SUBCELLULAR DISTRIBUTION OF 5-HYDROXYTRYPTAMINE IN THE RABBIT BRAIN

Kiyoshi Kataoka

Department of Physiology, Kyoto University School of Medicine
Kyoto, Japan

It was demonstrated by Baker (1958, 1959) that a large fraction of 5-hydroxytryptamine (5-HT) in duodenal mucosa was present in intracellular store granules denser than the mitochondria. On the other hand, Whittaker (1959) reported that the brain 5-HT was chiefly found as a bound form in subcellular fractions less dense than the typical mitochondrial one, suggesting the difference between the brain and intestinal 5-HT. He also described release and inactivation of this particulate 5-HT. Using the fraction action and bioassay method similar to those used by Whittaker, his result could be confirmed in our laboratory. But Kataoka (1962) found that substance P of rabbit brain showed the subcellular distribution and properties quite similar to those of 5-HT. Stating the Vane's rat fundus preparation (1957) used for bioassay for 5-HT in Whittaker's work was also sensitive to substance P, he cast some doubts on the nature of particulate 5-HT described by this investigator, since he omitted the acetone extraction of 5-HT prior to its bioassay. It seemed necessary, therefore, to re-examine whether or not neuronal 5-HT is mainly present in the so-called synaptic vesicle fraction. Then, using the acetone treatment, subcellular distribution and some properties of such a bound 5-HT in brain were re-investigated. This paper is chiefly concerned with this problem. A brief account of the results described has already been submitted to the publication (Inouye, Kataoka and Shinagawa, 1962).

MATERIALS AND METHODS

Materials: Rabbits of both sexes were usually used. The animal was killed by bleeding. When the whole brain was used, the cerebellum was omitted. In some experiments, the spinal cord and cerebellum were also used separately.

After removal of adhering water by filter paper, the wet tissue was weighed on a balance and subjected to the homogenation and then to the fractional centrifugation. All the preparation procedure was carried out in the cold room (4°C).

Homogenation and fractional centrifugation: The homogenation and centrifugation were carried out as described previously by Kataoka (1962) but with slight modification, their procedures being the same in their principles as those employed by Whitt-
TAKEI (1959): the nuclear fraction was usually suspended into 1.0 M sucrose and the washing fraction was separated and examined (PALLADIN, 1960), since considerable amount of 5-HT activity was found in the crude nuclear fraction (P1) in contrast to substance P. The diagram of fractionation was as follows.

**Diagram 1. Flowing diagram of subcellular fractionation.**

In some experiments, some of the subfractions thus separated were placed over solutions of hypertonic sucrose (0.8-2.4 M) layered in the centrifuge tube and centrifuged for 1 hr. at 140,000 g in an ultracentrifuge (Hitachi 40P type) with swing bucket rotor head as made by BLASCHKO, HAGEN and HAGEN (1957). The layered sucrose solutions of various concentration in the tube were usually used after kept at 0°C overnight. Each layer was carefully pipetted out and subjected to the assay for 5-HT and protein after adjusting its sucrose concentration to 0.32 M.

**Assay of 5-HT**: Using the method of AMIN, CRAWFORD and GADDUM (1954), the samples were extracted with acetone and assayed by rat's uterus as described by them or by rat's fundus preparation described by VANE (1957). The strip was suspended in aerated de Jalon's solution for the former, and Krebs's solution for the latter. To minimize its desensitizing effect, samples to be tested or standard 5-HT solutions were added to the bath at least 4 min after its prior administration and repeated washing.

In some experiments, however, acetone extraction was omitted and the samples were directly applied to the rat's fundus preparation as described by WHITTAKER in order to confirm that the 5-HT content reported by him was too large because of the presence of substance P. Otherwise acetone-insoluble fraction was assayed to estimate the extent of apparent 5-HT activity derived from substance P.

**Assay of substance P**: Activity of substance P (SP) was assayed on the isolated guinea-pig ileum in the presence of atropine, pyribenzamine and 5-HT, as described previously (INOUE, KATAOKA and TSUJIOKA, 1961). The standard SP was prepared from horse intestine by Euler's salting out method (EULER, 1942).

