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A FLUOROMETRIC MICROMETHOD FOR THE SIMULTANEOUS DETERMINATION OF SEROTONIN, NORADRENALINE AND DOPAMINE IN MILLIGRAM AMOUNTS OF BRAIN TISSUE*

MARGRET SCHLUMPF, WALTER LICHTENSTEIGER, HEINRICH LANGEMANN, PETER G. WASER and FRANZ HEFTI

Department of Pharmacology, University of Zürich, Zürich, Switzerland

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Abstract—A miniaturized method for the assay of serotonin, noradrenaline and dopamine in extracts from 1·5 to 5 mg of brain tissue (rat and mouse) was developed. The method permitted quantification and spectral analysis of pmole amounts of the amines. The method is derived from the solvent extraction technique and uses the principles of the trihydroxyindole and *o*-phthaldialdehyde methods for the development of fluorophores. The increase in sensitivity was accomplished mainly by volume reduction accompanied by changes of reagent concentrations. The small size of the tissue pieces requires a standardized dissection technique for the control of topographical variations. The problem of an appropriate reference system for the calculation of concentrations was also studied in detail. Protein weight was judged superior to wet weight for this purpose. The method was tested in a series of amine determinations on various areas of rat and mouse brain. Some examples of amine determinations as well as spectral analyses in various areas of single mouse brains are discussed.

IN FUNCTIONAL studies on central monoamine systems, it is often desirable to measure amine concentrations within comparatively small brain areas of individual animals. This also facilitates comparisons with results obtained with histochemical techniques.[1,2] The miniaturized method described here can be used to determine either serotonin (5-HT) or noradrenaline (NA) and dopamine (DA) in extracts from a few milligrams (1·5–5 mg) of tissue taken from circumscribed regions of single rat and mouse brains. When the amount of tissue and the extraction volumes (cf. below) are doubled, all three amines can be assayed in one extract.[3] The sensitivity of the method was found to lie in the pmole range. When amine determinations are carried out on brain samples of this size, slight variations in the topographical position of the tissue samples can influence considerably their content in monoamine neurones or terminals and hence the amine concentrations. In order to improve the topographical reproducibility of the excision procedure, we employed a special dissection technique that is described in more detail elsewhere [see refs. 3 and 18 (accompanying Short Communication, pp. 2447–2449 of this issue)].

METHODS

The sensitivity of fluorometric techniques can be increased considerably by volume reduction. Such a reduction is accomplished much more easily with methods

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based on the extraction of amines by organic solvents than with techniques using column chromatography. This prompted us to start from the extraction method originally described by Chang[4] and Maickel *et al.*[5] The dopamine estimation is based on the procedure of Carlsson and Waldeck.[6] Along with volume reduction, it also became necessary to change some solvent proportions and reagent concentrations in order to optimize the recovery and fluorescence yield.

Preparation of tissue extracts

Dissection Frozen rat or mouse brains were first cut on a cooled microtome (−20°) into frontal slices (about 1-mm thick) at predetermined antero-posterior levels. The frontal slices were subsequently placed on the cooled stage (−20°) of a punching apparatus where cylindrical tissue samples (usually 1 mm in dia, same thickness as the slice) were punched out of selected brain areas with a glass tube. The *x*- and *y*-coordinates of the center of the area were adjusted in a stereomicroscope, the occular of which contained cross lines that were concentric with the center of the glass tube (for details, see refs. 3 and 18). For weight determinations the tissue pieces were immediately transferred to pre-cooled microhomogenizers which were closed with a glass stopper and stored at −25°.

Extraction. The tissue (1·5–5 mg) was homogenized in 0·1 ml HCl–butanol (0·85 ml 37% HCl in 1 liter *n*-butanol for spectroscopy) for 1 min in a glass homogenizer made from a small centrifuge tube (vol. 1·5 ml). The total volume was considered to give 0·105 ml, taking account of the tissue volume (1 mg = 0·001 ml). The sample was then centrifuged for 10 min at 2000 *g*. An aliquot of the supernatant phase (0·08 ml) was removed and added to an Eppendorf reagent tube (vol. 1·5 ml) containing 0·2 ml heptane (for spectroscopy) and 0·025 ml HCl 0·1 M. After 10 min of vigorous shaking, the tube was centrifuged under the same conditions as above in order to separate the two phases, and the overlaying organic phase was discarded. The aqueous phase (0·02 ml) was then taken either for 5-HT or NA and DA assay. All steps were carried out at 0°.

