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Simultaneous Assay for L-Tryptophan, Serotonin, 5-Hydroxyindoleacetic Acid, Norepinephrine and Dopamine in Brain

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MARINI, J. L., S. P. WILLIAMS AND M. H. SHEARD. *Simultaneous assay for L-tryptophan, serotonin, 5-hydroxyindoleacetic acid, norepinephrine and dopamine in brain*. PHARMAC. BIOCHEM. BEHAV. 11(2) 183–187, 1979.—A routine simultaneous assay for the title compounds is described, which uses a cation-exchange resin for separations, and standard fluorometric methods for analyses. Practicability of the ion-exchange chromatography is enhanced by means of a novel apparatus, and the procedure has the flexibility to permit extension to other endogenous compounds using published techniques.

Brain Fluorometric assay Ion-exchange resin 5-Hydroxyindoleacetic acid Dopamine Norepinephrine Serotonin L-Tryptophan

SIMULTANEOUS (multiple) assays are useful in neuropharmacology when data are required on levels of several compounds in the same tissue. Such assay procedures typically extract acidic or basic substances from aqueous solutions of appropriate pH by means of organic solvents. These extraction methods are useful for isolation of the bases serotonin (5HT), dopamine (DA) and norepinephrine (NE) [3, 9, 12], or acids and bases, such as 5-hydroxyindoleacetic acid (5HIAA) and 5HT [7]. However, they generally cannot quantitatively isolate amphoteric substances such as amino acids, and cannot be used to determine the specific activity of chemically similar radiolabeled substances, for example, after *in vivo* administration of labeled tyrosine (Tyr) to form labeled DA and NE.

Ion-exchange resin methods, on the other hand, have sufficient specificity to isolate amphoteric as well as acidic and basic substances, and have been used successfully to separate labeled NE and DA from each other and labeled Tyr precursor [4]. Resin methods have the disadvantages of relatively elaborate apparatus and time-consuming chromatography procedures [2]. With either solvent extraction or resin procedures, the preliminary isolation of compounds is followed by manipulations for concentration and/or assay.

In this paper we report a simultaneous assay which interfaces methods from published Dowex 50W cation-exchange resin procedures for isolation of compounds [1, 2, 4], and employs standard fluorometric methods for their analysis. Costa *et al.* [4] used Dowex to separate tryptophan (Trp), Tyr, NE, DA and 5HT, but gave few details about their techniques. Atack and coworkers [1,2] have exhaustively studied the use of Dowex for separation of indoles and catecholamines, including 5-hydroxytryptophan and dopa, but employ a complex and time-consuming chromatography system [2]. Our modification uses a novel chromatography apparatus which is convenient, requires less than 1 m^2 of bench space, and is relatively rapid. For 30 tissue samples, one working day is required for sample preparation, elution of fractions, assays for 5HIAA and 5HT, and column cleaning. Fractions containing DA plus NE, or Trp, can be stored for later assay. Our procedure enhances the practicability of ion-exchange chromatography without sacrificing its flexibility for use with large numbers of endogenous compounds [1, 2, 4].

METHOD

Reagents

Distilled, deionized water is used in all cases. Chemicals are reagent grade whenever available. Two N ethanolic HCl (resin washing): 172 ml conc. HCl made up to 1 L with 95% ethanol. Homogenization reagent: 0.4 N $HClO_4$ plus 1 mg/ml sodium metabisulfite ($Na_2S_2O_5$), prepared on day of homogenization and kept at 0°. Sixty percent (v/v) methanol-water is prepared on day of separation. Five tenths (0.5) M Na_3PO_4 is prepared on day of separation from 19.0 g Na_3PO_4 plus 100 ml water. Calibration standards are prepared as concentrated stock solutions (expressed as free acid or base) which can be stored at 8° for up to 3 months: 5HIAA, 120 μg/ml water; Trp, 51 μg/ml 0.001 N NaOH; 5HT, 546 μg/ml water; NE, 1 mg/ml 0.01 N HCl; DA, 1 mg/ml 0.01 N HCl. Borate buffer: 15.5 g boric acid and 200 g NaCl are made up to 450 ml with water, pH is adjusted to 11.2–11.4 with 10 N NaOH, and the volume made up to 500 ml. Sufficient additional

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NaCl is added to saturate and pH is readjusted. Other reagents are described in the references cited below, or are prepared by standard methods.

