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AUTOMATED METHOD TO ESTIMATE CATECHOLAMINE AND INDOLEAMINE CONTENT AND TURNOVER RATES

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SUMMARY

A double-label isotopic method for estimation of the rate of formation of serotonin (5-HT) and dopamine (DA) in mouse striatum, hippocampus and cortex was standardized. Mice received an intravenous pulse injection of [^{3}H]tryptophan (TRP) and [^{3}H]tyrosine (TYR) at 2.5, 5, 10 or 20 min before sacrifice by microwave irradiation. Compounds of interest were separated by automated high-performance liquid chromatography and their contents were determined by electrochemical detection. Programmed collection of the TYR, DA, 5-HT and TRP peaks allowed determination of their radioactivity by liquid scintillation. Conversion of [^{3}H]TYR to [^{3}H]DA was nearly ten times greater in striatum than cortex, whereas the formation of [^{3}H]5-HT from [^{3}H]TRP was similar in striatum, cortex and hippocampus.

INTRODUCTION

Since brain monoamine concentrations are maintained during even rapid neuronal changes, sensitive assessment of monoamine metabolism requires some measure of synthesis. Accumulation of monoamine metabolites [1–3] and ratios of metabolite to precursor concentrations [4—7] provide some indices of rates of formation. Isotopic procedures that utilize incorporation of radiolabelled tyrosine (TYR) or tryptophan (TRP) into dopamine (DA) or serotonin (5-HT), respectively, may be more sensitive to various experimental and pharmacological manipulations [8]. However, these precursors are often infused over extended periods [9–11], which complicates experimental interpretations. The prolonged restraint that is required for isotopic infusion causes stress [12] which may alter brain metabolism and interfere with any treatment

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effects. Thus, in the present studies isotopes were administered in a pulse injection.

Simultaneous determination of neurotransmitter content and turnover by collection of peaks after separation by high-performance liquid chromatography (HPLC) and electrochemical detection has been reported [13—15]. The present method differs from these approaches in many important aspects. The times between isotope injection and animal sacrifice are minimized, which allows assessment of turnover during brief experimental and pharmacological treatments. In the present paper, turnover was measured during the linear increase in the conversion of [^{3}H]TYR and [^{3}H]TRP into [^{3}H]DA and [^{3}H]5-HT, respectively, rather than from the decline of specific activity of the products at 60 and 90 min after injection [13]. In the present study, large brain samples were used to obtain dpm values that are substantially above background, and longer analysis times minimized cross-contamination of peak radioactivity. Documentation of peak purity by injection and collection of ^{14}C-labeled standards assured the purity of the samples that were utilized for radioactivity measurements. Automatic injection of samples, as well as programmed collection of peak eluents, increased sample-to-sample reproducibility and decreased staff time.

EXPERIMENTAL

Animal treatment and tissue extraction

Tritium-labeled L-[ring-2,6-^{3}H]TYR (38.1 Ci/mmol) and L-[5-^{3}H]TRP (28.7 Ci/mmol; New England Nuclear, Boston, MA, U.S.A.) were dried under nitrogen in separate vials and reconstituted in saline (200 μCi per 150 μl) on the day before the experiment. In order to verify that the amount of isotope was constant from day to day, the radioactivity in each of these solutions was determined. A 10-μl aliquot of each was added to 20 ml of mobile phase and 10 μl of each diluted solution were counted. The two isotopes were then combined to give a final concentration of 100 μCi of each isotope in 150 μl saline. Male CD-1 mice (30—35 days old, 23—25 g) that were obtained from Charles River Breeding Labs. (Stone Ridge, NY, U.S.A.) were fasted overnight with free access to water. On the morning of the experiment, animals were placed in fenestrated plexiglass holders and the tail vein was quickly cannulated with a butterfly infusion set (27 gauge; Abbott Hospital, Chicago, IL, U.S.A.) connected to a stainless-steel Y by polyethylene tubing (PE-50, Clay Adams, NJ, U.S.A.). Once blood flowed back into the needle, a saline flush assured that the vein was properly cannulated. The combined isotope solution (150 μl per mouse) was administered with a 250-μl Hamilton syringe from one arm of the stainless-steel Y, and this was immediately followed with 0.1 ml saline from the other side of the Y. Animals were sacrificed by head-focussed microwave irradiation (2.2 kW, 0.35 s; Gerling Moore) at 2.5, 5, 10 or 20 min after injection. Brains were quickly removed and the striatum, hippocampus and cortex were free-hand dissected and weighed. Microwave fixation allows the three brain regions to be easily separated during the dissection. For recovery of radioisotopic compounds individual solutions of [^{14}C]TYR (56 mCi/mmol), [^{14}C]DA (56 mCi/mmol), [^{14}C]TRP (54 mCi/mmol) and [^{14}C]5-HT (55

