Biochemical Pharmacology, 1965, Vol. 14, pp. 831–835. Pergamon Press Ltd., Printed in Great Britain.

A SENSITIVE AND SPECIFIC FLUORESCENCE ASSAY FOR TISSUE SEROTONIN

SOLOMON H. SNYDER, JULIUS AXELROD and MARK ZWEIG*

Laboratory of Clinical Science, National Institute of Mental Health, National Institutes of Health, Bethesda, Md., U.S.A.

(*Received* 9 *November* 1964; *accepted* 30 *November* 1964)

Abstract—A sensitive and specific method for the estimation of serotonin in biological materials is described. In this method, serotonin is reacted with ninhydrin to form a product whose fluorescence is eight times more intense than the native fluorescence of serotonin in strong acid solution. With this method it is possible to measure serotonin in organs in which endogenous serotonin had not been previously detected and to study the subcellular distribution of this amine in the rat pineal and adrenal glands.

THE availability of specific and sensitive methods for the estimation of biogenic amines in biological material has facilitated rapid advances in studies of their metabolism, physiological disposition, and actions. The fluorometric method commonly used for serotonin, although relatively specific and sensitive, cannot accurately measure less than 0·3 μg of tissue serotonin.[1] Consequently this amine cannot be assayed in tissues such as the heart and adrenal gland in which the concentrations may be low. Recently, Jepson and Stevens[2] observed that small amounts of serotonin (0·02 μg/cm^2) could be detected on paper chromatograms after dipping into a ninhydrin solution and heating. On the basis of this reaction, Vanable[3] developed a quantitative procedure for the measurement of serotonin in aqueous solutions; in his study the specificity of the reaction was not determined and tissue measurements were not presented. This report describes a modification of the procedure of Vanable so that serotonin can be accurately measured in biological material. This method has been found to be specific and about eight times as sensitive as the procedure of Bogdanski *et al.*[1] As little as 10 mμg of tissue serotonin can be measured by the procedure described here.

METHODS

Serotonin creatinine sulfate, bufotenin, 5-hydroxyindole acetic acid, epinephrine, N,N-diethyltryptamine, tryptamine, N,N-dimethyltryptamine, norepinephrine, histamine, and all amino acids were obtained from the California Corp. for Biochemical Research. Melatonin, indole, 5-hydroxyindole, 5-methoxyindole, 5-methoxytryptamine, and N-acetylserotonin were purchased from the Regis Chemical Co. The following compounds were kindly donated by Dr. John Daly: 6-methoxyindole, 6-methoxytryptamine, 5-hydroxy-6-methoxytryptamine, 6-hydroxy-5-methoxytryptamine.

Aqueous solutions of 0·1 M ninhydrin (California Corp. for Biochem. Res., A grade) were made up in glass-distilled water. N-Heptane (spectranalyzed) and 1-butanol (reagent grade) were obtained from the Fisher Scientific Co. Epinephrine-^{3}H

*NIH Special Fellow, Washington University Medical School, St. Louis, Mo.

(5 c/mmole), norepinephrine-^{3}H (7 c/mmole) and serotonin-^{14}C (30 mc/mmole) were obtained from the New England Nuclear Corp.

The method involved extraction of serotonin into 1-butanol from a salt-saturated solution at pH 10. Serotonin was then returned to an aqueous solution, pH 7·0, by the addition of heptane and reacted with ninhydrin to yield a fluorescent product. All measurements were made on an Amino-Bowman spectrophotofluorometer.

Tissues were homogenized in 8 ml of ice-cold 0·4 N perchloric acid in a motor-driven glass homogenizer. Homogenates were centrifuged at 900 *g* for 10 min. A 4-ml aliquot of the supernatant fluid was adjusted to pH 10·0 with sodium hydroxide solutions and a glass electrode and then transferred to a 40-ml glass-stoppered centrifuge tube containing 0·5 ml of 0·5 M borate buffer at pH 10, 1·5 g sodium chloride, and 15 ml 1-butanol. Tubes were shaken for 10 min, centrifuged, and the aqueous phase removed by aspiration. The organic phase was then shaken for 3 min with 2 ml of 0·1 M borate buffer (pH 10), previously saturated with sodium chloride, and centrifuged. Ten ml of the organic phase was transferred to a 40-ml glass-stoppered centrifuge tube containing 1·4 ml of 0·05 M phosphate buffer, pH 7·0, and 15 ml of *n*-heptane. After shaking for 2 min, the tube was centrifuged and the organic phase carefully aspirated.