Animals and Procedures

Male albino Sprague-Dawley rats, 250–300 g, were housed in an air-conditioned room with 12 hr light/dark cycle. They were given free access to Purina Lab Chow and water. Animals were sacrificed by decapitation, brains were removed, and either divided mid-sagitally (Experiments 1 and 2, one brain half, ca. 0.95 g/column, used for assay), or into forebrain and hindbrain (Experiment 3, forebrain, ca. 1.15 g/column; hindbrain, ca. 0.51 g/column). In Experiments 1 and 3, animals were untreated. In Experiment 2, groups of 6 rats received either 5 ml/kg physiological saline or 0.4 M LiCl, IP, 2× daily for 4 days, with injections separated by 6.0 hr. On Day 5, animals received the first injection, and were sacrificed 5.0 hr later.

Resin Preparation

Dowex 50W-X4, 200–400 (H^+-ion form) must be thoroughly washed before use, but requires only routine washes for 1–2 yr thereafter. Excessive mechanical agitation should be avoided in order to minimize formation of fines, which should be removed after each preliminary washing step. With stirring in all steps, wash 70 g (wet weight) of resin 4× with 250 ml water (15 min/wash), 4× with 250 ml 2 N NaOH (1 hr/wash), followed by 5× with 250 ml water, allowing the resin to remain overnight in the last wash. Wash once with 250 ml water (pH of supernatant ca. 9), and once with 170 ml 2 N HCl in ethanol (4 hr). Rinse 4× with 250 ml water, allowing the resin to remain in the last rinse for 24 hr (supernatant pH ca. 3). With stirring, add NaOH to give a stable supernatant pH of 8–9, and decant from resin. Wash 2× with 250 ml 60% (v/v) methanol-water, standing 1 hr in the first wash, and overnight in the second. Wash 4× with 250 ml water and 4× with 0.1 M sodium phosphate buffer, pH 6.5. The washed resin is stored refrigerated (8°) under sodium phosphate buffer.

Column Preparation and Packing

Chromatography columns (Fig. 1) are made from Pyrex No. 5 0-ring joints (Corning 6780) by sealing sintered glass filter frits (fine porosity, 3 mm in length) into the stems; frits serve both to hold the resin and control flow rate. Untreated frits require high pressures for elution and are unsatisfactory. To obtain appropriate flow rates, the frits are treated by slowly drawing 1 ml aliquots of 5% (w/v) HF through the columns by means of a vacuum. After HF treatment, resin-packed columns should give flow rates of ca. 0.25 ml/min at 2–2½ psig with sodium phosphate buffer.

The columns are packed to a height of 36 mm with resin in the H^+-ion form, using a slurry in 2 N HCl. The height of the resin bed (H^+-ion form) should be maintained at 36 ± 2 mm for the life of the column. After packing, resin is converted to the Na^+-ion form with 10 ml of sodium phosphate buffer, and stored at 8° in the column under buffer. The height of the resin bed varies with pH or other ions, and changes during elutions are to be expected. Dowex 50W-X4, 200–400 mesh

