UPTAKE AND STORAGE MECHANISM OF 5-HYDROXYTRYPTAMINE IN RABBIT BRAIN STEM AND EFFECT OF RESERPINE

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Received for publication July 21, 1970

In a previous paper (1) to clarify the physiological significance of endogenous 5-hydroxytryptamine (5-HT) the subcellular distribution of 5-HT in rabbit brain stem has been studied and the electron microscopical structures of the fractions which contain 5-HT have been examined. 5-HT was principally found in P_2 -fraction which consisted largely of mitochondria and nerve-ending particles (NEPs). After density-gradient centrifugation 5-HT was found to be comparatively highly concentrated in NEPs-fraction (P_2B -fraction). On the other hand suspension of P_2 -fraction in hypo-osmotic medium and subsequent differential centrifugation resulted in high concentration of 5-HT in synaptic vesicles (sv) fraction although considerable amount of 5-HT was also found in P_2D - and P_2S -fraction. In general 5-HT in brain stem was not so highly concentrated in specific fraction as with the distribution of acetylcholine (ACh) (2, 3) but rather diffusibly distributed among several fractions.

In this paper the role of 5-HT in synaptic transmission, especially in central nervous system was investigated mainly through the experiments on 5-HT uptake by subcellular fraction of rabbit brain stem. The chemical transmission at synapses consists of the following consecutive steps: 1) release of transmitter by synaptic impulses. 2) combination of transmitter with postsynaptic receptor. 3) generation of impulses by postsynaptic potential. It appears that the study into the formation, uptake, storage, release and inactivation of 5-HT is necessary for full and accurate understanding of mechanism of chemical transmission, especially when there is no direct evidence for that 5-HT is synaptic transmitter in central nervous system. Segawa and Kuruma (4), Segawa et al. (5) in this laboratory have already shown that NEPs or sv of brain, when incubated in a medium containing 5-HT, could take up 5-HT from the medium and some drugs could inhibit the uptake of 5-HT. However we have as yet very little information as to whether NEPs or sv are specific uptake and storage site for 5-HT in situ. Also the mechanisms with which amine can be taken up by synapses and drug inhibits the uptake are poorly under-

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The results were briefly presented at a Regional Meeting of the Japanese Pharmacological Society at Kyoto on July, 13, 1968.

stood. The following is a systematic study concerning the mechanism of 5-HT uptake and drug effect.

MATERIALS AND METHODS

Male and female rabbits, weighing about 2.5 kg were used. One to three brain stems (ca. 2.5–7.5 g) were homogenized in ice cold 0.32 M sucrose using a Teflon pestle with two to three up-and-down motions of the mortar during preparation. The time for homogenization was not restricted. The method of fractionation was based on that previously employed in this laboratory (4, 5). The subcellular fractions were suspended in Krebs-Ringer phosphate buffer (pH 7.4) of the following composition: 140 mM-Na⁺, 6.0 mM-K⁺, 2.7 mM-Ca²⁺, 1.2 mM-Mg²⁺, 130 mM-Cl⁻, 18 mM-PO₄³⁻, 1.2 mM-SO₄²⁻, 10 mM-glucose. An aliquot (4.4 ml or 4.6 ml) of suspension was transferred to a 30 ml of Erlenmeyer flask and incubated with 0.2 ml of pheniprazine (final concentration of 5×10^{-8} M) for 30 minutes in air at 37°C with 70–80 shaking per minute. After incubation 0.2 ml of a solution of 5-HT and 0.2 ml of a solution of drug to be tested in Krebs Ringer phosphate buffer solution were added and incubated for a further 30 minutes at 37°C. Thereafter the mixtures were centrifuged as the method of subfractionation. The pellet was washed twice with 0.32 M sucrose solution at 0°C.

When P₂V (sv)-fraction was used directly in the experiment, the fraction was suspended in isotonic modified Krebs solution (NaCl 6.90 g; KCl 0.35 g; CaCl₂ 0.28 g; MgCl₂ 0.11 g; NaH₂PO₄ 0.14 g; glucose 2.00 g in 1000 ml of glass distilled water) without pre-incubation with pheniprazine.

The protein content was estimated by the method of Lowry et al. (6) with slight modification. 5-HT was extracted and assayed fluorimetrically by the method of Snyder et al. (7).

