigma (St. Louis, MO, U.S.A.), and DL-normetanephrine (NM) from Calbiochem-Boehring (San Diego, CA, U.S.A.). Sodium octyl sulfate was obtained from Eastman Kodak (Rochester, NY, U.S.A.). All other reagents and solvents were of analytical grade and used without further purification.

*Standards*

Stock standard solutions were made 1 m*M* in 0.01 *M* hydrochloric acid for each compound and stored at 4° C (stable for at least six months). Mixtures of working standards were prepared from stocks with 0.01 *M* hydrochloric acid according to the relative detector responses generally expected in the tissue samples: TYR and TRP were diluted to 8.0 $\mu M$, and all other compounds including epinine were made 3.0 $\mu M$. Working standard solutions were stored at 4° C and freshly prepared each week.

A 20-$\mu$l aliquot of the mixture of working standards was routinely chromatographed at the beginning and end of each day. Additionally, a standard containing TYR alone (8 $\mu M$) was routinely run following every second chromatographic analysis.

The efficacy of the extraction procedure in the presence of tissue was evaluated by estimation of the recovery of the components of the working standard solution. Cerebellar tissue was homogenized as described above and three 300-$\mu$l aliquots removed. Two aliquots were spiked with 50 $\mu$l of the working standard solution and the third served as a tissue blank. The three samples were then carried through the rest of the procedure in conjunction with the individual tissue samples. Recoveries were determined for each compound from the difference in peak heights of the spiked and unspiked samples in comparison to the peak heights from 20 $\mu$l of the mixture of working standards injected directly into the chromatograph.

*Data analysis*

Concentrations of each compound were determined by comparing sample peak heights with peak heights obtained from the mixture of working standards. All measures of content were corrected by the epinine recovery value for each sample. All radioactivity measures were corrected by the epinine recovery value for the sample or the $^{14}$C-recovery for the specific compound (if available), whichever was lower. Choice of the lower value is based on the rationale that accurate collection of eluent associated with a specific peak would contain the amount of radioactivity reflecting the epinine recovery value. However, human error in the manual collection of eluent representing a specific peak could lead to less than expected amounts of radioactivity due to incomplete collection. Therefore, employing the smaller of the two recovery values would compensate for this source of error. Concentrations were expressed as $\mu$g/g tissue weight and specific activities as dpm/nmol.

Neurotransmitter turnover was determined utilizing steady-state kinetics with the radiolabel assumed to disappear from a single open neuronal compart-

ment [59]. Neurotransmitter specific activities were used to determine a line on a log—linear plot across the sacrifice time points by computerized linear regression techniques. Turnover constants were then computed both with and without incorporation of the amino acid specific activities [45, 59].

## RESULTS AND DISCUSSION

### *Voltammetric responses*

The response of the electrochemical detector depends upon the applied voltage and the nature of the compound being oxidized as shown in Fig. 2. Catechols in comparison to the other compounds of interest were most readily sensed by the detector with current first noted at about +400 mV. Further increases in current response above +600 mV were small. The 3-methoxylated metabolites of the catecholamines were more difficult to oxidize with current initiated at an applied voltage of about +700 mV. This group of compounds reached a plateau in the voltammograms and displayed little additional response above +800 mV. The 5-hydroxyindoles differed from both these groups in their electrochemical response. Current was initiated at +450 mV and reached a plateau at about +700 mV before increasing again at +1000 mV. Similar voltammograms have been reported previously for 5-hydroxylated indoles [63]. TYR and TRP required higher applied voltages (+850—+900 mV) than the other compounds tested to initiate an electrode response. In addition, the current—potential curves for these amino acids did not plateau in the range of potentials tested. Other authors have reported $E_{1/2}$ values for TYR or TRP greater than +1000 mV [64].