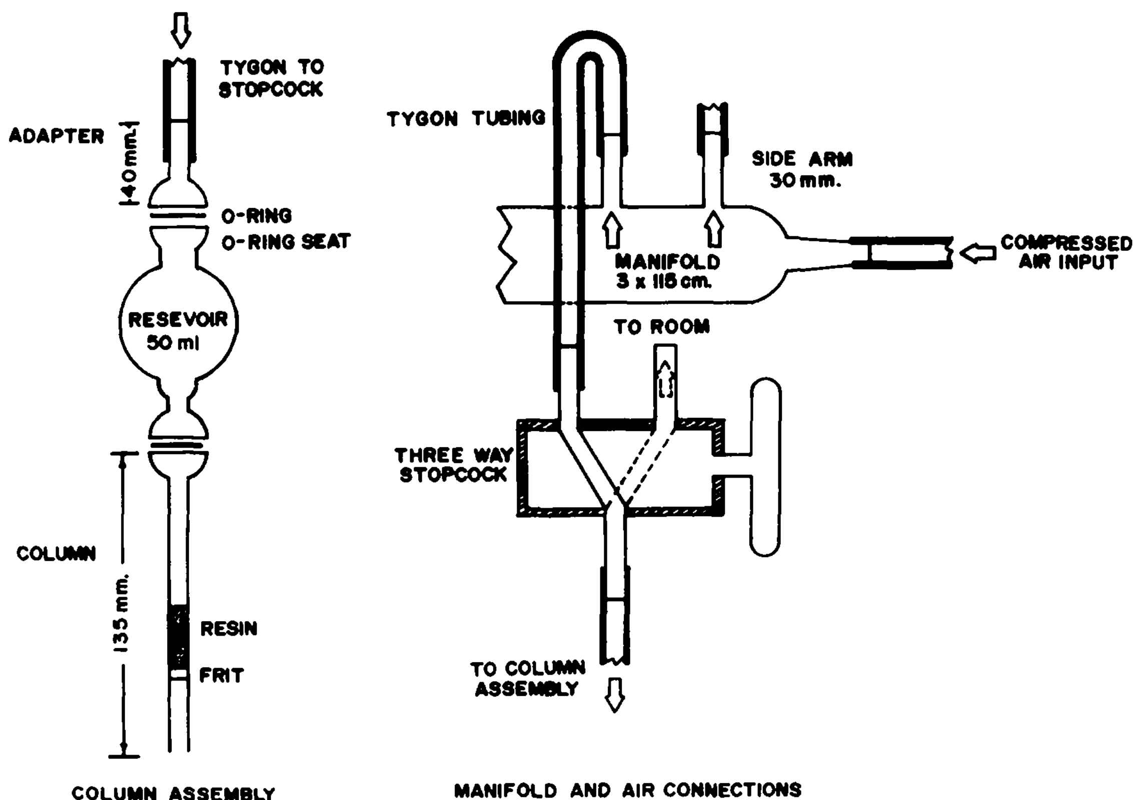

FIG. 1. Diagram of chromatography apparatus components.

Eluant Inputs	Effluent Outputs
1. Supernatant, pH 2–2.5.	1. Supernatant. Discard.
2. 7 ml 60% methanol-H_2O.	2. 5HIAA fraction for assay.
3. 2 ml sodium phosphate buffer, pH 6.5	3. Buffer wash. Discard.
4. 7 ml sodium phosphate buffer, pH 6.5.	4. Trp fraction for assay.
5. 2 ml 1 N HCl.	5. HCl wash. Discard.
6. 6 ml 4 N HCl + 1 mg/ml $Na_2S_2O_5$.	6. NE + DA fraction for assay.
7. 2 ml H_2O.	7. H_2O wash. Discard.
8. 6 ml 0.5 M Na_3PO_4.	8. 5HT fraction for assay.
9.–11. 5 ml washes with H_2O, 4N HCl, sodium phosphate buffer	9.–11. Discard washes.

FIG. 2. Flow chart for isolation of compounds from Dowex 50W-X4 resin.

size, becomes orange in strong base; the intensity of the color diminishes with use.

Prior to a separation, columns are washed with 5 ml of water, 60% methanol-water, water, 2 N NaOH, water, 4 N HCl, and sodium phosphate buffer. Immediately following a run, columns are washed with 5 ml of water, 4 N HCl, and sodium phosphate buffer.

Apparatus

The apparatus (Fig. 1) employs compressed air to pressurize a glass manifold. Manifold pressure is transmitted through Tygon tubing to individual stopcocks and then to the column assemblies, 15 to each side of the manifold. The apparatus is mounted on a 60×120 cm table, and housed in a Plexiglas (9 mm) framework, 18×120 cm × 75 cm high. Eluants are added to the resevoirs by removing the adapters. The stopcock connections are made so that one position connects the manifold to the column assembly, and the other connects the column assembly to ambient pressure. The manifold (3×115 cm Pyrex tubing) has a connection to the compressed air source, and 2 adjacent rows of 15 side arms (8×30 mm Pyrex), spaced 8 cm apart. Compressed air from a regulated source is fed to the manifold through an air filter (Turbo Flo Filter, Model M, Watts Regulator Co.), followed by a pressure gauge. Gauge (manifold) pressure can be regulated readily to within 0.5 psig by adjusting the bleed valve in the filter.

When the manifold is pressurized, "opening" the 3-way stopcocks drives eluant through the resin at a constant rate. "Closing" the stopcocks vents the column assemblies to ambient pressure. Since the columns incorporate sintered glass filters, they have a negligible flow rate at ambient pressure, and serve as passive valves for eluant flow. The compressed air input arrangement described assures that, when all columns are closed to the pressurized manifold, its pressure will not rise above the set pressure.