Materials used were 5-hydroxytryptamine creatinine sulfate (Nakarai Chemical Co.), pheniprazine (Chugai Seiyaku, Co.), 5-hydroxytryptophan (Nakarai Chemical Co.) and reserpine (Nihon Shinyaku, Co.).

RESULTS

1. Subcellular distribution of 5-HT after administration of 5-hydroxytryptophan (5-HTP) and pheniprazine

Our findings reported previously (1) have shown that 5-HT was not so highly concentrated in P₂B-fraction which consisted almost entirely of NEPs, although concentration of 5-HT per protein in this fraction increased when this fraction was re-centrifuged. From this result it is difficult to draw the conclusion that NEPs are the specific storage site for 5-HT. Therefore in an attempt to obtain more evidence for this problem 5-HT synthetic ability of each fraction was examined.

The subcellular distribution of endogenous 5-HT in rabbit brain stem was presented in Table 1, Column 1. The effect of pheniprazine (3 mg/kg, i.v., 15 minutes) on the concentration and distribution of 5-HT in brain stem was given in Table 1, Column 2. Col-

TABLE 1. Subcellular distribution of 5-HT in rabbit brain stem after intravenous administration of pheniprazine or pheniprazine and 5-HTP.

Fraction	Treatment		Ratio (II/I)	Treatment		III-II I
	I: None	II: MAOI*		III: MAOI+5-HTP**	III-II	
	(ng/g tissue)			(ng/g tissue)		
P ₁	26.6	46	1.72	90	44	1.69
P ₂	242.5	392	1.61	2360	2068	8.50
P ₃	36.5	158	4.33	660	502	13.70
S ₃	152.5	580	3.80	4620	4040	26.50
P ₂ -A	64.9	194	2.99	851	621	9.58
P ₂ -B	76.8	155	2.02	1320	1165	15.20
P ₂ -C	31.9	45	1.42	56	11	0.35

* Pheniprazine 3 mg/kg

** 5-HTP 40 mg/kg

umn 4 in Table 1 showed the results of the experiment in which rabbit was treated with 5-HTP (40 mg/kg, i.v.) 5 minutes after the administration of pheniprazine (3 mg/kg, i.v.) and was killed 20 minutes later. The value of II/I shown in Table 1, Column 3 represented relative increase in 5-HT content after monoamine-oxidase inhibitor (MAOI) and this was thought to give some indication of 5-HT synthetic ability of each fraction.

The value of III-II/I given in Table 1, Column 6 which was considerably high in P₃-, S₃- and P₂B-fraction and relatively high in P₂A-fraction was regarded as the uptake- and synthetic-ability of 5-HT.

2. 5-HT uptake by subcellular fractions

The primary fractions, P₁, P₂ and P₃ were suspended in Krebs Ringer phosphate buffer

FIG. 1. Uptake of 5-HT by subcellular fractions from rabbit brain stem homogenates. Each fraction was incubated with 5 μ g/ml of 5-HT.

2XW: washed twice with 0.32 M sucrose solution at 0°C.

* P₁-fraction was incubated for 60 minutes at 37°C with 5-HT and was re-incubated after 2 times washing with ice cold 0.32 M sucrose solution.

solution (pH 7.4) and were incubated with 5-HT (final concentration of 5 $\mu\text{g/ml}$). As was shown in Fig. 1 P_1 -fraction took up practically same amount of 5-HT both at 4°C and 37°C in air and at 37°C in nitrogen gas. Furthermore nearly all of 5-HT taken up at 37°C could be readily removed by washing twice with cold 5-HT-free 0.32 M sucrose and again they could take up almost the same amount of 5-HT when they were incubated with 5-HT. These observations suggest that the uptake of 5-HT by P_1 -fraction in vitro was non-specific in nature.

On the other hand P_2 -fraction could take up much more quantity of 5-HT at 37°C than at 4°C (Fig. 1) and this could not easily be removed by washing at 4°C. This result would indicate that the uptake of 5-HT by P_2 -fraction was rather specific. P_3 -fraction also took up 5-HT in similar fashion. This result is in good agreement with those of Inouye et al. (8), Gillis et al. (9) and Robinson et al. (10). However as P_3 -fraction consisted of microsomal particles and was devoid of NEPs the physiological significance of this uptake and the mechanism which involved in this uptake must be different from that of P_2 -fraction. Because NEPs was abundant in P_2 -fraction it is reasonable to presume that large proportion of 5-HT which was taken up by P_2 -fraction was incorporated into NEPs. The question now arises as to the possible source of energy for the uptake of 5-HT by P_2 - or P_3 -fraction because the uptake was unaffected by N_2 gas. Further studies into this problem are necessary.