mCi/mmol; Amersham, IL, U.S.A.) were prepared in 1 *M* formic acid. Individual aliquots (5 μl) of each ^{14}C-radioactive compound were pipetted into separate scintillation vials and used as a reference for radioactive recoveries. These solutions were mixed so that a 20-μl aliquot of the combination contained the following: [^{14}C]TYR (10 000 dpm), [^{14}C]TRP (10 000 dpm), [^{14}C]DA (2500 dpm) and [^{14}C]5-HT (500 dpm).

Although some procedures allow direct injection of an acid extract onto the HPLC column [15, 16], the large sample in the present method required pre-purification. The striatum (about 30 mg) and hippocampus (about 25 mg) were homogenized using a Teflon pestle with a polypropylene tube that contained 0.7 ml of ice cold 1 *M* formic acid—acetone (15:85) with 3,4-dihydroxybenzylamine (DHBA) (final concentration of 218 pmol/ml) as the internal standard. The cortex (about 120 mg) was homogenized in 1.2 ml of formic acid—acetone with the same concentration of DHBA. For each region, the homogenate was centrifuged (15 000 *g* at 0°C for 10 min) and 275 μl of the supernatant were added to 5-ml conical tubes with the ^{14}C-radioactive standards.

Three volumes (825 μl) of cold heptane—chloroform (8:1) were added to the supernatant and the contents were mixed and centrifuged. The organic layer was aspirated and the aqueous layer was dried with nitrogen and stored at −20°C until assay. The dried extract was reconstituted in 110 μl of mobile phase, vortexed for 60 s and 100 μl were analyzed.

Chromatogaphy and HPLC quantitation

Mobile phase was prepared by initially making 900 ml of 0.066 *M* chloroacetic acid, 0.046 *M* dibasic sodium phosphate, 0.06 m*M* Na_2 · EDTA, 1.66 m*M* octanesulfonic acid sodium salt monohydrate. Then 75—125 ml of methanol were added to this 900 ml and the pH was adjusted to 3.2 with 1 *M* hydrochloric acid. The varying amounts of methanol helped to compensate for decreased retention times due to column aging. Progressive batches of mobile phase were prepared with less methanol. The mobile phase was filtered through a 0.22-μm membrane filter and further degassed by sonication. The flow-rate for new columns was set at 1.2 ml/min. As the column deteriorated and peaks began to fuse, flow-rate was decreased by as much as 33% to maintain good resolution.

The stationary phase was a μBondapak (10 μm particle size) 30 cm × 3.9 mm I.D. C_{18} reversed-phase column and a radial compression separation system (RCSS) guard-pak disposable C_{18} precolumn insert (Millipore, Waters, Milford, MA, U.S.A.). Column temperature was maintained at 27°C with a temperature control unit, Model III (Waters).

The detector was an LC-4B electronic controller (Bioanalytical Systems, West Lafayette, IN, U.S.A.) and LC-17 flow cell with a TL-5 glassy carbon working electrode. The applied potential was +0.875 V versus an Ag/AgCl reference electrode. The sensitivity was 20 nA/V.

Samples were injected onto the column with a Model 710B WISP autosample injection module and the pump was a 6000A solvent delivery system (Waters).

Quantitation was based on peak areas and external calibration. The exact concentration of the reference compounds differed for the three brain areas

TABLE I

CONCENTRATIONS OF REFERENCE COMPOUNDS FOR EACH BRAIN REGION

Different concentrations of reference compounds (100 μl) that were optimized for each brain region were injected onto the HPLC column. The values that were used to calculate the concentration in the initial tissue extract were 0.4 times these, since 275 μl of the regional brain samples were dried and reconstituted in 110 μl of mobile phase before injection onto the HPLC column.