Tissue Preparation

Tissue is homogenized in 0.4 N $HClO_4$ plus $Na_2S_2O_5$ at 0°, using a glass and Teflon apparatus. Five ml of $HClO_4$ is used per g of tissue, with a minimum volume of 5 ml. After standing at 0° for 10 min, homogenate is centrifuged (14,000× g and 0° for 10 min), the supernatant is decanted into a beaker on ice, and its pH adjusted to 2–2.5 with 5 N KOH. After standing on ice 5 min to allow complete precipitation of $KClO_4$, the mixture is filtered with vacuum, and the filtrate is added to the solvent reservoir, as is a 1 ml water rinse of the receiver.

Elution

The top of the resin should remain wet at all times. If the resin becomes "dry," air channels may form which can reduce its efficiency. Resin containing air bubbles may be resuspended with 0.5–1.0 ml of the appropriate eluant and gentle stirring without affecting the results. Flow rates should be between 0.25–0.5 ml/min.

Supernatant is passed through the resin and discarded (Fig. 2). The tips of the columns are rinsed with water and blotted dry before collecting the next fraction; this routine should be used throughout. 5HIAA is eluted in 7.0 ml 60% methanol-water. Columns are rinsed with 2.0 ml sodium phosphate buffer (discard), and Trp eluted in a subsequent 7.0 ml. The columns are rinsed with 2.0 ml 1 N HCl (discard), and NE plus DA eluted in 6.0 ml 4 N HCl (1 mg $Na_2S_2O_5$/ml). (Alternatively, after collecting Trp, NE is eluted in 10.0 ml 1 N HCl with $Na_2S_2O_5$, and DA in a subsequent 4.0 ml 4 N HCl with $Na_2S_2O_5$.) After eluting the catecholamines, the columns are rinsed with 2.0 ml water (discard), and 5HT eluted in 6.0 ml 0.5 M Na_3PO_4.

Assays

The fluorometric assays are for the most part identical to published methods. *5HIAA* (1.0 ml aliquots of thoroughly-mixed methanol-water effluent) is assayed by the oxidized blank, native fluorometric method of Atack and Lindqvist [1]. The assay is usually performed on the day of separation, but samples are stable for at least 24 hr at 8°. Average recovery of 450 ng 5HIAA added to supernatant before adjusting pH is 55%. Standards are made up in 60% methanol-water and assayed as for column effluent. *Trp* (2.0 ml

TABLE 1

RESULTS OF THE SIMULTANEOUS ASSAY ON RAT BRAIN LEVELS OF 5-HYDROXYINDOLEACETIC ACID (5HIAA), L-TRYPTOPHAN (TRP), SEROTONIN (5HT), NOREPINEPHRINE (NE) AND DOPAMINE (DA). LEVELS ARE REPORTED IN ng/g ± S.E.M., AND ARE CORRECTED FOR RECOVERY

Substance	Exp. 1 Control rats, whole brain. N = 6 for all data	Exp. 2 Five-day LiCl vs. NaCl, whole brain. N = 6 for all data	
	Level	Level, NaCl	Level, LiCl¶
5HIAA	372 ± 2.38	394 ± 25.8	425 ± 20.8
TRP	4590 ± 175	5070 ± 301§	6150 ± 303§
5HT	425 ± 20.4	322 ± 13.3	323 ± 16.7
NE	364 ± 17.5	346 ± 15.5	373 ± 13.3
DA	727 ± 62.1	649 ± 18.0	708 ± 41.8

¶Serum Li (N = 6), 0.93 ± 0.098 mEq/L; brain Li (N = 6), 0.81 ± 0.041 mEq/kg.
§Independent t-test (2-tailed), $t = 2.77$, df = 10, $p<0.02$.