**Estimation of protein content**: Protein content of samples was determined by Lowry's, method (LOWRY, ROSENBURGH, FARR and RANDALL, 1951).
**Inactivation of 5-HT:** Each subcellular fraction and the known quantity of 5-HT (10 ng/ml) were incubated (usually 30 min), and boiled for about 10 minutes. Amount of each particulate suspension applied to the incubation was always adjusted to correspond to the same weight of the original tissue. Loss in 5-HT activity was measured by applying the cooled incubation mixture directly on the rat’s fundus preparation, because the particulate suspension incubated was so small that the contraction of test preparation due to its 5-HT and SP was negligible.

**RESULTS**

I. The subcellular distribution of 5-HT. The 5-HT activity found in each subcellular fraction of whole brain was illustrated in Fig. 1. When it was extracted as in the method of Amin, Crawford and Gaddum (1954), the results obtained were in good agreement with each other irrespective of the test preparation, the rat’s uterus or rat’s fundus (the average of six observations).

Subcellular distribution of 5-HT of the spinal cord and cerebellum expressed in % was also quite similar to that of the brain, only its absolute amount being different. The total sum of 5-HT in the seven fractions would provide an estimate of 5-HT per gram wet tissue of brain, cerebellum and spinal cord. As presented in Table 1, the 5-HT content of these three parts of the central nervous system shows good agreement with the results reported by previous investigators (Amin, Crawford and Gaddum, 1954; Paasonen and Vogt, 1956). As described below, acetone treatment liberates particulate 5-HT completely, so that the high 5-HT content of whole homogenates as well as of particulate fractions of brain reported by Whittaker should be ascribed to some plain muscle stimulant other than 5-HT. At least substance P (SP) could not be excluded because of his omitting acetone treatment (Amin, Crawford and Gaddum, 1954).

Indeed, the subcellular distribution of 5-HT presented in Fig. 1 is evidently different from that of fundus-contracting action of samples untreated with

---

**Fig. 1.** Subcellular distribution of 5-HT.

Ordinates: recovered activity (ng/g wet tissue). N: nuclear, W: washing, M: mitochondria, V: synaptic vesicular, A: amorphous, Ms: microsomal and C: cytoplasmic supernatant. Each value was averages of 6 determinations made by parallel bioassay with rats uterus (black block) and rat’s fundus preparation (white block). Numerals in parentheses represent % recovery of 5-HT activity of acetone soluble sample, full recovery being referred to the difference between 5-HT activity observed on acetone-untreated and acetone-insoluble ones.
Table 1. Regional distribution of 5-HT in the rabbit brain.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>5-HT (µg/g, wet tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Our results</td>
</tr>
<tr>
<td>Cerebrum</td>
<td>0.174</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>0.009</td>
</tr>
<tr>
<td>Spinal cord</td>
<td>0.078</td>
</tr>
</tbody>
</table>

* From the paper of Amin, Crawford and Gaddum (1954).

acetone and their acetone-insoluble aliquots (Fig. 2). The latter two are quite similar to the subcellular distribution of SP (Kataoka, 1962), the higher activity being found in Whittaker's synaptic vesicle (V) fraction and the microsomal (Ms) fraction, but the nuclear (N) and mitochondria (M) fraction considerably lower than these two. In contrast, the distribution of neuronal 5-HT rather resembles that of intestinal 5-HT reported by Baker (1959), only a small amount was found in the lighter mitochondrial particles (i.e. our V fraction), the higher activity being found in the nuclear (our P1) and typical mitochondrial (i.e. M) fractions.

Fig. 2. Fundus-contracting activity of acetone-untreated samples and acetone insoluble aliquots.