Reference system (a) *Weight determinations* were performed on a microbalance (Mettler) with frozen tissue pieces contained in the microhomogenizers, as the risk of degradation of the amines did not permit waiting until the tissue had reached room temperature. In order to control disturbing effects from condensation as well as evaporation of water, all determinations were carried out on closed homogenizers (carried on dry ice), and a standardized procedure was followed: homogenizers were wiped for 30 sec, then exposed to 24° for 60 sec (in the dark). After subsequent wiping for another 60 sec, a first weight determination was made followed 30 sec later by a second determination.[3]

(b) *Protein determination*: after centrifugation of the butanol-homogenate the residue was dissolved in 0·1 M NaOH and assayed fluorimetrically for protein with fluorescamin according to the method described by Böhlen *et al.*[7] In previous series of assays, the method of Lowry *et al.*[8] was also used for comparison.

Fluorometry

Equipment. Fluorometry was performed in a Farrand spectrofluorometer with a 1-P 21 photomultiplier. Variations in intensity caused by instability of the xenon arc

were largely eliminated by an arc stabilizer. The stability of the system was controlled continuously by recording on paper.

The round microcuvettes supplied by Farrand did not give reproducible readings. Therefore, special square quartz microcuvettes with parallel inner and outer faces were constructed (o.d. *ca.* 6·5 mm, i.d. 1·5 mm; total volume *ca.* 70 μl, reading volume 25–30 μl; supplied by Hellma, Germany). These proved to give highly reproducible readings for a given sample. In our set-up, the temperature of the sample cannot be controlled during fluorometry; a temperature-controlled chamber would probably be of advantage with the small samples.

Serotonin assay. As mentioned earlier, some modifications in reagent concentration became necessary, together with changes in the proportions of the solvents, in order to obtain a good fluorescence yield with the reduced volumes. For 5-HT determination, the *o*-phthaldialdehyde method was employed. From the OPT reagent (20 mg% in conc. HCl) 0·025 ml were added to 0·02 ml of the HCl extract. The fluorophore was developed by heating to 100° for 10 min. After the samples reached equilibrium with the ambient temperature, excitation/emission spectra or intensity readings at 360–470 nm were taken in the microcuvette described above.

Noradrenaline and dopamine assay. The assay represents a miniaturization of the trihydroxyindole method. To 0·02 ml of the HCl phase, 0·005 ml 0·4 M HCl and 0·01 ml EDTA/sodium acetate buffer (pH 6·9) were added, followed by 0·01 ml iodine solution (0·1 M in ethanol) for oxidation. The reaction was stopped after 2 min by addition of 0·01 ml Na_2SO_3 in 5 M NaOH (0·5 *g* Na_2SO_3 in 2 ml H_2O + 18 ml 5 M NaOH). Acetic acid (0·01 ml, 10 M) was added 1·5 min later. The solution was then heated to 100° for 6 min. When the sample again reached room temperature, excitation and emission spectra were read in the microcuvette as with 5-HT; in some cases, the readings were limited to the excitation/emission maxima (395–485 nm for NA, 330–375 nm for DA, uncorrected instrument values).

Standards, blanks and recoveries. The small tissue amounts and volumes of the method make it difficult to produce a tissue standard. Therefore we compared the tissue values (fluorescence of tissue extract minus fluorescence of tissue blank) with an internal reagent standard (fluorescence of internal reagent standard minus fluorescence of internal reagent blank). Tissue blanks for the catecholamine assay were prepared by adding the reagents of the oxidation step in reversed order (Na_2SO_3 before I_2). For serotonin tissue blank, 0·025 conc. HCl without OPT was added. Internal reagent standards were obtained by adding 10 ng 5-HT or 10 ng NA and 20 ng DA in 0·005 ml bidistilled water to 0·1 ml HCl-butanol, which was then carried through the entire extraction procedure. For the internal reagent blank 0·005 ml water was added to 0·1 ml HCl–butanol. Spectral studies were also performed on standards containing all three amines in twice the volume mentioned above.[3] No cross interference was noted. Corrected recoveries ranged from 60 to 70 per cent for all three amines.

