ISOLATION OF 5-HYDROXYTRYPTAMINE CONTAINING VESICLES AND OF SYNAPTIC MEMBRANES FROM RAT BRAIN

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1. Introduction

In our research about the physiological importance of 5-hydroxytryptamine we have isolated and analyzed vesicles and synaptic membranes from rat brain in order to get information for the possible classification of this amine as a synaptic transmitter. In this letter we present evidence that one vesicle fraction and the synaptic membranes contain the highest amount of 5-HT * from all brain fractions analyzed. These results are compatible with the concept that 5-HT – as a possible transmitter substance like acetylcholine and norepinephrine – must be stored or synthesized in the presynaptic terminals and must be bound to the subsynaptic membranes.

2. Methods

Vesicles and synaptic membranes were isolated from rat brain by differential- and sucrose gradient centrifugation as described in fig. 1. This is a modification of the methods first published by De Robertis [1–3] and Whittacker [4,5]. Protein was assayed with the biuret reaction [6], 5-HT fluorimetrically by the method of Bogdansky [7], and SDH with cytochrome *c* as electron acceptor according to Kadenbach [8]. Sialic acids were determined with thiobarbituric acid [9] as *N*-acetyl-neuraminic acid after 1 hr hydrolysis with 0.1 N H_2SO_4 at 80° using *N*-acetylneuraminlactose as reference. The number and size of vesicles were determined exponentially from electron micrographs of ultrathin sections by means of a "Teilchengrössenzählgerät" from Zeiss. Vesicle suspensions containing 0.3–0.6 mg protein/ml were incubated with 5-hydroxytryptamine-3-^{14}C-creatinine sulphate (0.025 μC/ml; 4.9 mc/mmole; the Radiochemical Centre, Amersham, Bucks.) at 37°C, in 0.01 M potassium phosphate–0.15 M KCl buffer (pH 8). At different times aliquots of the incubation mixture were centrifuged and the loss of radioactivity in the supernatant was taken as a measure of 5-HT uptake by the vesicles.

Thin-layer chromatography was achieved on Kieselgel G, Merck, coated plates with $CHCl_3$/Me/12.5% NH_3 (60:40:9) and Propanol/12.5% NH_3 (7:3) as solvents.

3. Results and discussion

The analysis of all fractions obtained during the isolation of the vesicles (table 1) shows that the 106,000 × *g* precipitate – the "crude" vesicle fraction (fraction 6) – still contains SDH activity which is due to mitochondrial fragments. After further purification of this fraction by centrifugation on a simple H_2O/0.4 M sucrose gradient a vesicle fraction free of SDH activity was collected just below the water/sucrose interface. This "purified" vesicle fraction contains 0.31 nMol 5-HT/mg protein. Thus the 5-HT concentration is raised 6-fold by this purification step, while the NeuNAc concentration falls by about 40%.

* Abbreviations: 5-HT = 5-hydroxytryptamine (serotonin); NeuNAc = *N*-acetylneuraminic acid; SDH = succinate dehydrogenase (E.C. 1.3.99.1).