A 1·2-ml aliquot of the aqueous phase was transferred to a small tube containing 0·1 ml of 0·1 M ninhydrin solution and the tubes heated for 30 min at 75°. One hour after heating, the solution was transferred to a quartz cuvette and the fluorescence measured at 490 mμ after activation at 385 mμ (uncorrected). The activation and fluorescent spectra were essentially the same as described by Vanable.[3] The fluorescence increased about 10% in the first 20 min after heating but was then stable for at least 6 hr. A small reagent blank was obtained by carrying 4 ml of 0·4 N perchloric acid through the above procedure. The fluorescence intensity was proportional to serotonin concentration over the range 0·005–0·5 μg/ml. Serotonin added to tissues was recovered to the extent of 90–100% after correcting for 85% extraction.

RESULTS AND DISCUSSION

Fluorescence of a variety of compounds after reacting with ninhydrin

The capacity of a number of biologically occurring substances and indole derivatives to form fluorescent compounds when heated with ninhydrin at pH 7·0 as described above, but without prior butanol extraction, was tested (Table 1). The fluorescence was highly specific for serotonin. The fluorescence of bufotenin was about 2% and that of 5-hydroxytryptophan about 1% of an equal concentration of serotonin, while other compounds tested produced negligible fluorescence. When tested at 0·1 M concentrations, leucine and methionine gave appreciable fluorescence but with a fluorescent peak at 450 mμ and an activation peak at 380 mμ. Tyrosine at 0·1 M concentrations produced fluorescence with an activation peak at 400 mμ and fluorescent peak at 520 mμ. However, none of the amino acids studied was extracted to any appreciable extent.

Estimation of serotonin in tissues

The development of a sensitive method for serotonin made it possible to measure this biogenic amine in tissues, such as heart and adrenal gland, in which it could not be

detected by chemical methods previously used. Measurable amounts of serotonin were found in the adrenal glands and hearts of rats (Table 2). In all tissues, the activation and fluorescent spectra were the same as that of authentic serotonin. This amine was also assayed in the rat pineal gland, large intestine, spleen, lung, and brain by the

TABLE 1. RELATIVE FLUORESCENCE OF SEROTONIN AND OTHER COMPOUNDS AFTER HEATING WITH NINHYDRIN

Activating peak 385 mμ. Fluorescent peak 490 mμ.

Compound	Units
Serotonin	850
Bufotenin	20
5-Hydroxytryptophan	10
5-Hydroxyindoleacetic acid	2
Epinephrine	2
Histidine	2
5-Hydroxyindole	1

Compounds that gave no measurable fluorescence: 6-hydroxytryptamine, 4-hydroxytryptamine, 5-methoxytryptamine, N,N-diethyltryptamine, tryptamine, indole, N,N-dimethyltryptamine, 6-methoxytryptamine, 6-methoxyindole, 5-methoxyindole, 5-hydroxy-6-methoxytryptamine, 6-hydroxy-5-methoxytryptamine, melatonin, norepinephrine, proline, cysteine, alanine, leucine, methionine, tyrosine, histamine, glutamic acid, tryptophan, N-acetylserotonin.

All compounds tested at 3×10^{-6} M concentrations.

TABLE 2. SEROTONIN CONTENT OF SEVERAL TISSUES

Tissue	Serotonin content (μg/g)	
	Method of Bogdanski *et al.*	Ninhydrin method
Rat pineal gland	62·2 ± 6·5	64·0 ± 7·1
Rat adrenal gland	0·00	0·45 ± 0·05
Cat superior cervical ganglion	0·00	0·00
Rat large intestine	1·75 ± 0·20	1·78 ± 0·29
Rat spleen	3·09 ± 0·30	3·05 ± 0·22
Rat heart	0·00	0·19 ± 0·02
Rat brain	0·44 ± 0·06	0·45 ± 0·06
Rat lung	1·01 ± 0·20	1·10 ± 0·14

Groups of 3 to 6 rats were used for each tissue. Data are presented as means ± S.E.M.

ninhydrin method and by the method of Bogdanski *et al.*[1] Close agreement in the serotonin concentration was obtained by both methods. Serotonin could not be detected in 200 mg of cat superior cervical ganglia by the ninhydrin method, although as little as 10 mμg would have been measured by this method.

Subcellular distribution of serotonin in adrenal and pineal glands

The various subcellular fractions of the adrenal gland were separated by the procedure of Potter and Axelrod[4] and their serotonin content measured by the ninhydrin method in two separate experiments. The largest fraction of serotonin was present in

the chromaffin granules (Fig. 1). These granules also took up ^{3}H-epinephrine and ^{14}C-serotonin after the i.v. administration of these compounds. Since there are large quantities of epinephrine normally present in adrenal chromaffin granules, the possibility of interference by this compound was examined. The amount of epinephrine present in the aliquot of adrenal gland used for the assay of serotonin was carried through the complete extraction procedure and reaction with ninhydrin. This amount of epinephrine, equivalent to 1·2 mg/g adrenal tissue, gave no detectable fluorescence.