RESULTS AND DISCUSSION

Sensitivity and linearity

Aqueous standards showed a linear relation between concentration and fluorescence intensity over a considerable concentration range (Fig. 1). With internal standards the relation between concentration and fluorescence intensity was also found

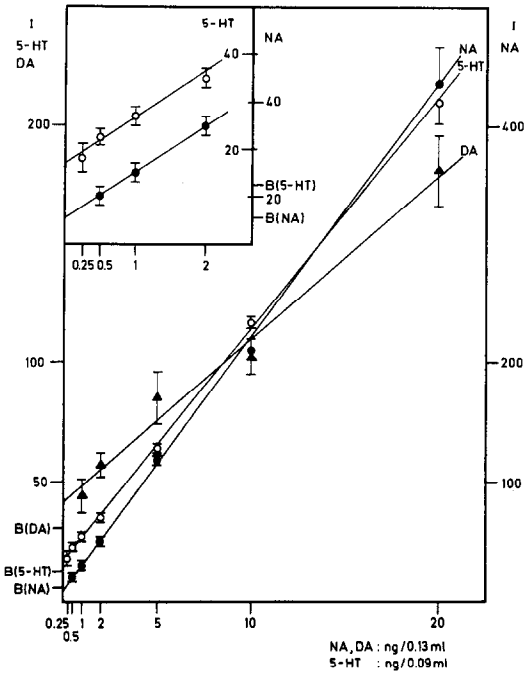

FIG. 1. Relation between fluorescence intensity and concentration of serotonin (5-HT, open circles), noradrenaline (NA, closed circles) and dopamine (DA, triangles) in aqueous standards prepared according to the micromethod (means and S.D. from four determinations). Ordinate: fluorescence intensity in mA (instrument readings); B (5-HT), B (NA) and B (DA): intensity of the respective blanks of the amine assay. Abscissa: concentrations of amines (ng) in the final assay volumes, which in this case were twice those usually employed[3] (0·13 or 0·09 ml). It should be noted that the actual reading volume was 0·025–0·030 ml. The inset shows the lower concentration ranges of 5-HT and NA (ng in 0·09 and 0·13 ml, respectively), with the respective blank values. There exists a good linearity in the relation between the two parameters, which was shown to exist up to 100 ng/0·09 ml for 5-HT and 100 ng/0·13 ml for NA and DA.

to be linear. The limit sensitivity of internal standards was shown to be about 0·5 ng of amine added to the solution before the extraction procedure. In order to obtain reliable data from tissue extracts, the signal to tissue blank ratio should be 2:1. This ratio was obtained when the following absolute amounts of amines were present in the homogenate before extraction: 2·4 ng 5-HT, 1·0 ng NA, and 2·5 ng DA. The resulting sensitivity levels allowed us to perform spectral analyses and determinations of amine concentrations in extracts from two tissue cylinders (1·5–5 mg, cf. below). In such determinations, we observed signal to tissue blank ratios of, e.g., 2·5:1 for 5-HT in mouse raphe region, 6:1 for Na in the area preoptica (mouse) or 7:1 for DA in the caudate (mouse) (cf. Tables 1 and 2).

Excitation and emission spectra from standards and tissue extracts

When excitation and emission spectra were investigated, the spectra from tissue extracts were compared with those of an internal reagent standard containing 10 ng 5-HT or 10 ng NA and 20 ng DA at the beginning of the extraction procedure. For tissue extracts two cylindrical pieces of tissue (1 mm dia.) from the corresponding areas of both sides of a frontal brain slice (1 mm thick, cf. Table 1) were extracted.

In addition, spectra were taken from tissue blanks and internal reagent blanks. As shown in Figs. 2a–c, excitation and emission spectra of tissue extracts from various brain regions correspond closely to the spectra of the three amines as observed in the standards. Sometimes, in regions with relatively low amine concentrations (e.g. NA and DA in substantia nigra of mice and rats), a slight shift of the fluorescence maxima could be observed. the maxima were always displaced towards the position of the tissue blank peak (shorter wavelength with 5-HT and NA, higher wavelength with DA). When the spectra of the tissue blank were subtracted, the peak of the amine fluorescence of the tissue extract again coincided with that of the standard, which indicates that the shift was due to a superposition of amine and tissue blank spectra. The sum of intensities of aqueous standard plus tissue extract (read at peak emission after subtraction of the blank) was found to equal the intensity of corresponding internal tissue standards.[3] This further supports our assumption that quantification can be reliably performed in small tissue samples.