An operating potential of +950 mV was set for all analyses to obtain

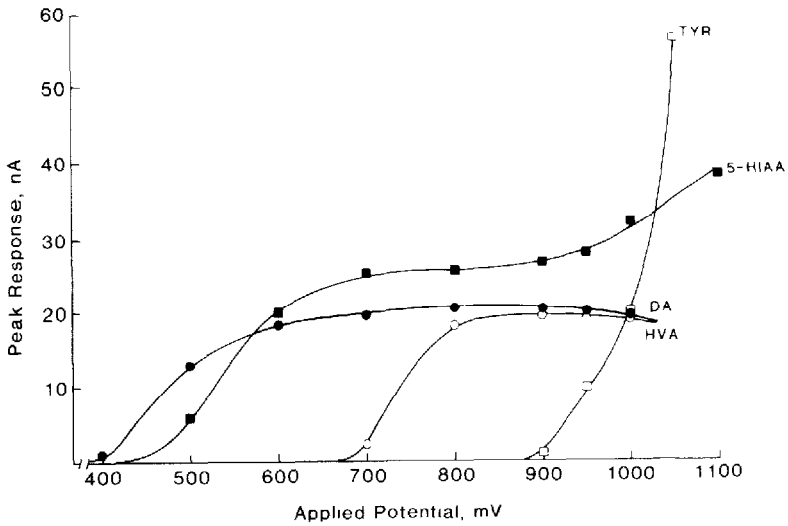


**Fig. 2. Current—potential curves for standard compounds. DA is representative of catechol compounds (NE, DOPA, DOPAC), HVA represents the 3-methoxylated catecholamine metabolites (3-MT, NM), and 5-HIAA is indicative of the 5-hydroxyindoles (5-HT, 5-HTP). The curve for TRP is similar to that for TYR. All curves were generated from the current response to 80 pmol of each compound.**

sufficient current responses for TYR and TRP. At this voltage the detector responses of all other compounds had attained plateaus. Higher potentials were impractical because of the background noise generated.

*Linearity of detector response*

The linearity of detector response for each compound of interest was confirmed by injecting amounts of 1—400 pmol into the chromatograph. Fig. 3 shows the resulting curves for representative compounds demonstrating linearity for amounts spanning more than two orders of magnitude. Correlation coefficients determined by regression analysis of the amount injected and the detector response were all greater than 0.996. The detection limits based on a signal-to-noise ratio of 2:1 ranged from 51 fmol for 5-HIAA to 6.5 pmol for DOPAC. The variation in repeated injections of the same amount of standard was less than ± 2%.

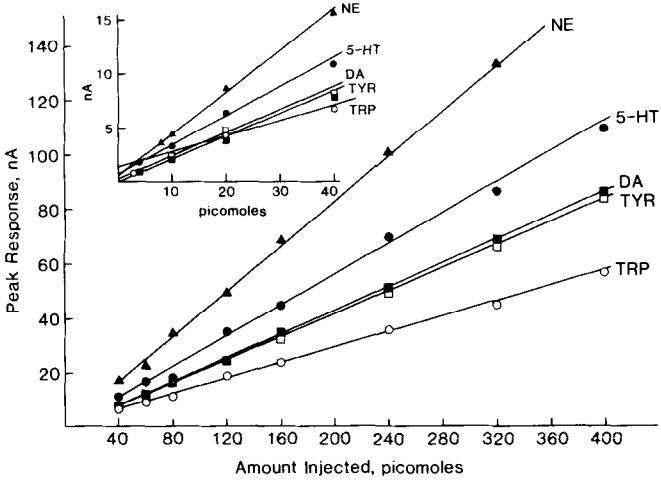


Fig. 3. Linearity of response curves for representative standard compounds (1—400 pmol of each compound in 20 μl) at an applied potential of +950 mV. The ratio of the slope of the standard curve for each compound relative to the slope of the standard curve for epinine was: NE = 2.05; TYR = 1.02; DOPAC = 2.08; DA = 1.05; HVA = 0.90; 5-HIAA = 2.32; 5-HT = 1.34; TRP = 0.68.

*Chromatographic performance*

The relatively large number of peaks in each chromatogram required a mobile phase which could be manipulated to maintain optimal resolution. Changes in pH altered the retention times of the acids in the chromatogram in a consistent manner without substantially affecting the amines. Increased mobile phase acidity increased retention of these compounds through increased protonation of the carboxyl groups and subsequent increased ion-pair formation with the octyl sulfate. The elution time of TRP (the last peak) was particularly sensitive to pH and effectively established the lower pH limit that could be utilized. Similarly, TYR resolution required a mobile phase of pH 3.00 since above pH 3.10 and below pH 2.95 it was eluted with neighboring peaks (Fig. 1). Changes in the acetonitrile component also altered the chromatography consistently. Increased organic solvent decreased the retention

times of all the compounds. In contrast, additional sodium octyl sulfate selectively increased the retention of the amines without substantially affecting the acids. The mobile phase constituents thus comprised a flexible and versatile system that could be altered as column performance warranted.

The separation of a mixture of standards is shown in Fig. 1A. Generally, the catechol compounds were eluted before the indoles and the acidic metabolites before the 3-methoxylated metabolites. Fig. 1B demonstrates the effectiveness of the chromatographic conditions for simultaneous determination of these compounds from nucleus accumbens.