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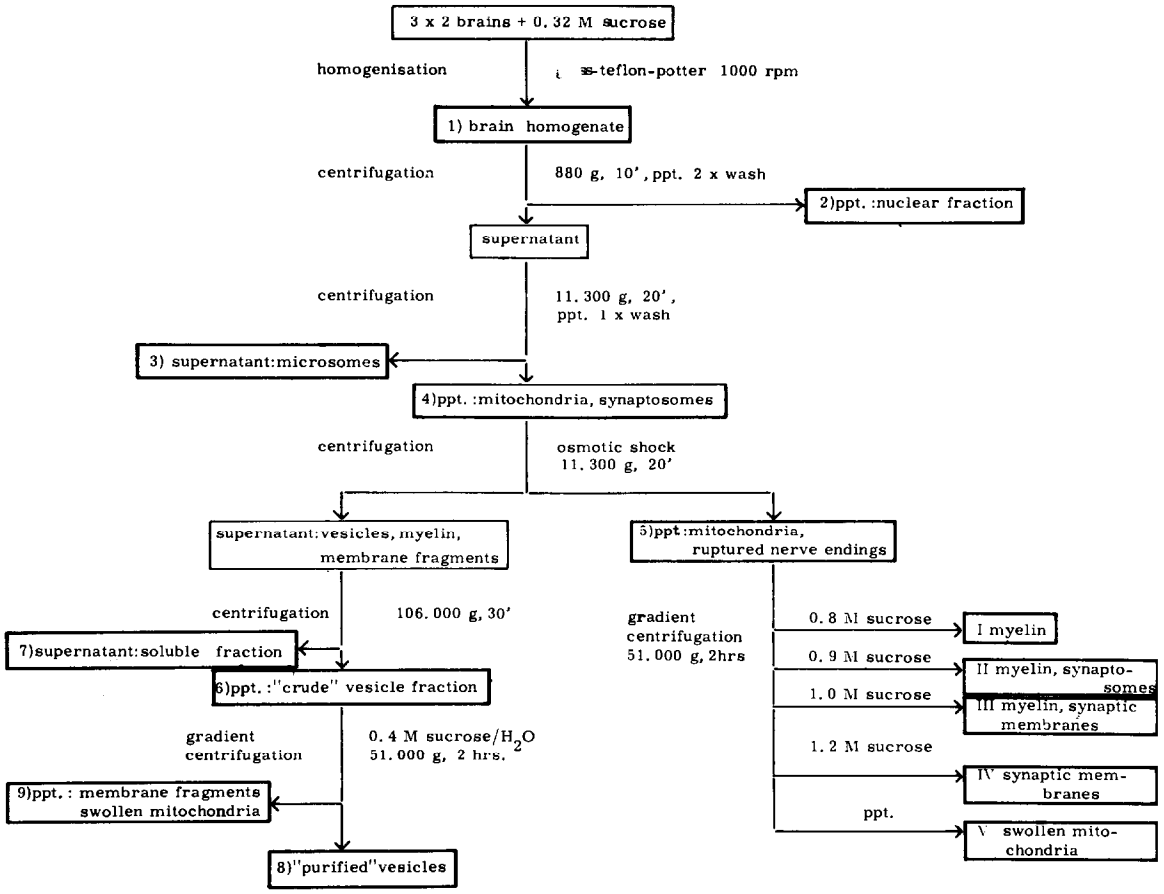
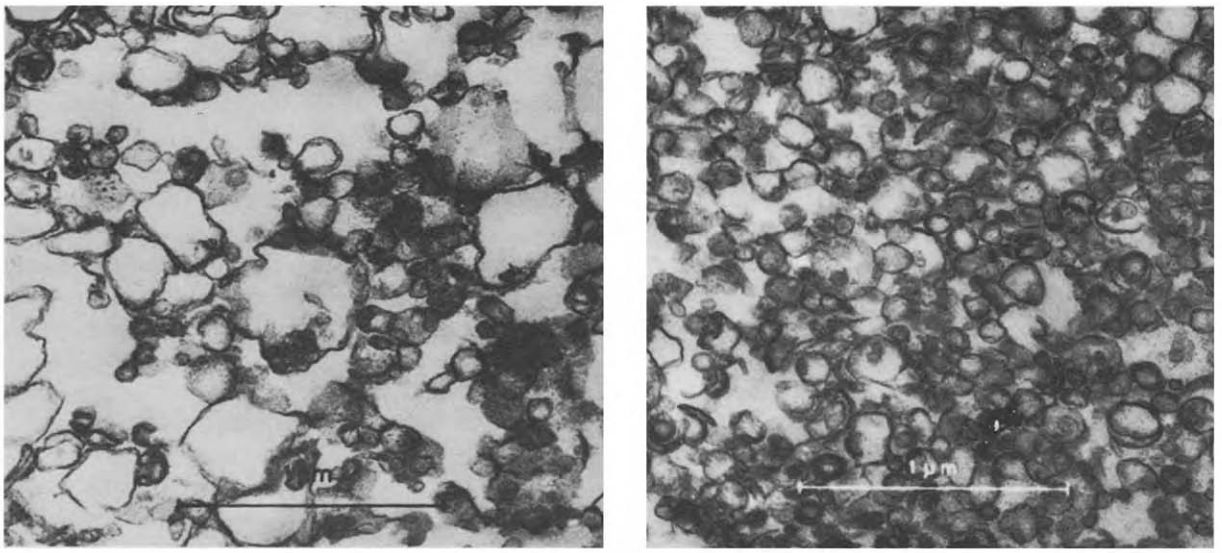