The existence of serotonin could also be demonstrated in extracts from tissue cylinders by the typical shift of its native fluorescence from 370 nm in 0·1 M HCl to 550 nm in 3 M HCl (excitation at 295 nm). The method of Bogdanski *et al.*[9] may thus be used on a microscale as an additional tool to identify this amine.

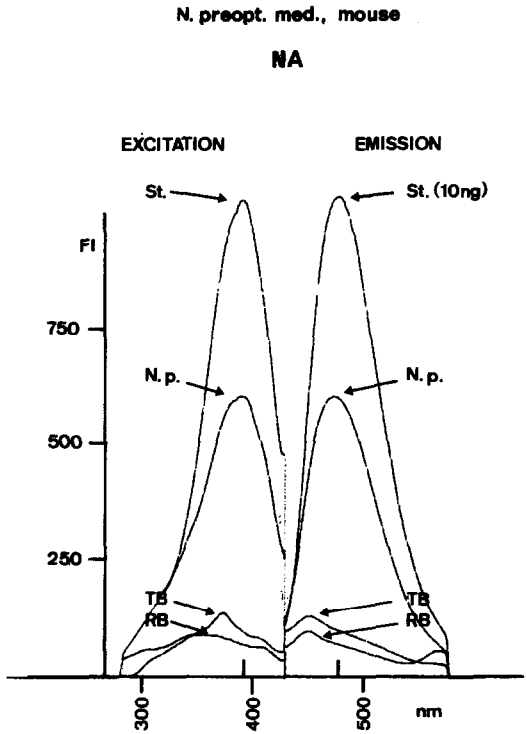

FIG. 2(a). Excitation and emission spectra of noradrenaline in standard and extract from preoptic area of mouse. A combined standard containing 10 ng NA and 20 ng DA at the beginning of the assay was carried through the entire extraction procedure and served as internal Standard (St.). The tissue extract (N. p.) was prepared from two tissue cylinders (*ca.* 2 mg) of preoptic area of a single brain. Tissue blank (TB) was prepared by adding in reversed order the reagents of the oxidation step. RB = internal reagent blank. Ordinate: fluorescence intensity in mA (instrument readings), abscissa: wavelength in nm.

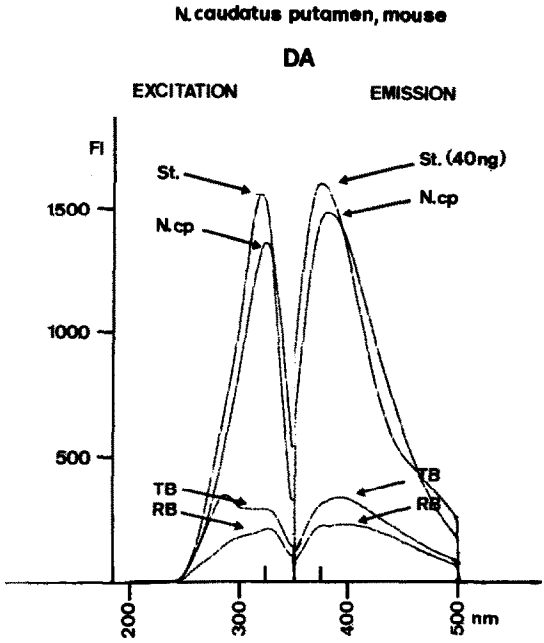

FIG. 2(b). Excitation and emission spectra of dopamine in standard and extract from caudate nucleus of mouse. Internal standard (St.), tissue extract (N. cp), tissue blank (TB) and internal reagent blank (RB) were prepared as for Fig. 2(a). Ordinate and abscissa as in Fig. 2(a).