Compound	Concentration (pmol/ml)		
	Striatum	Hippocampus	Cortex
NE	352	422	422
TYR	6400	6400	12800
DA	4262	128	512
TRP	2000	1920	3200
5-HT	355	425	922
HIAA	325	195	390
DOPAC	445	178	298
HVA	685	205	412
DHBA	545	545	545

and approximated the levels of neurotransmitters and metabolites in each brain region (Table I). Automatic recalibration of the instrument with the mixture of standards after every four samples compensated for any loss of detector sensitivity to the compounds of interest. Data reduction were performed with a Model 730 data module and a Model 721 programmable system controller (Waters). The data module automatically integrated the chromatographic peaks. The amount of each component was calculated and corrected for recovery of DHBA. The amounts of the ^{14}C-radioactive tracers added to each brain tissue supernatant were converted to pmol/ml values and subtracted from the concentrations of the respective neurotransmitter.

Fraction collection and liquid scintillation counting

The radioactivity in the TYR, DA, 5-HT and TRP peaks was determined after collection of each peak in mini glass scintillation vials with a Gilson Model 201 fraction collector. The fraction collection program was determined from the retention times of standards. The dead-space volume of the tubing from the flow cell to the fraction collector was determined and converted to a delay time by dividing by flow-rate (ml/min). This delay time was added to the number of minutes before the collection of the first peak and the timing of the collection of the remaining peaks was shifted accordingly. The fraction collection program was readjusted after every fourth sample if the change in the retention times of the compounds of interest exceeded 5%. The volumes of the collected peaks were brought to 3.5 ml by addition of mobile phase. Samples were then mixed with 3.5 ml of Aquasol 2 counting cocktail (New England Nuclear). This combination of scintillation cocktail and mobile phase produced a stable counting mixture at temperatures between 0 and 30°C. Samples were counted in a Beckman LS 9000 liquid scintillation system with a

dual-label external standard quench correction program. Tritium dpm values were corrected for radioactive recovery by dividing the ^{3}H dpm of the compound of interest by the percentage of ^{14}C recovered for that same compound.

The estimated conversion rate (ECR) of the precursor amino acids, TYR and TRP, into DA and 5-HT, respectively, was calculated as follows:

$$ECR = \frac{\text{dpm/mg of tissue in product (DA or 5-HT)}}{\text{precursor specific activity (dpm/pmol) (TYR or TRP)}} \text{/time (min).}$$

RESULTS AND DISCUSSION

The elution profiles of standards (Fig. 1A) or striatal samples (Fig. 1B) show clear resolution of all but the norepinephrine (NE) peak. Although NE is clearly separated in the standard mixture, the peak is not adequately resolved in brain samples because of its close proximity to the much longer solvent front. The isolation of NE by alumina [17] successfully resolved the peak, but incorporation of radioactivity was very low and was not analyzed in subsequent experiments.

Radioactive cross-contamination of various peaks by other standards was assessed (Table II). The ^{14}C-standards that were used to estimate recovery were added individually to the tissue and collections were made before and after the TYR, DA, 5-HT and TRP peaks to determine the extent of radioactive overlap. Contamination of the DA peak by radioactive TRP was evident and prompted a more selective approach to the collection of the DA peak to obtain a purer DA sample (Table III). Six equal segments of the DA peak were collected. Since segments 2—4 did not have [^{14}C]TRP contamination, they were collected routinely. Even with the restriction on the collection of the DA peak, approximately 90% of the [^{14}C]DA recovered was found in segments 2—4 (dpm of segments 2—4 divided by dpm of segments 1—6). Because of the DA results, TYR, TRP and 5-HT peaks were collected in a similar manner to obtain the purest possible samples.