Peaks in the chromatogram were identified by co-elution with authentic standards and by peak superimposition, that is, by addition of known amounts of standards to tissue samples and comparison of the increased peak heights at the corresponding retention times. DOPA, 5-HTP, and 4-hydroxy-3-methoxyphenylene glycol (MHPG) were not routinely identifiable in tissue samples. Only trace amounts of NM could be normally detected and only in certain brain regions. 3-MT was observable only in DA-rich areas such as nucleus accumbens and corpus striatum with levels apparently resulting largely from postmortem enzymatic degradation of DA [65].

Other potential chromatographic interferences were also checked. Epinephrine was eluted immediately after NE, octopamine immediately following DOPAC, and tyramine and tryptamine between HVA and 3-MT, but these peaks were not observed in tissue samples. Metanephrine, the 3-methoxylated metabolite of epinephrine, was eluted with DA but would not have been present in sufficient amounts in tissue to cause significant interference. The catecholamine metabolites vanillylmandelic acid, dihydroxymandelic acid, and dihydroxyphenylene glycol were eluted in the solvent front.

The occurrence of unknown peaks is a concern when unpurified brain extracts are being chromatographed. A few peaks of this nature were clearly identified in specific brain regions, but they were generally small and could not be characterized as monoamine-related. Moreover, these peaks contained no appreciable radioactivity. With the relatively high potential applied to the detector and the crude nature of the brain extracts it is possible that one or more of these peaks represented small TYR- or TRP-containing peptides.

*External and internal standardization*

The formic acid—acetone homogenization medium employed in these procedures extracts a large number of compounds of neurochemical interest [66] in a single unpurified fraction. However, the specificity of the electrochemical detector in combination with the high-efficiency column makes the use of a non-specific extraction procedure feasible.

The relatively high voltage applied and the large number of endogenous compounds oxidized resulted in a gradual decay in detector sensitivity over the course of a day. Consequently, a mixture of working standards was run immediately before the first and after the last tissue samples each working day. The results from analysis of this data indicated that slight changes in retention times (and peak heights) were also a factor. The mean difference in the peak heights for all compounds in the working standards mixture except TYR and TRP from the beginning to the end of the day was 3.0 ± 1.4% with no values

greater than ± 6—7%. The amino acid changes were greater. TRP declined on the average 14.4% and TYR 41.5% from the beginning to the end of the day. A TYR standard was therefore run after every second tissue sample throughout the day to more closely monitor this change. The basis for these latter changes in electrode response can be seen in the voltammogram in Fig. 2. Since neither amino acid has attained a plateau on the current—potential curve at +950 mV, relatively little fouling of the electrode would markedly depress the peak response. The changes in peak responses from beginning to the end of the day for all the working standards were assumed to occur linearly with respect to time, and external standard values relative to a specific tissue sample were interpolated accordingly.

The use of internal standards in quantitative analytical procedures is generally considered to be superior to direct calibration because it provides an inherent correction factor. Analyses of the monoamines by liquid chromatography with electrochemical detection have frequently relied upon dihydroxybenzylamine (DHBA) or other compounds such as N-methyl-5-hydroxytryptamine added to tissue samples before extraction and chromatographic separation. Such compounds mimic the chemical behavior of the catechols and/or indoles both in extraction and chromatographic characteristics. Hence, they effectively control for variable extraction or injection volumes and variations in detector performance within a day. In this work epinine was used as the internal standard.

Spiked and unspiked cerebellar homogenates were employed as tissue blanks to compare the recovery of epinine as internal standard to the recovery of added amounts of each compound to be measured. Epinine was chosen as internal standard because it is not detectable in brain tissue of normal animals

TABLE II

COMPARISON OF INTERNAL STANDARD RECOVERY WITH RECOVERY OF INDIVIDUAL COMPOUNDS IN THE PRESENCE OF TISSUE

Cerebellar homogenates were split and some of the replicates were spiked with known amounts of authentic standards of the compounds of interest. All replicates were then carried through the procedure (see Experimental) and the rates of epinine (EPN) recovery compared with the rates of recovery of the other compounds. The percent recovery values reported are the means ± S.D. with $n$ = 16 for EPN and $n$ = 25—26 for all other compounds. Ratios were computed by dividing the mean spike recovery for an individual compound by the mean EPN recovery.