Hence the sensitivity of rat's fundus preparation to 5-HT and SP was examined and it confirmed that this test preparation was sensitive to the amount of SP contained in our samples tested. Rough estimation based on dose-response curves of rat's fundus to SP and 5-HT showed that about 6-7 units of SP is nearly equivalent to 0.01µg of 5-HT. The 5-HT activity of our samples obtained without acetone extraction is, therefore, nearly the same order of magnitude of fundus contracting action expected from the amount of SP and 5-HT present in them. When the 5-HT content presented in Fig. 1 is compared with that calculated from difference between the 5-HT equivalence obtained on acetone-untreated and acetone-insoluble samples, the 5-HT activity
recovered in the former is, as presented in Fig. 1, 81-105% of the latter except for the nuclear fraction. In the last-named one, the presence of fundus-stimulating substance other than 5-HT and SP would be suggested.

Even after the nuclear fraction was washed with 0.32 M sucrose twice, still remains considerable amount of 5-HT activity in the sediment. In contract to SP, 5-HT found in this fraction seems not to be merely due to particulate contaminants. According to Palladin (1960), therefore, the nuclear fraction was resuspended in 1 M sucrose with the aid of a homogenizer after washed twice with 0.32 M sucrose and then submitted to centrifugation at $1\times10^4 g$ for 10 min to separate his “washing fraction (W)” the sediment being the proper nuclear (N) fraction. Thus about a half of 5-HT in the original nuclear fraction could be recovered in W fraction. Hence N fraction here is somewhat different from the crude N fraction in the previous paper (Kataoka, 1962), the former being less sticky.

II. Ultracentrifugal subfractionation in the density gradient of hypertonic sucrose solution. The above-stated results indicated the neuronal 5-HT is also present, as that of intestinal mucosa (Baker 1959), rather in the heavier particles than in the lighter ones. Therefore, the P$_2$ fraction or the combined fraction of P$_2$ and Ms was subjected to the ultracentrifugal subfractionation in the density

![Diagram of distribution of 5-HT and SP in the subfractions obtained from the combined P$_2$+Ms suspension and W fraction.](image)

(a) SP distribution in the P$_2$+Ms suspension determined on acetone-insoluble aliquots and evaluated with unit/g. wet tissue.

(b) 5-HT distribution in the P$_2$+Ms suspension determined on acetone-soluble aliquots (ng/g. wet tissue).

(c) 5-HT distribution in the W fraction (ng/g. wet tissue).

As shown in the right side, subfractions were separated by ultracentrifugation in the density gradient prepared by layering 1.2, 1.6, 2.0 and 2.4 M sucrose solution in the centrifuge tube. The particulate suspension in 0.32 M sucrose was layered on the top. The activity of 5-HT and SP found in each subfraction is presented by the horizontal bar at the corresponding height in the right side. Solid lines show their activity-protein ratio with arbitrary scale.
gradient. The sucrose concentration of layered hypertonic solution for making up a density gradient was varied between 1.2-2.4 M. An example of such experiment was illustrated in Fig. 3. As stated above, the 5-HT equivalence of the acetone-insoluble aliquot of each subfraction could be safely regarded as indicative of their SP activity (Fig. 3a), while the fundus contracting activity of acetone-soluble aliquot would provide the proper estimate of 5-HT (Fig. 3b). All five observations showed always that the SP activity was highest in 0.32 M subfraction, the 5-HT being highest in 1.6 M subfraction. The protein content was determined on each fraction and subfraction (Fig. 4), their highest activity-protein ratio for SP was found in 0.32 M sucrose, that for 5-HT in 1.6 M.

When the subcellular distribution of protein (Fig. 4a) is compared with that of PALLADIN (1960), the protein content of our W fraction is found to be considerably lower than his values (about 23% for his 42%). This would be probably because 0.32 M sucrose used for washing of the crude nuclear fraction was not included, but unfortunately we did not measured its recovery; such a waste of protein in our preparing W frac tion being assumed as 20% of total protein, our results on other fractions show fairly good agreement with that of PALLADIN. So the protein content of our P$_2$ or P$_2$+Ms fraction might be not so unreasonable. The protein distribution among the subfraction of (P$_2$+Ms) or W (Fig. 4b) is quite similar to each other, the only difference being the higher protein content of 2.0-2.4 M subfraction in the latter, a fact which probably suggests the presence of incompletely homogenized tissue debris in W fraction.