Substance	Exp. 3. Control rats, forebrain and hindbrain, N = 6 for all data except 5HT (N = 5)	
	Forebrain	Hindbrain
5HIAA	268 ± 19.2*	415 ± 18.7*
5HT	357 ± 14.0	360 ± 21.5
NE	421 ± 34.1†	300 ± 17.7†
DA	800 ± 104‡	96 ± 23‡

Paired t-tests (2-tailed, df = 5), * $t = 7.90$, $p<0.001$; † $t = 3.29$, $p<0.05$; ‡ $t = 7.56$, $p<0.001$.

aliquots of thoroughly-mixed buffer effluent) is assayed by published methods [4,5]. Trp in buffer effluent is stable for at least 48 hr at −9°. Average recovery of 1300 ng Trp is 90%. Standards in phosphate buffer are assayed as for samples of effluent. *5HT*: A 4.0 ml aliquot of the well-mixed Na_3PO_4 effluent is thoroughly mixed with 1.5 g NaCl, 2.0 ml borate buffer and 16 ml ethyl acetate. The sample is shaken 10 min, centrifuged, and 13 ml ethyl acetate transferred to a tube containing 1.5 ml 0.1 N HCl and 25 ml cyclohexane. After shaking 10 min and centrifuging, the organic phase is aspirated and a 1.0 ml aliquot of the 0.1 N HCl removed. After adding 0.05 ml 6% (w/v) L-cysteine hydrochloride and 0.30 ml conc. HCl, and mixing well, the aliquot is assayed by native fluorescence [4]. The assay is usually performed on the day of separation, but the 0.1 N HCl extract can be stored at least 24 hr at −9°. Average recovery of 200 ng 5HT is 55%. Standards are treated as for 0.1 N HCl extract. *NE and DA* are assayed after alumina chromatography on columns prepared with 1.0 g alumina on the day of assay [6]. A 3.0 ml aliquot of thoroughly-mixed 4 N HCl effluent is mixed with 0.5 ml 2 M Tris (pH 7.8) and 0.6 ml 10% (w/v) Na_2EDTA, and stored at 0° prior to adjusting pH. The cold aliquot is transferred to a vessel on ice which is fitted with magnetic stirrer and pH electrode, and pH is adjusted to 8.0 ± 0.1 with NaOH. The solution is immediately added to the alumina column, and the effluent discarded. The alumina is rinsed with 3× 25 ml water (discard), blown dry, and the catecholamines eluted with consecutive 1.5 ml aliquots of 0.2 N $HClO_4$ and water. The combined effluent is thoroughly mixed and stored at 4°. Two 1.0 ml aliquots are assayed as described by Shellenberger and Gordon [12]. The 4 N HCl effluent from the Dowex resin can be stored at least 72 hr at −9°. Average recoveries of 250 ng NE and DA are 60%. Standards are prepared in 0.05 N $HClO_4$ and assayed as for alumina column effluent.

RESULTS AND DISCUSSION

Thorough washing of the resin results in negligible "blank column errors" [1] for all assays described. This is most significant for the 5HIAA fraction, since resin-derived fluorescence is eluted by methanol-water.

With some practice it is easy to prepare columns which give satisfactory flow rates. Using 9–12 resin-packed columns, methanol-water gave flow rates of 0.2, 0.25 and 0.4 ml/min at 2, 3 and 4 psig, respectively, while 1 N HCl gave 0.25, 0.55 and 0.75 ml/min rates at the same pressures. Flow rates at ambient pressure were less than 0.05 ml/min. Flow rates used for assays were similar to those of Atack *et al.* [1,2].

Recovery of eluant added to the reservoirs is quantitative (N=14; volume added, 9.6 ± 0.05 ml; volume recovered, 9.6 ± 0.08 ml). Flow rates with sodium phosphate buffer at 2 psig are independent of eluant volume between 1 and 8 ml, but volumes greater than 10 ml give increasing flow rates due to the weight of the fluid. Flow rates for individual columns may be adjusted by incorporating a valve or clamp between the manifold and stopcock.

Minimum linear range for the assays (ng/column) are: 5HIAA, 100–480; Trp, 2550–5100; 5HT, 175–900; NE, 150–500; DA, 150–750. Within-assay reproducibility was

determined from triplicate assays of brain homogenate pool run with Experiment 2, Table 1; data are mean ng/column ± SEM, uncorrected for recovery or weight of tissue: 5HIAA, 85.5 ± 2.88; Trp, 4950 ± 40.8; NE, 144 ± 6.32; DA, 387 ± 29.6; 5HT, 141 ± 7.56.