Once the methods for separation and collection of compounds were standardized, the in vivo conversions of TYR into DA and TRP into 5-HT were determined. DA specific activity (dpm/pmol) differed in the three brain regions (Fig. 2A). In the striatum, the increase in DA specific activity was linear for 20 min, whereas cortical specific activity displayed a steeper increase up to the 10-min point. Present methods were not sensitive enough to detect hippocampal DA concentrations. The decline of TYR specific activity from 2.5 to 20 min was similar in all three regions (Fig. 2B). Striatal 5-HT specific activity increased in a linear fashion throughout the time course, whereas that in cortex and hippocampus were only linear for 5 min (Fig. 2C). Steeper decreases in TRP specific activity were evident in cortex and hippocampus than in striatum (Fig. 2D).

For the most part, the concentrations of the amino acid precursors, monoamines and metabolites showed little change at various times after isotope administration (Table IV). Concentrations of all compounds were generally highest in the striatum.

The monoamine precursors dpm/mg values declined with time (Table V).

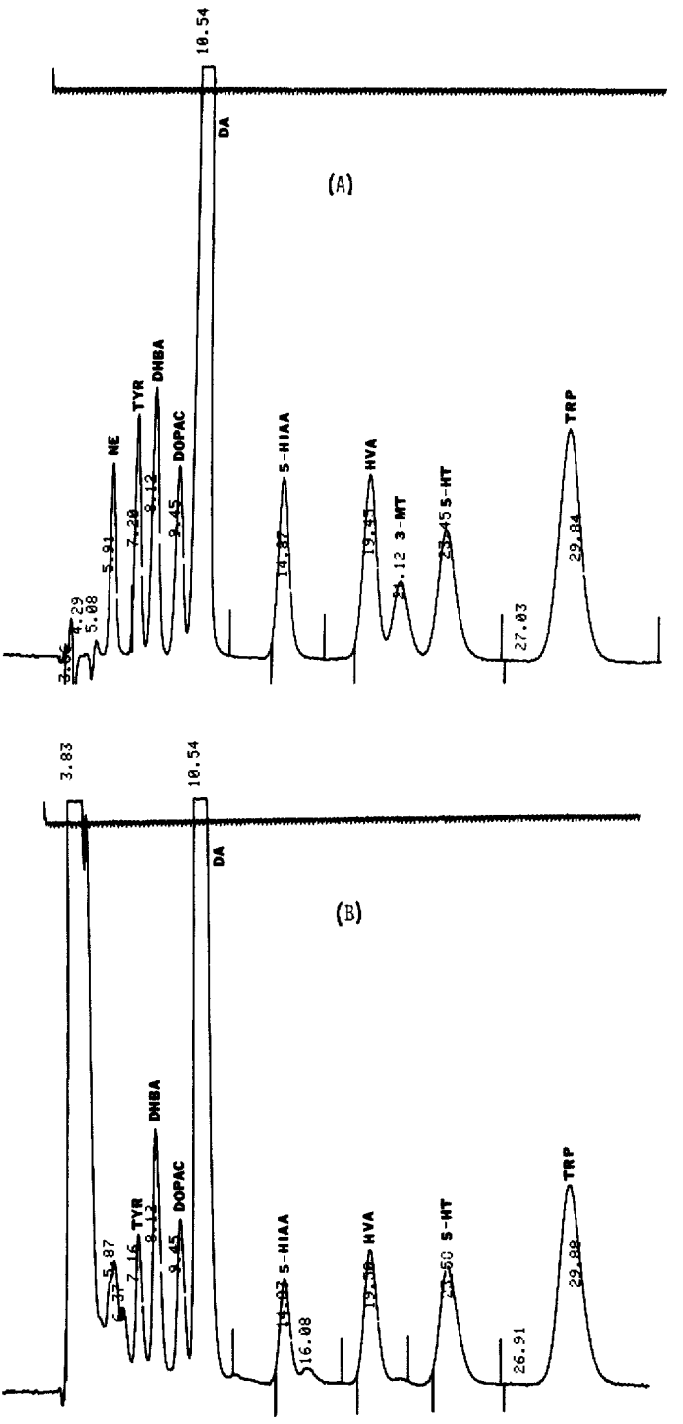

Fig. 1. The elution profile of (A) a mixture of standards for a striatal assay at the concentrations specified in Table I and (B) striatal brain tissue. Chromatographic conditions are as described in Experimental. The retention time for each compound is printed on the chromatogram.