![Fig. 4. Protein distribution in subcellular fractions of rabbit brain.](image)

(a) Protein content of each subcellular fraction as % of the total sum.
(b) Protein content of each subfraction separated from W (white block) and from P$_2$+Ms (black block) as % of that of whole suspension.
In the (b), protein contents of whole W and P$_2$+Ms suspension were taken as 100% respectively.

As stated above, W fraction contains considerable amount of 5-HT, and so the ultracentrifugal subfractions of this fraction was also attempted. As illustrated in Fig. 3c, the heavier granules showed the highest 5-HT-protein ratio though the amount of 5-HT recovered in 0.32 M subfraction was highest. As pointed out above, loss in protein content of our W fraction might be considerably large due to waste of 0.32 M sucrose washing, but the 5-HT activity found in this washing solution was fairly small. So the 5-HT-protein ratio
of lighter granules (mainly 0.32 M fraction) would be considerably lower than (about a half of) those in Fig. 3c, while the presence of debris would contribute to the 5-HT and protein in the heaviest subfractions (2.0–2.4 M). Taking such factors into account, our results appear to indicate that the composition of W fraction with respect to the particulate 5-HT is similar to that of the whole subcellular particles excluding nuclei and so about a half of 5-HT activity found in the crude nuclear fraction would originate from the cytoplasmic contaminant.

III. Liberation of the particulate 5-HT in the brain. As stated above, 5-HT was found in the heavy granule fraction in contrast to the particulate SP. To demonstrate that this 5-HT is bound in the particles, its release from the granules by various treatment was examined.

Since 5-HT in P₂ fraction was largely derived from that in the heavy granules, P₂ fraction was used without separating the heavier granule fraction. The P₂ fraction suspended in 0.32 M sucrose was treated as follows: As shown below, hardly any 5-HT destroying activity was found in this fraction, addition of iproniazid was safely omitted.

1. The suspension was kept at 4°C, 20°C, 37°C and 60°C for 15–60 minutes.
2. The pH of suspension was adjusted to 7.0 (with 1/10 volume of 0.1 M sodium phosphate buffer), 5.0 (with 1/10 volume of 0.1 M sodium acetate buffer) and 3.0 (with 1/10 volume of 0.1 M citrate buffer) and they were kept at 4°C for 15–60 minutes.
3. The sucrose concentration of suspensions was adjusted to 0.03, 0.08, 0.16, 0.32 and 1.2 M and they were kept at 4°C for 15–60 min.
4. After KCl (10–200 mM), NaCl (10–200 mM), CaCl₂ (10–200 mM) or MgCl₂ (10–200 mM) was added to the suspension, it was kept at 4°C for 30–60 min.
5. After the suspension was shaken with 9 volumes of ether for 15–20 min at room temperature and separated from the ether layer, it was freed from trace of ether by evaporation in vacuo.
6. To the suspension, 9 volumes of acetone were added. After being kept at 4°C for 30–240 min.
7. After reserpine (50 μM/ml) was added to the suspension, it was kept at 4°C for 30–240 min.

After such treatments, the suspensions were centrifuged at 10,000 g for 30 min and the 5-HT content was determined on the sediment and the supernatant respectively. Comparing the sum of 5-HT content of both portions with that of the original suspension, its nearly full recovery (90–100%) was confirmed, while the released particulate 5-HT in % was estimated from the decrease in the 5-HT content of sediment. Some of the results obtained are illustrated in Table 2 (average of four observations). These results appear to indicate, as in the particulate ACh (Whittaker, 1959) or SP (Kataoka, 1962), the presence of 5-HT bound to the subcellular particles.