The results of three independent assays are shown in Table 1. Brain tissue levels of the compounds, and the precision of the results, agree well with other methods. In Experiment 2, a 5-day lithium regimen resulted in significantly elevated Trp, and elevated 5HIAA, as has been reported in other studies using the same regimen [8, 10, 11, 13]. For Experiments 1 and 2, half brains, ca. 1.15 g tissue, were used per column. To determine the suitability of the assay for smaller tissue samples, Experiment 3 employed forebrain (1.15 g/column) and hindbrain (0.51 g/column), and assays were performed only for the substances present in lowest concentration. The results (Table 1) showed that the method works well with 500 mg of tissue, and revealed significant regional differences in 5HIAA, NE and DA. Smaller samples can be used, since the volumes of most eluant aliquots (Trp, NE, DA, 5HT) can be increased, and since more sensitive methods for indoles eluted from Dowex have been described [1]. We have found that overall recoveries of standards added to supernatant before adjusting pH (Trp, 90%; 60% for the others) agree well with reported values [1, 2, 4], and correspond to approximately 90% recovery of substances from the Dowex resin column.

EDTA is unnecessary in the homogenizing reagent, and blocks the Trp assay. The elution procedures we report are similar or identical to published methods [1, 2, 4], and we confirmed the profiles of elution volume vs. sample recovery using radiolabels. We elute 5HT with Na_3PO_4 [4] rather than ethanolic HCl [1,2] because the former is faster, and the latter reagent can affect Trp recovery under some conditions. It is unnecessary to collect the Na_3PO_4 effluent in organic solvent [4], since 30 or 60 min standing in 0.6 M Na_3PO_4 results in no loss of 5HT. Finally, ethyl acetate is superior to n-butanol for extracting 5HT [4], a single extraction giving a 75% recovery, as compared to 60% for butanol.

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REFERENCES

1. Atack, C. and M. Lindqvist. Conjoint native and orthophthaldialdehyde-condensate assays for the fluorometric determination of 5-hydroxyindoles in brain. *Naunyn-Schmiedeberg's Arch. Pharmac.* **279:** 267–284, 1973.
2. Atack, C. and T. Magnusson. A procedure for the isolation of noradrenaline (together with adrenaline), dopamine, 5-hydroxytryptamine and histamine from the same tissue sample using a single column of strongly acidic cation exchange resin. *Acta pharmac. tox.* **42:** 35–57, 1978.
3. Ciarlone, A. E. Modification of a spectrophotofluorometric method of analyzing serotonin, norepinephrine and dopamine in one brain sample. *Microchem. J.* **21:** 349–354, 1976.
4. Costa, E., P. F. Spano, A. Groppetti, S. Algeri and N. H. Neff. Simultaneous determination of tryptophan, tyrosine, catecholamines and serotonin specific activity in rat brain. *Atti. Accad. Med. Lombardo* **23:** 1100–1104, 1968.
5. Denckla, W. D. and H. K. Dewey. The determination of tryptophan in plasma, liver, and urine. *J. Lab. clin. Med.* **69:** 160–169, 1967.
6. Euler, U. S. von and F. Lishajko. The estimation of catecholamines in urine. *Acta physiol. scand.* **45:** 122–132, 1959.
7. Giacolone, E. and L. Valzelli. A spectrophotofluorometric method for the simultaneous determination of (serotonin) and 5-hydroxyindol-3-yl acetic acid in the brain. *Pharmacology* **2:** 171–175, 1969.
8. Iwata, H., H. Okamoto and I. Kuramoto. Effect of lithium on serum tryptophan and brain serotonin in rats. *Jap. J. Pharmac.* **24:** 235–240, 1974.
9. Jacobowitz, D. M. and A. S. Richardson. Method for the rapid determination of norepinephrine, dopamine and serotonin in the same brain region. *Pharmac. Biochem. Behav.* **8:** 515–519, 1978.
10. Perez-Cruet, J., A. Tagliamonte, P. Tagliamonte and G. L. Gessa. Stimulation of serotonin synthesis by lithium. *J. Pharmac. exp. Ther.* **178:** 325–330, 1971.
11. Poitou, P., F. Guerinot and C. Bohuon. Effect of lithium on central metabolism of 5-hydroxytryptamine. *Psychopharmacology* **38:** 75–80, 1974.
12. Shellenberger, M. K. and J. H. Gordon. A rapid, simplified procedure for simultaneous assay of norepinephrine, dopamine, and 5-hydroxytryptamine from discrete brain regions. *Analyt. Biochem.* **39:** 356–372, 1971.
13. Young, S. N., P. Etienne and T. L. Sourkes. Relationship between rat brain and cisternal CSF tryptophan concentration. *J. Neurol. Neurosurg. Psychiat.* **39:** 239–243, 1976.