Our previous results (4, 5) in which NEPs and sv were found to be able to take up 5-HT from the medium do not per se mean that these structures are the storage sites for 5-HT. To observe how much proportion of 5-HT which was taken up by P_2 -fraction was associated with sv fraction the following experiments were performed. The P_2 -frac-

FIG. 2.. Accumulation of 5-HT in P_2 -fraction after incubation with 5-HT (5 $\mu\text{g/ml}$) or 5-HTP (5 $\mu\text{g/ml}$). P_2 -fraction was subfractionated into P_{2V} -, P_{2S} - and P_{2D} -fraction after incubation.

tion suspended in Krebs Ringer phosphate buffer solution (pH 7.4) was incubated with 5-HT (final concentration of $5 \mu\text{g/kg}$) at 37°C for 30 minutes, centrifuged at $11,500 \text{ g}$ for 20 minutes and the yielded pellet was washed with 0.32 M sucrose at 0°C . The pellet was then submitted to hypo-shock and then subfractionated in the usual manner. The results in Fig. 2, Section A showed that the concentration of 5-HT in P_2V -fraction containing sv was highest among three subfractions.

Rate of uptake of 5-HT by sv as a function of time was shown in Fig. 3. The uptake was fast: after 5 minutes of incubation the amount of 5-HT in the fraction was 75% of that after 30 minutes. In Figs. 4 and 5 the uptake of 5-HT by P_2V - and P_2S -fraction was plotted against the concentration of 5-HT in the medium. At low 5-HT concentration in medium the amounts of 5-HT taken up by the fractions were almost proportional to the concentration of exogenous 5-HT but showed the tendency to approach the plateau in the concentration range of $5 \mu\text{g/ml}$. There was similarity between 5-HT uptake by P_2V - and P_2S -fraction in relation to the absolute quantity of 5-HT in fraction and the rate of uptake of 5-HT as a function of 5-HT concentration in medium although 5-HT concentration per protein in P_2V -fraction was slightly higher than in P_2S -fraction. This result might be in conflict with the

FIG. 3. Rate of uptake of 5-HT as a function of time by P_2V -fraction. P_2 -fraction was incubated with 5-HT ($5 \mu\text{g/ml}$) and then subfractionated into P_2V -fraction.

FIG. 4. Uptake of 5-HT by P_2V -fraction. P_2 -fraction was incubated with 5-HT ($5 \mu\text{g/ml}$) and then subfractionated into P_2V -fraction.

FIG. 5. Uptake of 5-HT by P_2S -fraction. P_2 -fraction was incubated with 5-HT (5 $\mu\text{g/ml}$) and then subfractionated into P_2S -fraction.

view that sv is the specific storage site for 5-HT. However it should be taken into account that the fraction was treated with MAOI which inhibited the metabolic destruction of any 5-HT taken up into axoplasm. Without MAOI 5-HT concentration in P_2S -fraction should decrease.

P_2 -fraction was also subjected to incubation with 5-HTP (final concentration of 5 $\mu\text{g/ml}$) and was then subfractionated into P_2V -, P_2S - and P_2D -fraction. It was found that less amount of 5-HT was taken up than in the experiment in which P_2 -fraction was incubated with 5-HT (Fig. 2, Section A, B). If the endogenous amount of 5-HT was subtracted from 5-HT concentration after incubation the remaining value was almost negligible, indicating that actually no uptake occurred (Fig. 2, Section B). This indicates that originally 5-HT, but not 5-HTP can be taken up into the synaptic region and that accumulation of 5-HT at sv is taken up as 5-HT itself.

3. Effect of pH on 5-HT uptake by P_2V -fraction

The isolated P_2V -fraction was suspended in isotonic modified Krebs solution of various pH with 5-HT at 37°C for 60 minutes. The result in Fig. 6 showed that the uptake was greatly affected by pH, it increased with increasing pH of the medium (pH 8.5) and decreased with decreasing pH of the medium (pH 5.7). After large amount of 5-HT was taken up by P_2V -fraction at pH 8.5 the incubation medium was centrifuged to yield P_2V -pellet. Thereafter the pellet was either washed twice with ice-cold amine free-medium of pH 8.5 or of pH 5.7 or incubated in the medium of pH 5.7 at 0°C for 30 minutes. No apparent difference in the remaining quantity of 5-HT in P_2V -pellet was detected. From this result it is suggested that the uptake is not a non-specific ionic binding of 5-HT to the surface of sv.