In one experiment, the microsomal fraction was treated in similar way; the supernatant separated from P₂ fraction was centrifuged at 108,000 g for 60 min, the sediment was resuspended in 0.32 M sucrose and then subjected to
the above-stated treatments, after which the mixture was centrifuged again at 108,000 g. The 5-HT content being determined on the sediments and the supernatant. The results obtained were, generally speaking, similar to those obtained on the P₂ fraction. The only difference being that the total recovery of 5-HT was less than the P₂ suspension (60-90%), especially in the raising temperature, a fact which suggests the presence of 5-HT inactivating enzyme in the microsome (see below).

**Table 2.** Effect of various treatments on the liberation of particulate 5-HT.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>5-HT liberated (in %)</th>
<th>Treatment</th>
<th>5-HT liberated (in %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raised temperature</td>
<td></td>
<td>KCl (10–40 mM) 10–25</td>
<td></td>
</tr>
<tr>
<td>(centigrade) (30 min)</td>
<td>60 91</td>
<td>NaCl (10–200 mM) &lt;25</td>
<td></td>
</tr>
<tr>
<td></td>
<td>37 75</td>
<td>CaCl₂ (10–200 mM) 0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20 33</td>
<td>MgCl₂ (10–200 mM) 0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4 4</td>
<td>Inorganic salt (4°C, 30 min) 47–62</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ether (9×19′) 94</td>
<td></td>
</tr>
<tr>
<td>pH (30 min)</td>
<td>3.0 84</td>
<td>Acetone (9×15′) 99</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.0 24</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>7.0 3</td>
<td>30′ 10</td>
<td></td>
</tr>
<tr>
<td>Osmotic pressure (sucrose in M) (30 min)</td>
<td>0.03 66</td>
<td>60′ 16</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.08 56</td>
<td>120′ 20</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.16 44</td>
<td>240′ 39</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.32 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.20 16</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**IV. Effect of 5-HT inactivating enzyme on the particulate 5HT.** A crude enzyme preparation was prepared from pig serum by the procedure described by Blaschko and Levine (1960), in which the later purification stage was omitted. It was confirmed, however, even our crude preparation could inactivate 5-HT of such a concentration as found in the P₂ or M₀ suspension on incubation for 15–30 min. So the P₂ fraction suspended in 0.32 M sucrose was incubated with somewhat large amount of enzyme at 37°C for 30 min, the incubation mixture was boiled at pH 4 to stop the enzyme action and to liberate 5-HT remaining in the granules and then it was applied on the rat’s fundus preparation after neutralization. As the control run, suspension was boiled immediately after adding the enzyme and then incubated, while the 5-HT solution was also incubated simultaneously with the enzyme to check the enzyme activity.

On incubation with the crude enzyme solution, the activity of inducing a rather rapid response of the rat’s fundus found in the original suspension was apparently lost, but a somewhat slower contraction with display of 60–90 sec was observed (Fig. 5). Such a slow contraction observed on incubation with the crude enzyme was considerably higher than the boiled control one. On the other hand, the boiled control gave the usual rapid response of 5-HT while the incubated 5-HT solution showed only a slight activity.
FIG. 5. Effect of a crude monoamine oxidase preparation on the particulate 5-HT.

Rat's fundus in 10 ml of aerated de Jalon solution with atropin (10⁻⁶) and pyribenzamine (10⁻⁶).

At the arrows, the samples were added into the bath.

a and f: control response to 5-HT (5 ng).

b and e: P₂ suspension boiled immediately before incubation with the enzyme.

c: P₂ suspension incubated with the enzyme.

d: 5-HT solution (5 ng) incubated with the enzyme.

(The records on the smoked drum were retouched).

Such a result appears to indicate at least that the particulate 5-HT was not unaffected by our enzyme preparation, probably inactivated, even though the origin of slow contraction remains to be determined.

V. Uptake of 5-HT by subcellular particles. To the P₂ or microsomal fraction suspended in 0.32 M sucrose, a known quantity of 5-HT solution (10-50 ng/ml) was added. After kept at 4°C for 30-60 min, 5-HT was determined on the sediments separated by centrifugation.