Fig. 1. Isolation of vesicles and synaptic membranes from rat brain.



(a) (b)

Fig. 2. Electron micrographs of the vesicles isolated from rat brain.
(a) "Crude" vesicle fraction. (b) "Purified" vesicle fraction; 10,000 X 3½.

Table 1
Analysis of the different brain fractions (obtained according to fig. 1).

Fraction	Protein (mg/g fresh)	SDH †		5-HT †		NeuNAc †	
		nMol/min mg protein	r. sp. a. *	nMol mg protein	r. sp. c. **	nMol mg protein	r. sp. c. **
1) homogenate (total brain)	100 (32) †	10.8 (26)	1	0.02 (26)	1	23.4 (23)	1
2) nuclear fraction	10.9 (18)	8 (13)	1.2	0.03 (13)	0.7	13.9 (10)	0.6
3) microsomes	47.2 (18)	0.8 (13)	0.1	0.02 (13)	0.8	25.4 (10)	1.1
4) mitochondria, synaptosomes	24.9 (18)	20 (13)	1.5	0.02 (13)	1.3	24.3 (10)	1.1
5) mitochondria, ruptured nerve endings	11.9 (31)	49.2 (26)	3.5	0.02 (22)	0.8	17.4 (23)	0.7
6) "crude" vesicle fractiob	4.4 (18)	10 (13)	3.1	0.05 (13)	1.1	33.0 (10)	1.8
7) soluble fraction (supernatant 6)	5.9 (18)	– (13)	–	0.05 (13)	1.9	10.0 (10)	0.5
8) "purified" vesicles	0.26 (26)	– (20)	–	0.31 (20)	21	20.6 (21)	0.7
9) membrane fragments, swollen mitochondria	2.1 (18)	9 (13)	2.6	0.02 (13)	1.1	34.2 (10)	0.5

* r. sp. a. = relative specific avtivity = %SDH/%protein
** r. sp. c. = relative specific concentration = % substrate/% protein
† The number of experiments is given in parentheses

Table 2
Analysis of the synaptic membranes

Fraction	Protein (mg/g fresh)	SDH		5-HT		NeuNAc	
		nMol/min mg protein	r. sp. a. *	nMol mg protein	r. sp. c. **	nMol mg protein	r. sp. c. **
1) homogenate (total brain)	100 (32) †	10.8 (26)	1	0.02 (26)	1	23.4 (23)	1
5) mitochondria, ruptured nerve endings	11.9 (31)	49.2 (26)	3.53	0.02 (22)	0.8	17.4 (23)	0.7
I) 0.8 M sucrose	2.8 (14)	1.9 > (13)	0.18	0.02 (13)	1.1	12.7 (13)	0.5
II) 0.9 M sucrose	1.0 (14)	2.4 > (13)	0.24	0.02 (13)	1.9	15.7 (13)	0.5
III) 1.0 M sucrose, myelin, synaptic membranes	0.7 (14)	2.4 > (13)	0.18	0.04 (13)	2.2	15.3 (13)	0.6
IV) 1.2 M sucrose synaptic membranes	1.1 (14)	8.8 > (13)	0.70	0.05 (13)	4.5	11.0 (13)	0.3
V) precipitate swollen mitochondria	3.9 (14)	146.7 (13)	10.94	0.01 (13)	0.7	5.7 (13)	0.2

* r. sp. a. = relative specific activity = % SDH/% protein
** r. sp. c. = relative specific concentration = % substrate/% protein
† The number of experiments is given in parentheses.