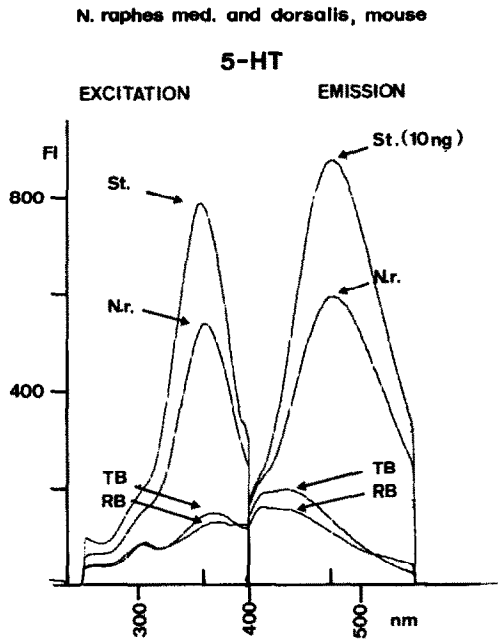

FIG. 2(c). Excitation and emission spectra of serotonin in standards and extracts from the raphe region of the mouse. An internal reagent standard (St.) containing 10 ng 5-HT, the tissue extract (N. r.) and the internal reagent blank (RB) were prepared as in Fig. 2(a). The tissue blank (TB) was prepared by adding all reagents to the extract without *o*-phthaldialdehyde. Ordinate and abscissa as in Fig. 2(a).

TABLE 1. MONOAMINE CONCENTRATIONS IN VARIOUS REGIONS OF MOUSE BRAIN (MEAN ± S.D.) AS DETERMINED IN EXTRACTS FROM SINGLE BRAINS

Brain regions	No. of samples*	Protein wt§ (mg) mean ± S.D.	Serotonin ng/mg protein	Serotonin Absolute intensity readings	Noradrenaline ng/mg protein	Noradrenaline Absolute intensity readings	Dopamine ng/mg protein	Dopamine Absolute intensity readings
Nuc. raphes medialis and dorsalis*	8	0·150 ± 0·039	19·9 ± 4·1	58 ± 5	18·9 ± 4·6	31 ± 4	—†	30 ± 5
Substantia nigra	10 (8)‡	0·131 ± 0·030	26·9 ± 10·9	70 ± 19	13·7 ± 6·7	21 ± 4	31·5 ± 8·3	37 ± 3
Preoptic area¶	8	0·148 ± 0·037	31·4 ± 8·2	67 ± 7	29·4 ± 6·5	56 ± 10	43·4 ± 10·6	64 ± 12
Caudate-putamen	8	0·144 ± 0·041	—†	36 ± 5	—†	11 ± 1	122·5 ± 28·4§	141 ± 27

* One sample consisted of the extract from two tissue cylinders (1 mm dia.) of corresponding areas of both sides of a single brain slice of 1 mm thickness (male mouse), with the exception of the raphe region where one of the two cylinders was centered on nuc. raphe medialis, the other on nuc. raphe dorsalis.

† Fluorescence readings for these samples were below the limit sensitivity of the method defined as signal to tissue blank ratio of 2:1.

‡ Noradrenaline and dopamine determinations were made with ten samples, serotonin determinations with eight samples.

§ In our material 0·074 ± 0·013 mg protein were found in 1 mg of wet weight. Referred to wet weight DA concentration in caudate is thus obtained as 9·1 ng/mg.

¶ Cylinder bordered medially by the third ventricle and including, in the mouse, medial and part of lateral preoptic area.

TABLE 2. EFFECT OF MORPHINE ON THE SEROTONIN CONCENTRATION OF THE RAPHE REGION OF SINGLE MOUSE BRAINS

Controls		Morphine (40 mg/kg s.c. 20 min before decapitation)	
Protein wt* (mg)	Serotonin* (ng/mg protein)	Protein wt* (mg)	Serotonin* (ng/mg protein)
0·142	28·9	0·096	35·5
0·107	28·9	0·107	39·9
0·107	35·4	0·118	44·7
0·091	41·6	0·107	50·7
0·183	24·6	0·094	47·0
0·129	32·5	0·107	47·8
0·121	22·6	0·113	37·8
0·126 ± 0·030	30·6 ± 6·5	0·106 ± 0·009	44·7 ± 4·9†

* Values of individual samples (extracts of two tissue cylinders from one brain) and mean ± S.D. The two tissue cylinders correspond to the areas described in Table 1. The fourteen samples of control and morphine-treated male mice were prepared in one assay series.

† Morphine-treated animals are different from controls for $P < 0{\cdot}01$ according to the Wilcoxon rank sum test.