As the control, the particles were separated immediately after adding 5-HT, their 5-HT content being determined (exposure time 0 min). Their values were slightly larger (at most 10%) than that of the original particulate suspension. Such an insignificant increase would be probably due to adhering 5-HT-rich supernatant. Referring the 5-HT recovered in the sediment to control value thus obtained, we could not find appreciable uptake of added 5-HT by the
TABLE 3. Uptake of 5-HT by subcellular particles (P₂+Ms fraction)

<table>
<thead>
<tr>
<th>Time of exposure to 5-HT rich media (min)</th>
<th>5-HT concentration in the suspension 10 (ng/ml)</th>
<th>50 (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0'</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>30'</td>
<td>105</td>
<td>109</td>
</tr>
<tr>
<td>60'</td>
<td>96</td>
<td>109</td>
</tr>
</tbody>
</table>

Each value was estimated from recovered activity in the sediment after exposure to 5-HT rich sucrose (10 and 50 ng/ml), that found on the sediment separated immediately after adding 5-HT being taken as 100%.

particles. It might be said, therefore, neither the crude mitochondria nor the microsomes takes up added 5-HT significantly at 4°C, only a slight increase of 5-HT within the limit of assay error being observed.

VI. The subcellular distribution of 5-HT destroying enzyme. Using 5-HT solution (10 ng/ml) in 0.32 M sucrose as substrate, the 5-HT-inactivating power of each fraction was examined. The amount of each fraction subjected to the incubation with 5-HT was adjusted to be equivalent to each other in reference to the original wet tissue weight.

As illustrated in FIG. 6, only the microsomal fraction inactivated 5-HT significantly, the cytoplasmic supernatant feebly and other fractions showed hardly any inactivating action. When the particulate suspensions were boiled for 10-20 min prior to incubation, all the fraction showed no destroying activity. On the other hand, iproniazid added to the incubation mixture (4 mM/ml) inhibited the inactivating action of the Ms fraction. Such a result suggests an enzymic nature of 5-HT inactivating power found in microsomes.

Whittaker (1949) reported that the ether treatment enhanced the inactiv-
5-HT IN RABBIT BRAIN

5-HT in rabbit brain. After treated with ether as described in the previous section, however, we failed to confirm such an enhanced 5-HT inactivating power of microsomal fraction (FIG. 6).

It seems worthy to note here that the rat's fundus not only showed no contraction but also often responded with relaxation, when the incubation mixture of the microsome suspension and 5-HT was added to the organ bath, a fact suggesting the formation during incubation (or at least the presence) of a plain muscle relaxing substance in the suspension.

DISCUSSION

The data presented in this paper demonstrate that the particulate 5-HT of rabbit brain was found in the heavier granule as well as in the microsome, the so-called "synaptic vesicle" fraction (Whittaker, 1959) showing rather weak 5-HT activity, a finding in sharp contrast with that reported by Whittaker. Such a fact is probably attributable, as stated above, to his omitting acetone-extraction in his 5-HT determination. As for the accurate bioassay of 5-HT, CorreaLe (1958) and Costa (1960) described that not only the acetone-extraction to isolate it from practically acetone-insoluble SP, but also incubation with chymotrypsin to destroy SP contaminant completely was required. In the present experiments, the latter procedure was omitted and the possibility of slight contamination of SP might not be completely excluded. As shown above, however, the amount of neuronal 5-HT found by us is in good agreement with those obtained by careful study by Amin, Crawford and Gaddum (1954). Moreover, the fundus stimulating activity of acetone-insoluble aliquots expressed by the 5-HT equivalence shows good parallelism with their SP activity and its subcellular distribution is quite similar to that of SP (Kataoka, 1962), and of ACh (Whittaker, 1959), while the distribution of 5-HT found by us is rather similar to that of intestinal mucosa (Baker, 1959). Therefore, contribution of SP contamination would be only to a negligible extent if present.