TABLE II

EXAMINATION OF THE CROSS-CONTAMINATION OF PEAKS BY ADDITION OF RADIOACTIVE STANDARDS TO STRIATAL TISSUE

The indicated amount of radioactivity was added to the acid extract from a striatum (approximately 30 mg). Pre- and post-peak samples were collected for approximately 2 min. [^{3}H]TRP contaminated the DA peak similarly (results not shown). Each value represents the mean of a triplicate. Values less than 25 dpm are considered background and S.E.M. are not shown.

	[^{14}C]TRP	[^{14}C]TYR	[^{14}C]DA	[^{14}C]5-HT
Total dpm added	8302	10000	2098	460
Pre-TYR	129 ± 13	95 ± 26	18	17
TYR	110 ± 21	7363 ± 231	6	14
Post-TYR	104 ± 2	121 ± 57	56	10
Pre-DA	32 ± 5	5	20	10
DA	39 ± 3	1	1376 ± 100	8
Post-DA	231 ± 16	3	222 ± 25	12
Pre-5-HT	18	11	10	22
5-HT	5	11	12	275 ± 59
Post-5-HT	12	7	8	12
Pre-TRP	73 ± 41	6	6	7
TRP	5957 ± 461	9	9	19
Post-TRP	43 ± 9	6	5	4
Percentage recovery in primary peak	72	74	66	60

TABLE III

PURIFICATION BY PEAK SUBDIVISION OF THE DOPAMINE THAT WAS COLLECTED

Values (dpm per fraction) were obtained by addition of trace amounts of [^{14}C]DA (2098 dpm) or [^{14}C]TRP (8302 dpm) to striatal samples. The DA peak was divided into six 0.3-min segments. The post-DA collection included four 0.3-min segments. Each value represents the mean of two samples.

Name	Segment	[^{14}C]DA	[^{14}C]TRP
DA	1	65	11
DA	2	543	8
DA	3	584	5
DA	4	217	3
DA	5	39	5
DA	6	14	16
Post-DA	1	36	37
Post-DA	2	88	73
Post-DA	3	74	78
Post-DA	4	39	183

After 20 min, the TYR dpm/mg values were approximately 47 and 41% of the 2.5-min value in the striatum and cortex, respectively. For TRP, the dpm/mg values decreased to approximately 32, 24 and 30% of the 2.5-min value in the striatum, cortex and hippocampus, respectively. The DA dpm/mg value in the striatum greatly exceeded cortical values and increased three-fold from