| Compound | Percent recovery | Ratio to EPN |
|---|---|---|
| EPN | 84.6 ± 12.3 | 1.00 |
| TYR | 95.6 ± 16.0 | 1.13 |
| NE | 85.1 ± 10.4 | 1.01 |
| DA | 91.1 ± 12.6 | 1.08 |
| DOPAC | 93.1 ± 12.0 | 1.10 |
| HVA | 83.5 ± 10.6 | 0.99 |
| TRP | 87.3 ± 11.2 | 1.03 |
| 5-HT | 86.7 ± 11.4 | 1.03 |
| 5-HIAA | 92.0 ± 12.8 | 1.09 |

TABLE III

REGIONAL CONCENTRATIONS OF MONOAMINE PRECURSORS, NEUROTRANSMITTERS, AND METABOLITES IN RAT BRAIN

Brain regions were dissected as described in Experimental. Values represent means ± S.E.M. for 12—13 determinations expressed as μg/g tissue. No differences in content were observed among any of the time points after radiolabeled precursor administration. N.D. = not detectable.

| Brain region | TYR | NE | DA | DOPAC | HVA | TRP | 5-HT | 5-HIAA |
|---|---|---|---|---|---|---|---|---|
| Nucleus accumbens | 11.69 ± 0.77 | 1.03 ± 0.06 | 6.32 ± 0.20 | 1.36 ± 0.07 | 0.55 ± 0.03 | 4.77 ± 0.96 | 1.18 ± 0.02 | 0.68 ± 0.02 |
| Corpus striatum | 11.42 ± 0.76 | 0.29 ± 0.02 | 10.41 ± 0.03 | 1.41 ± 0.04 | 0.84 ± 0.04 | 4.79 ± 0.96 | 0.78 ± 0.02 | 0.71 ± 0.03 |
| Hypothalamus | 11.17 ± 0.83 | 2.26 ± 0.06 | 0.53 ± 0.02 | 0.11 ± 0.01 | N.D. | 4.40 ± 0.95 | 1.08 ± 0.03 | 0.60 ± 0.02 |
| Hippocampus | 10.95 ± 0.78 | 0.43 ± 0.03 | 0.21 ± 0.03 | N.D. | N.D. | 4.40 ± 0.89 | 0.54 ± 0.01 | 0.53 ± 0.02 |
| Parietal cortex | 10.04 ± 0.64 | 0.40 ± 0.02 | 0.56 ± 0.03 | 0.10 ± 0.01 | 0.10 ± 0.01 | 4.27 ± 0.82 | 0.46 ± 0.01 | 0.33 ± 0.01 |

TABLE IV

REGIONAL MONOAMINE NEUROTRANSMITTER TURNOVER RATES COMPUTED WITH AND WITHOUT INCLUSION OF TYR AND TRP SPECIFIC ACITIVITIES

NE, DA and 5-HT specific activities were utilized to compute turnover constants in two different ways: (1) (*HL*) the half-life of the decrease in neurotransmitter specific activity was derived from the decline portion of the curve; (2) (*AA*) TYR and TRP specific activities were included with the slope of the decline in neurotransmitter specific activity in the equation shown in Results and discussion. Turnover rate is the product of the turnover constant and the concentration of neurotransmitter. The turnover rates in this table are based upon 10—13 specific activity determinations with animals treated as described in Experimental. Values are expressed as μg/g tissue/h ± S.E.S. (standard error of the slope).

| Brain region | NE | | DA | | 5-HT | |
|---|---|---|---|---|---|---|
| | *HL* | *AA* | *HL* | *AA* | *HL* | *AA* |
| Nucleus accumbens | 0.14 ± 0.10 | 0.69 ± 0.51 | 2.21 ± 0.30 | 6.12 ± 0.82 | 0.33 ± 0.07 | 1.09 ± 0.24 |
| Corpus striatum | 0.02 ± 0.02 | 0.08 ± 0.10 | 2.71 ± 0.42 | 9.16 ± 1.43 | 0.21 ± 0.06 | 0.73 ± 0.20 |
| Hypothalamus | 0.11 ± 0.26 | 2.84 ± 6.72 | 0.19 ± 0.05 | 0.53 ± 0.14 | 0.31 ± 0.07 | 0.95 ± 0.22 |
| Hippocampus | 0.04 ± 0.05 | 0.17 ± 0.21 | 0.07 ± 0.02 | 0.14 ± 0.04 | 0.16 ± 0.04 | 0.44 ± 0.12 |
| Parietal cortex | 0.10 ± 0.07 | 0.22 ± 0.15 | 0.19 ± 0.05 | 0.45 ± 0.13 | 0.13 ± 0.07 | 0.33 ± 0.17 |

and because it had a more acceptable retention time in the elution pattern than the more frequently employed DHBA. Table II gives the mean recovery of epinine in the unspiked tissue blanks and the average recoveries of the other compounds in the spiked homogenates. The ratio of the recoveries of the other compounds to the epinine recovery range from 0.99 to 1.13 and indicate that epinine is a valid internal standard in representing the chemical behavior of the precursors, neurotransmitters, and metabolites quantified in this assay.