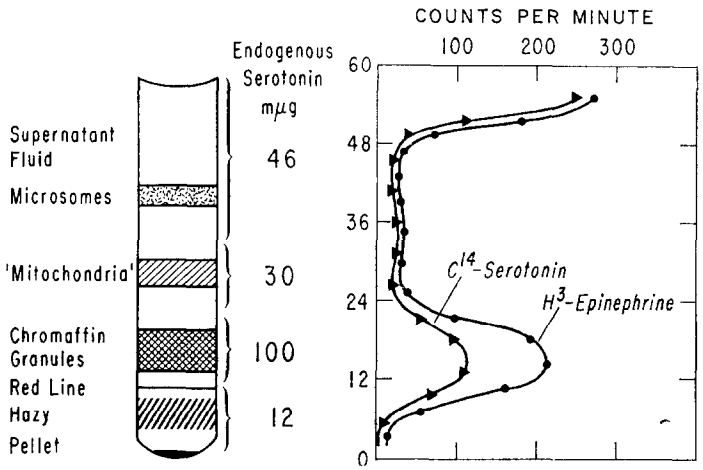

FIG. 1. The subcellular distribution of serotonin in the rat adrenal gland. Adrenal glands from 6 rats weighing 400 mg were homogenized in 0·5 ml of 0·25 M sucrose, centrifuged in a 0·25–2·2 M exponential sucrose gradient at 125,000 *g* for 60 min, and the resultant subcellular fractions assayed for serotonin. In separate experiments adrenal homogenates from rats injected i.v. 1 hr earlier with 50 μc ^{3}H-epinephrine or 15 μc serotonin-^{14}C were centrifuged in identical sucrose gradients.

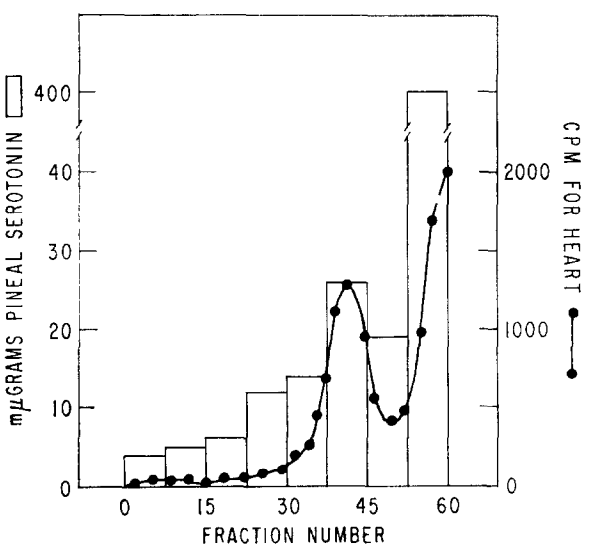

FIG. 2. The subcellular distribution of serotonin in the rat pineal gland. Pineal gland or heart homogenates were centrifuged in a gradient prepared by diluting 1·17 M sucrose exponentially with water.

Histochemical techniques[5] have shown that a large fraction of the serotonin content of the rat pineal gland is localized in sympathetic nerves. In subcellular studies using a continuous sucrose gradient, it was found that ^{3}H-norepinephrine in the pineal gland is localized in small particles which migrate with the "microsomal" fraction.[4] To see whether serotonin in the pineal gland is localized in the same or similar subcellular fractions, pineal glands from 10 rats were homogenized in 0·5 ml of isotonic potassium chloride layered on a sucrose density gradient as described earlier,[6] and centrifuged for 1 hr at 125,000 *g* (Fig. 2). About 90% of the serotonin was confined to the soluble supernatant fraction. However, there was a small peak of serotinin in the subcellular fraction which, in the heart,[5] salivary gland, and vas deferens (S. Snyder, unpublished observations) is associated with the norepinephrine storage granules. In a separate experiment, a rat was injected with 200 μc ^{3}H-norepinephrine and killed after 1 hr. Its heart was homogenized in 5 volumes of isotonic potassium chloride and centrifuged over a sucrose gradient identical with that used for the pineal homogenates described above. The peak area of radioactivity (Fig. 2) coincided with the small peak noted for pineal serotonin.

REFERENCES

1. D. F. Bogdanski, A. Pletscher, B. B. Brodie and S. Udenfriend, *J. Pharmacol. exp. Ther.* **117**, 82 (1956).
2. J. B. Jepson and B. J. Stevens, *Nature, Lond.* **172**, 772 (1953).
3. J. W. Vanable, *Analyt. Biochem.* **6**, 393 (1963).
4. L. T. Potter and J. Axelrod, *J. Pharmacol. exp. Ther.* **142**, 291 (1963).
5. C. Owman, *Int. J. Neuropharmacol.* **2**, 105 (1964).
6. S. H. Snyder, I. A. Michaelson and J. Musacchio, *Life Sci.* **3**, 965 (1964).