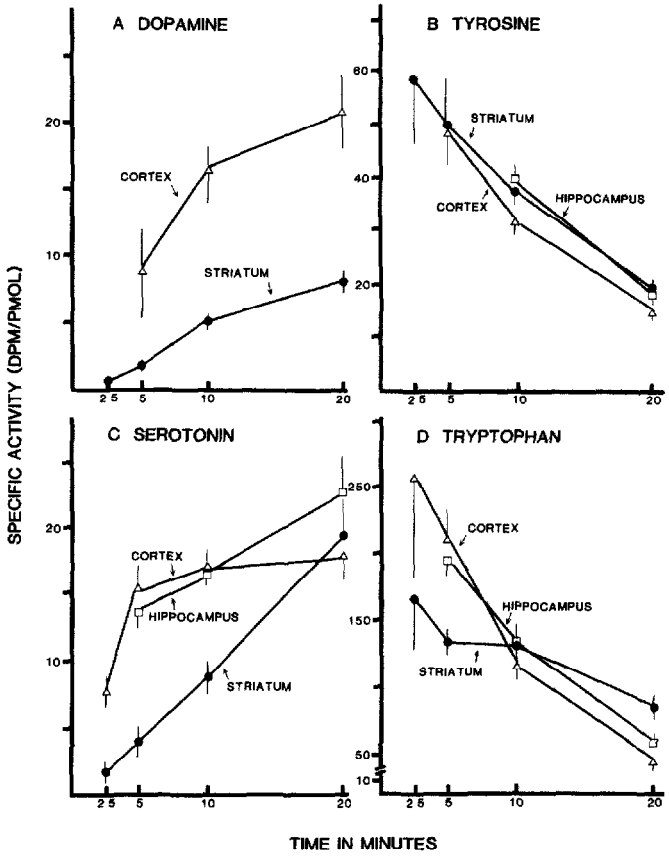

Fig. 2. Specific activities (dpm/pmol) of (A) [^{3}H]DA, (B) [^{3}H]TYR, (C) [^{3}H]5-HT and (D) [^{3}H]TRP in mouse brain regions at various times after isotope administration. Mice that had been starved overnight were injected with a combined isotope solution of [^{3}H]TRP (28.7 Ci/mmol, 100 μCi per mouse) and [^{3}H]TYR (38.1 Ci/mmol, 100 μCi per mouse) in 150 μl saline and sacrificed by microwave irradiation at 2.5, 5, 10 or 20 min. Values represent the mean ± S.E.M. of four to eight mice at each time point.

2.5 to 5.0 min and two-fold between 5.0 and 10.0 min. In the cortex, the dpm/mg value for DA increased only 1.5 times between 5.0 and 10.0 min. For 5-HT, the increase in the dpm/mg value was linear in the striatum. In the cortex and hippocampus, 5-HT dpm/mg values were higher at 2.5 min and in the cortex values did not differ significantly after 5.0 min.

The ECR varied with neurotransmitter and region. The ECR of TYR to DA in the striatum was ten times that measured in the cortex. The present approach assumes that there is uniform mixing of radiolabeled TYR and TRP into the precursor pool for the respective monoamines so that the measured dpm/pmol value is the specific activity of the precursor pool. The change in the ECR in the striatum with time demonstrates one of the limitations of this measurement as an estimate of turnover. Ideally, the ECR should be based on the integral of the decline of precursor specific acitivity with time. The incorporation of [^{3}H]TRP to [^{3}H]5-HT was greater in cortex than hippocampus or striatum. ECR values (TRP → 5-HT) remained constant across time in all regions.

TABLE IV

PRECURSOR REGIONAL AMINO ACID, MONOAMINE AND METABOLITE CONCENTRATIONS AT VARIOUS TIMES AFTER ISOTOPE ADMINISTRATION

Results are means ± S.E.M. of the number of animals in parentheses: striatum, 2.5 min (7); 5.0 min (8); 10.0 min (12); 20.0 min (5); cortex, 2.5 min (3); 5.0 min (8); 10.0 min (12); 20.0 min (4); hippocampus, 5.0 min (4); 10.0 min (8); 20.0 min (5).

Compound	Time (min)	Concentration (pmol/mg of brain)		
		Cortex	Striatum	Hippocampus
TYR	2.5	36.6 ± 8.6	38.3 ± 9.2	
	5.0	31.6 ± 1.9	34.0 ± 3.1	30.8 ± 3.2
	10.0	35.4 ± 3.6	34.6 ± 3.7	32.9 ± 4.2
	20.0	38.5 ± 4.4	37.6 ± 2.8	34.5 ± 2.8
DA	2.5	1.96 ± 0.26	46.1 ± 3.0	
	5.0	1.10 ± 0.09	46.8 ± 1.8	
	10.0	1.08 ± 0.13	41.5 ± 1.9	
	20.0	0.84 ± 0.09	37.4 ± 2.5	
TRP	2.5	9.28 ± 2.80	11.7 ± 1.7	
	5.0	9.52 ± 0.74	13.0 ± 1.1	8.48 ± 1.20
	10.0	11.30 ± 0.87	11.7 ± 1.5	8.67 ± 1.00
	20.0	10.70 ± 0.88	7.1 ± 0.6	8.45 ± 0.45
5-HT	2.5	2.42 ± 0.19	2.75 ± 0.27	
	5.0	2.20 ± 0.07	2.99 ± 0.25	2.07 ± 0.14
	10.0	2.33 ± 0.11	2.81 ± 0.18	1.95 ± 0.10
	20.0	2.10 ± 0.17	2.24 ± 0.21	
HIAA	2.5	0.76 ± 0.08	1.82 ± 0.15	
	5.0	0.68 ± 0.04	1.90 ± 0.11	1.15 ± 0.05
	10.0	0.82 ± 0.04	1.92 ± 0.12	1.32 ± 0.07
	20.0	0.74 ± 0.05	1.42 ± 0.11	1.33 ± 0.05
DOPAC	2.5	0.51 ± 0.05	3.60 ± 0.67	
	5.0	0.51 ± 0.03	3.59 ± 0.30	
	10.0	0.49 ± 0.03	3.47 ± 0.17	
	20.0	0.44 ± 0.07	3.79 ± 0.23	