Across more than 500 tissue samples analyzed in this laboratory the epinine recovery averaged 84.3 ± 8.0%. This value is higher than that of most other compounds used as internal standards employing similar methods (see Table I) and represents good reproducibility. These attributes ensue from the simple extraction procedure employed in which little loss of sample occurred through manipulations to separate or isolate individual compounds.

Recovery of radioactivity from collection of NE, DA, 5-HT, TYR, and TRP peaks was calculated by comparing the counts of the $^{14}$C-labeled compound in an eluate fraction to the counts added in the initial $^{14}$C-spike. When peak collection for a particular fraction was complete, the $^{14}$C-radioactivity recovery was within 2–3% of the epinine recovery value. Background radioactivity was consistently low in both the $^{3}$H- and $^{14}$C-channels in the range of 12–15 dpm.

*Monoamine turnover utilizing TYR and TRP*

Determinations of precursor, neurotransmitter, and metabolite concentrations in representative brain regions are shown in Table III. These values are in excellent agreement with reported values for similarly dissected brain areas (e.g., refs. 42, 45, 66, 67).

Table IV gives the turnover rates for NE, DA, and 5-HT in the same brain areas calculated both with and without incorporation of simultaneous changes in TYR or TRP specific activities. For the turnover rates expressed without inclusion of the amino acid precursors, half-lives ($t_{1/2}$) were calculated for NE, DA, and 5-HT from the regression lines of the declines in specific activity in each brain region. The $t_{1/2}$ values were then substituted into the equation $k = \ln 2/t_{1/2}$ to derive a turnover constant. The product of this constant times the neurotransmitter concentration in that brain area equals the turnover rate.

The development of computational procedures to incorporate TYR or TRP specific activities into calculation of the turnover constant has been reported by Neff et al. [59]. These methods were derived to more closely simulate the results of an intravenous pulse injection of radiolabeled precursor. Incorporation of label into the neurotransmitter is initially rapid, but since a portion of the labeled amino acid recirculates in the bloodstream for some time, synthesis of [$^{3}$H]neurotransmitter continues at a decreasing rate. If this continued synthesis is not accounted for, then the computed turnover constant (as calculated for Table IV above) will be less than the true value. The amino acid specific activities were included in the calculation with the decline in neurotransmitter specific activity by the following equation:

$$k = \frac{2 \times M}{(AA_{t_1} + AA_{t_2} - NT_{t_1} - NT_{t_2})}$$

where $k$ = turnover constant; $M$ = slope of the regression line for neurotrans-

mitter specific activity; $AA$ = TYR or TRP specific activity at time $t$; $NT$ = neurotransmitter specific activity at time $t$. These values are also shown in Table IV. As can be seen, the inclusion of the TYR or TRP specific activities results in most cases in a two- to four-fold increase in neurotransmitter turnover rates. This magnitude of change has also been observed by Neff et al. [59].

Concentrations of biogenic amine metabolites are currently thought to provide appropriate indices of the amount of functional neurotransmitter released [54, 57], and their turnover rates have been reported [28, 56, 68]. The procedure described in this report can also provide data on tissue content and specific activity of these compounds. Since metabolite formation lags behind neurotransmitter synthesis, care should be taken that the data points used to determine the specific-activity regression lines for the metabolites are only those occurring after the attainment of peak-specific activity, that is, are on the declining portion of the specific-activity curve.

The described procedure has merit in that it permits the accurate and precise determination in discrete rat brain regions of the content and specific activity of monoamine-related substances from precursor amino acids through metabolites. Furthermore, the inclusion of the amino acid specific activities in the calculation of the neurotransmitter turnover rates produces a more accurate biochemical measure. Several studies have demonstrated the effects of brain TYR and TRP availability on the synthesis of their respective neurotransmitters [69–71]. The present technique combines ease of sample preparation, internal standardization, and sensitivity to fulfill the need for simultaneous determination of monoamine turnover for correlation with behavioral observations and for the study of transmitter interactions.

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