Amine determination in various brain regions

Tables 1 and 2 show preliminary results of amine determinations in selected regions of single mouse brains. Table 2 illustrates that an analysis of drug effects on monoamines in small brain areas is possible with this technique. The determinations were performed on extracts from the two corresponding tissue cylinders of both sides. While the sensitivity of the method was found to be largely sufficient for amine determinations in these small tissue pieces, the choice of an appropriate reference system presented some problems. Several series of determinations were based on weight determination (cf. ref. 3 and Methods). Yet, this type of reference proved to be both laborious and rather variable. Therefore, subsequent analyses utilized protein weight as a reference according to either the method of Lowry *et al.*[8] or, for the data presented herein, the fluorimetric method of Böhlen *et al.*[7] The latter is more sensitive and particularly convenient because the fluorimetric equipment can be used. Variations were also encountered when protein weight was used as a reference (S.D. about 20–25 per cent of the mean as compared to 35 per cent with wet weight determination). Yet, when the relative concentrations calculated for typical brain regions were compared, there seemed to be a better correlation with results from previous determinations in pooled samples or (as far as NA and DA are concerned) with histochemical data.[10,17]

Certain general features appear from values shown in Tables 1 and 2. As already mentioned, there seems to exist a relatively good correlation between amine levels and topography of monoamine systems. This is especially evident in regions known to contain certain types of terminals (e.g. preoptic area as compared to caudate, cf. Table 1). The considerable amount of NA found in the raphe region points to the existence of NA terminals in this area (cf. ref. 10). Interestingly, the concentrations are relatively low in regions containing nerve cell bodies (raphe and substantia nigra, Table 1). This agrees with that which would be expected from histochemical fluorescence studies (Andén *et al.*,[11] Lichtensteiger[2] and unpublished observations). The

relatively important amounts of DA found in the preoptic region of mice may have resulted from contamination by the olfactory tubercle or the nuc. accumbens septi, but it also seems conceivable that in addition NA systems contain a certain amount of DA. Similarly, earlier determinations in pooled samples of rat locus coeruleus (ten tissue cylinders from five rats per sample[3]) also yielded comparatively high DA concentrations (NA 2·7 ± 0·3 ng/mg wet weight (n = 4 pools) and 1·7 ± 0·6 ng/ml (n = 4); DA 6·1 ± 1·4 ng/mg (n = 4) and 2·1 ± 0·5 ng/mg (n = 4) in two different series). The occurrence of DA in locus coeruleus was also discussed by Gérardy *et al.*[12] In this context, one should not overlook the possibility that DA values are to some extent contaminated by DOPA. We found that DOPA is carried through the extraction procedure and can interfere with the dopamine assay, as recently observed also by Waldmeier and Maitre.[13] Although DOPA was not detected in extracts from normal whole brain,[14] it may occur in limited amounts in certain regions, depending eventually upon the functional state. Recent studies with thin-layer chromatography tend to support this view (Schlumpf and Lichtensteiger, unpublished observations, cf. ref. 15). It becomes evident from this discussion that the omission of a purification step eliminating, *inter alia*, amino acids, may create certain problems especially with regard to DA. This abbreviated procedure originally introduced by Maickel *et al.*,[5] proved to be very suitable for development of a highly sensitive micromethod because of the reduced number of steps and of small volume losses. We are presently investigating whether another fluorimetric method eliminating amino acids, which was applied to invertebrate ganglia,[16] can be adapted for amine determination in small pieces of mammalian brain and used for control in DA determination.

CONCLUSIONS

As shown in the present investigation, 5-HT, NA and DA can be determined in two pieces (1·5–5 mg) of small corresponding areas from a single mouse or rat brain by fluorometry when the sensitivity of the method is increased by volume reduction throughout the entire assay procedure, in combination with some additional adjustments of reagent concentrations. In the case of the monoamines, the principle of the solvent extraction method,[4] proved to be more appropriate for this purpose than column chromatography. As mentioned above, the omission of certain steps which adds to the sensitivity of the method, may cause certain problems especially with regard to DA. The present method is sensitive enough to allow routine spectral analysis in these small samples, which increases the reliability of the assay. If the micromethod is to be applied to the study of different brain areas, it is, however, necessary to employ a highly standardized excision technique (see refs. 3 and 18) in order to limit topographical variations. Protein weight proved to be most suitable as reference system.

The present method represents a useful tool for the elucidation of certain functional problems, especially when combined with histochemical microfluorometry. Some problems are easier to study (on a quantitative basis) with the extraction method, such as levels of 5-HT (which is difficult to quantify in microfluorometry) or monoamine concentrations in areas with nerve terminals. In regions containing catecholamine nerve cell bodies, the comparison of results obtained with both techniques can provide a valuable means of control as well as additional information in case

of discrepancies.[15] The fact that brain pieces from single animals can be investigated, is certainly of advantage for functional studies.

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