FIG. 6. Effect of medium pH on uptake of 5-HT by 5-HT P_2 V-fraction.

FIG. 7. Effect of pH of washing- and re-incubation-medium on uptake of 5-HT by P_2 V-fraction. P_2 V-fraction was incubated with 5-HT ($5 \mu\text{g/ml}$) for 60 minutes at 37°C in pH 8.5 isotonic modified Krebs solution and was treated in the following manner.

- (1): washed two times with pH 8.5 solution.
- (2): washed two times with pH 5.7 solution.
- (3): re-incubated for 30 minutes at 0°C in pH 5.7 solution.

FIG. 8. Effect of reserpine on uptake of 5-HT. P_2 -, NEPS- and SV-fraction were incubated with 5-HT ($5 \mu\text{g/ml}$) in the presence of reserpine ($20 \mu\text{g/ml}$). P_2 -fraction was then treated in the usual manner to obtain SV- and P_2S -fraction.

4. *Effect of reserpine*

As to the site of action of reserpine many authors (11–14) have favoured the view that reserpine selectively blocks the incorporation of 5-HT into storage granule although there is objection (15) to this view. To obtain more direct information about the site of reserpine action P_2 -fraction was incubated with 5-HT in the presence of reserpine (final concentration of 20 $\mu\text{g/ml}$) and was then treated in the usual manner to yield P_2V - and P_2S -fraction. The results in Fig. 8, Section C, D showed that the uptake by P_2V -fraction was significantly inhibited by reserpine while that of P_2S -fraction was not greatly affected. These results, together with our previous findings (4, 5) (presented in Fig. 8, Section A, B) give more clear evidence that reserpine acts mainly at the level of sv. As presented in Fig. 8 5-HT uptake was inhibited more prominently in NEPs-fraction than in isolated P_2V -fraction. This may be explained in two ways: 1) reserpine needs some components in axoplasm in order to exhibit its action. 2) reserpine sensitive site which is supposed to localize at synaptic vesicle membrane is injured by subfractionation process.

DISCUSSION

If one make an attempt to identify some substance as chemical transmitter in central nervous system one should take the following two problems into account: 1) the question whether there exists regional specificity of the corresponding transmitter uptake and storage in a brain. Can 5-HT-, noradrenaline (NE)-, dopamine (DA)- and ACh-neurons have a high specificity in respect of the uptake and storage of the corresponding amines? 2) the question whether there is regional specificity within one neuron with respect to site and form of amine storage, mechanism and site of amine uptake. The present study was undertaken mainly to examine the latter problem.

Unfortunately the brain stem homogenate used in this experiment is by no means homogeneous. It contains not only serotonergic but catecholaminergic terminals as well. Therefore the accurate results are not expected unless more homogeneous preparation is available. In an attempting to approach the problem 1) described above, in a previous paper (16) the crude mitochondrial fractions derived from hypothalamus, corpus striatum and cerebellum of rabbit were incubated with 5-HT, together with or without NE or DA. It was found that there was a considerable degree of regional specificity in the uptake of 5-HT by 5-HT terminal since exogenous 5-HT was mostly taken up by terminals from area rich in endogenous 5-HT. Also the uptake of 5-HT by terminals containing NE and DA was found to be inhibited by NE and DA considerably. From this result it may be implied that the uptake of 5-HT by serotonergic nerve terminal *in vitro* is specific in nature although it was inhibited with NE or DA to some degree. This result also could give some answer to the question 1) mentioned above.

There were marked increases in 5-HT concentration in P_3 -, S_3 -, P_2A - and P_2B -fraction when rabbit was treated with pheniprazine alone or pretreated with pheniprazine followed by 5-HTP. P_3 - and S_3 -fraction are largely derived from cell body and axon. P_2B -fraction consists almost entirely of NEPs while P_2A -fraction comprizes myelin frag-

ments with or without attached axons in which vesicular structures were observed (1).

When P_2 -fraction was incubated with 5-HTP only negligible amount of 5-HT was found to be accumulated in P_2 -, P_2V - and P_2S -fraction. This result, together with Segawa's result (17) in which intravenous injection of 5-HTP resulted in largest increase in S_3 -fraction in 5-HT concentration followed by the increase in P_3 -, P_2 - and P_1 -fraction supports the view that 5-HTP from circulation is first accumulated in the cell body, decarboxylated to 5-HT and then transported down to the terminal.