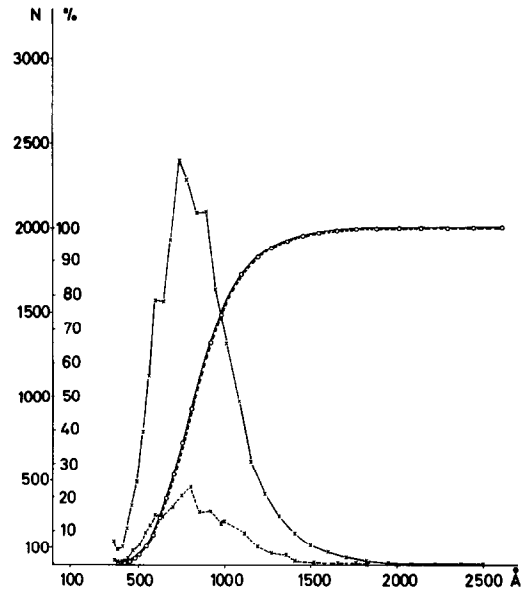


Fig. 3. Size distribution and summation curve of "purified" vesicles. The number of particles which had been counted was 7589 (———) and 1370 (------). x=====x size distribution curve, O=====O summation curve.

Extraction of the vesicles with chloroform/methanol (2:1) followed by centrifugation yielded a NeuNAc free supernatant. The total amount of NeuNAc was found in the precipitate in a bound form. Gangliosides were not detected on thin-layer chromatograms of the purified vesicle fraction. These results exclude the presence of gangliosides in the vesicles isolated by the procedure described above. We assume that NeuNAc is bound to a glycoprotein. Our findings are in good agreement with recent results from Whittacker [10] and Wiegandt [11]. In contrast to Burton et al. [12,13] these authors did not find any or almost no ganglioside-bound NeuNAc in their vesicle fractions. Though the protein content of the crude vesicle fraction – 4.4 mg/g fresh weight – agrees rather well with the results of other authors – De Robertis [14]: 4.4 mg/g fresh weight; Burton [12]: 2 mg/g fresh weight – the purified vesicles contain only about 1/10 of this concentration.

After resuspending and centrifugation of the precipitate containing mitochondria and ruptured nerve endings [see fig. 1 (fraction 5)!] in a discontinuous sucrose gradient ranging from 0.8–1.2 M 4 fractions and a precipitate of swollen mitochondria are obtained (table 2). Fraction IV contains synaptic membranes and a few small mitochondria as demonstrated by electron-microscopy and SDH activity.

Electron-micrographs of the "crude" (fig. 2a) and "purified" vesicle fraction (fig. 2b) are in accordance with the described analytical differences. The more uniform appearance of the "purified" vesicles as compared with the "crude" fraction is due to an enrichment of vesicles. The size distribution curve (fig. 3) of the "purified" vesicles ranges from about 500–1300 Å revealing a maximum at 735 Å with two shoulders at 600 and 890 Å which indicates three main particle sizes. According to the summation curve (fig. 3) about 3% of the "purified" vesicles fall in the range 300–500 Å contrasted with about 55% of the "synaptic" vesicles as described in the literature [14]. About 40% of the "purified" vesicles have a diameter between 700 and 900 Å. These results clearly indicate a difference of chemical composition and morphology between both vesicle preparations.

The binding of 5-HT to the vesicle fraction was studied with 5-hydroxytryptamine-3-^{14}C (5×10^{-6} M). It is time-dependent and reveals two plateaus: the first plateau was obtained after 10 min of incubation where a total of 0.44 nmole 5-HT/mg protein was incorporated. After a further increase in the rate of binding a second plateau was reached where a total of 0.85 nmole 5-HT/mg protein was incorporated after 120 min of incubation. Further work has to clarify whether these two plateaus are due to different binding constants of one vesicle species or whether 5-HT is incorporated into different kinds of particles of the vesicle fraction.

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