Most available analytical methods that employ HPLC with electrochemical detection use minute sample amounts which require high instrument sensitivity. In order to obtain dpm values that were consistently above background, especially in the case of 5-HT, the present procedure required increased sample size and decreased detector sensitivity. This explains why DA could not be detected in the hippocampus. Although direct injection of the supernatant was not used in the current method, the sample clean-up was not extensive and helped to prolong the life of the column.

Estimation of turnover in the mouse offers two advantages over larger animals. The amount of isotope that is required for each study is considerably less. Focussed microwave irradiation inactivates enzymes more rapidly in mice

TABLE V

TIME COURSE OF RADIOACTIVE CHANGES AND ESTIMATED CONVERSION RATES

Values are means ± S.E.M. of the number of animals in parentheses.

Region	Time* (min)	dpm/mg				Estimated conversion rate × 100	
		Tyrosine	Dopamine	Tryptophan	Serotonin	TYR → DA	TRP → 5-HT
Striatum	2.5	1609 ± 142 (4)	32.9 ± 9.0 (4)	1850 ± 317 (3)	5.7 ± 3.3 (3)	32.9 ± 9.8 (4)	2.2 ± 1.3 (2)
	5.0	1431 ± 50 (4)	91.9 ± 20.9 (4)	1728 ± 196 (4)	11.5 ± 3.0 (4)	46.4 ± 8.9 (4)	2.0 ± 0.5 (4)
	10.0	1228 ± 108 (9)	206.6 ± 10.4 (9)	1192 ± 74 (8)	23.2 ± 2.7 (8)	68.2 ± 3.8 (9)	2.3 ± 0.3 (8)
	20.0	760 ± 92 (5)	304.1 ± 25.6 (5)	587 ± 46 (5)	42.7 ± 7.2 (5)	93.7 ± 9.7 (5)	3.0 ± 0.4 (5)
Cortex	2.5			1966 ± 234 (3)	18.2 ± 2.8 (3)		4.1 ± 1.3 (3)
	5.0	1432 ± 29 (4)	8.1 ± 3.0 (4)	1961 ± 257 (4)	32.6 ± 3.3 (4)	4.4 ± 1.8 (4)	3.8 ± 0.4 (4)
	10.0	1066 ± 94 (8)	13.2 ± 0.8 (8)	1250 ± 113 (8)	39.8 ± 2.8 (8)	5.0 ± 3.1 (8)	4.4 ± 0.4 (8)
	20.0	580 ± 87 (4)	17.1 ± 1.5 (4)	476 ± 27 (4)	37.0 ± 2.3 (4)	7.1 ± 1.0 (4)	5.0 ± 0.3 (4)
Hippocampus	5.0			1628 ± 159 (4)	29.0 ± 4.3 (4)		3.7 ± 0.8 (4)
	10.0	1177 ± 76 (5)		1115 ± 63 (8)	33.4 ± 3.4 (8)		3.2 ± 0.5 (8)
	20.0	662 ± 84 (5)		482 ± 14 (5)	43.2 ± 4.8 (5)		4.5 ± 0.3 (5)

*Times are minutes after isotope administration.

Within- or between-experiment reliability was maintained by automated injection of samples and programmed collection of peak eluents. In addition to controlling for detector cell sensitivity loss, automatic recalibration of the data module with reference compounds made it possible to monitor changes in retention times of the compounds of interest. Accuracy of peak collection could then be further optimized by reprogramming of the fraction collector. The use of a fraction collector allowed collection and counting by liquid scintillation for prolonged periods. For the level of radioactivity, a flow cell would not have allowed an adequate number of dpm to be counted.

The radioactive pulse label method described in this report involved minimal stress to the animal and allowed for efficient use of time and mice due to the simultaneous administration of both radioactive precursors in a single injection. It should provide the opportunity to examine a variety of treatment conditions in mice that produce subtle alterations of brain function that may remain undetected by examination of endogenous levels or comparisons of the ratio of precursors and products.

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