Robinson et al. (10) have reported that when NEPs were incubated with C^{14} -5-HTP in a medium which contained brain supernatant fraction (equivalent to S_3 -fraction in our experiment) less 5-HT was taken up than in experiment in which C^{14} -5-HT was added. They explained that part of this was due to the addition of the brain supernatant fraction. Our experiment, however, showed that NEPs in Krebs solution could not take up large amount of 5-HTP. Therefore some additional mechanism must be involved.

As with P_2 -fraction, P_3 -fraction could also take up 5-HT in a similar fashion. At present it seems difficult to draw the conclusion that the mechanism involved in 5-HT uptake by P_3 -fraction is different from that by P_2 -fraction. However Segawa's report (17) in which when MAOI was injected into the reserpine treated rabbit, remarkable restoration of increase in 5-HT concentration after 5-HTP administration was obtained in P_2 -fraction but not in P_3 -fraction might suggest that some different mechanism exists.

The effect of medium pH on 5-HT uptake by P_2 -fraction has been discussed by many authors. Wise et al. (18), Robinson et al. (10) have found that the uptake increased with increasing pH of the medium. Furthermore Robinson et al. (10) have shown that the uptake of 5-HT by NEPs was inhibited by increasing concentrations of hydrogen ions, divalent and monovalent cations and they suggested that cations were competing with the positively charged amine for binding sites on the NEPs, analogous to an ion-exchange process. However in the system of Wise and Ruelius (18) NE and histamine (both are cations at pH 8.0) were bound to a considerably lesser extent than 5-HT. Our results in which 5-HT taken up by P_2V -fraction at pH 8.5 could not be removed by the medium of pH 5.7 or by incubating in this medium at 0°C for 30 minutes appear to be at variance with the view that the binding of 5-HT is solely ion-exchange process. Furthermore as pH 8.5–10 is far from physiological condition the binding of 5-HT in such pH seems not significant physiologically.

Electronmicroscopic observations revealed that synaptic vesicles have vesicle membrane (1, 19, 20). There are two possible mechanisms for the binding of 5-HT to sv: 1) 5-HT is solely bound to the outer surface of vesicle membrane electrically or chemically. 2) 5-HT is taken up within sv through vesicle membrane and is stored there in a free form or in a binding form with specific substance. In adrenal medullary granule 5-HT has been found to be bound to ATP (21–24) while in mast cell granule it has been known to be bound to heparin (25–29). From our present results it is suggested that mechanism 2) is involved in the uptake of 5-HT by sv.

Two different amine uptake mechanisms have been presented in adrenergic neurons:

active transport through neuronal membrane and subsequent incorporation into storage granules (11, 30, 31). The same may be said of the uptake of 5-HT by sv: transport through membrane of sv and concentration (storage) within sv. Thus, irrespective as to whether sv take up 5-HT by passive diffusion- or active transport-process 5-HT will be taken up and stored within sv to its storage capacity. The details of this mechanism remain to be determined.

SUMMARY

1. When rabbit was treated with MAOI alone or pretreated with MAOI followed by 5-HTP marked increase in 5-HT concentration in P_3 -, S_3 -, P_2A - and P_2B -fraction from brain stem was observed.

2. P_1 -fraction could take up 5-HT in vitro but the uptake was temperature insensitive and nearly all of 5-HT taken up at 37°C could be readily washed out.

3. The uptake of 5-HT by P_2 -fraction was temperature dependent and the amine taken up at 37°C could not easily be removed by washing. P_3 -fraction also took up 5-HT in similar fashion.

4. 5-HT bound to P_2 -fraction in vitro was found to be mainly associated with P_2V -fraction and was more concentrated than in P_2 -fraction.

5. The rate of uptake of 5-HT by sv was fast. At low 5-HT concentration in medium the amount of 5-HT taken up by P_2V - and P_2S -fraction was almost proportional to 5-HT concentration in medium.

6. P_2 -fraction, when incubated with 5-HTP took up less amount of 5-HT.

7. The uptake of 5-HT by P_2V -fraction was affected by pH, it increased with increasing pH of the medium, decreased with decreasing pH of the medium.

8. Reserpine was found to act mainly at the level of sv.

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