Whittaker's findings that the particulate 5-HT is liable to be released and such an inactivation is not inhibited by iproniazid but enhanced by ether treatment are also correspondent to our observations on the neuronal particulate SP (Kataoka, 1962). On the other hand, the microsomal fraction was proved to have a significant 5-HT inactivating action on incubation with 5-HT, which could be inhibited by iproniazid and appears to be attributable to the presence of a monoamine oxidase in this fraction (cf. Blaschko and Levine, 1960). Furthermore, the disappearance of fundus-contracting activity of our particulate fraction on incubation with a crude 5-HT oxidizing enzyme preparation would be regarded as indicative of our observing true 5-HT activity in the brain homogenate. In view of these facts, the results on the brain 5-HT
reported by Whittaker might be said to be chiefly on SP in the brain.

As shown in the subfractionation of the washing fraction of nuclei, about a half of the nuclear 5-HT could be interpreted as contamination of 5-HT-containing granules. The 5-HT activity found in the cytoplasmic supernatant, a part of it at least, could originate from liberation of such a particulate 5-HT during preparation procedure, since homogenation was carried out in the absence of iproniazid and so the free 5-HT might be destroyed. In fact, as shown above, the particulate 5-HT could be liberated with relative ease by changes in the temperature, pH and osmotic pressure of suspension media. From the data presented here, however, it is impossible to deny the existence of free 5-HT other than the granule-bound one. The present experiments could provide hardly any data on the nature of 5-HT found in the N fraction, the only thing to be noted here being its unsatisfactory recovery compared with other subcellular particles (Fig. 1). It might be said from those results that, putting aside nuclei, the particulate 5-HT of brain is chiefly of heavy granular origin and of microsomal origin. If the presence of a substance in the so called synaptic vesicle fraction be one of the criteria for its playing a physiological role as a transmitter substance, therefore, 5-HT would have hardly any significance as a central transmitter.

Exactly speaking, the nomenclature of the subcellular fraction separated by centrifugal fractionation is, as discussed by Hebb (1959), not always corresponding to the morphological one. When examined electromicroscopically, the synaptic vesicle fraction, our V fraction, contains many kinds of granules, solid and vesicular, small (300-400Å) and large (2000-4000Å) as well as detached myelin lamellae and membranous structures of presumably mitochondrial origin (Shinagawa, et al., to be published). Gray and Whittaker (1960) as well as De Robertis and his co-workers (1960) showed the very fine electromicrograph in which the intact nerve endings detached from cell body as well as the numerous synaptic vesicles could be clearly observed. We could also confirm their results. Judging from their sizes, the former might be expected to be precipitated in P₂ fraction and recovered as heavier granules or vesicle fraction, while the latter in the microsomal one. In fact the particles quite similar to the synaptic vesicles in their size and appearance could be found in the Mb fraction (the microsomal granules), prepared by Hanzon-Toschi's method (1960). Hence it seems not impossible that the heavier 5-HT containing granules are the detached nerve ending in which numerous 5-HT-containing vesicles are enclosed and the latter appear in the microsomal fraction when the nerve endings are disintegrated by homogenation. This would be also applicable to the particulate SP of brain reported by KATAOKA. Such a comment may be only a speculation. There still remain some question as to whether the heavier particles are the storage granule as suggested by Baker and the lighter ones found in Ms fraction are their functional state, or whether these two kinds of
The particulate 5-HT is liberated by hypotonic solution or by the treatment with organic solvents as if it escapes through the lysed membrane. Even after repeated washings and being kept at 4°C for 1 hour, most part of its activity can be recovered in the sediment. It is apparent, therefore, that it is enclosed in the particles. But, there is some doubt as to whether it is present in the "bound" form as the particulate ACh claimed by Whittaker. The latter is not destroyed by choline esterase while the former seems to be inactivated by monoamine oxidase. Such a circumstance is quite similar to that of particulate SP. Since our enzyme preparation is crude, the above result may be ascribed to other enzyme or enzymes present in the crude preparation, which can destroy the particle membrane and disrupt the bonding in its bound form to liberate as its free form. It seems worthy to note here that only the potassium ion is effective in liberating the particulate 5-HT, other ions such as Na+, Ca++, and Mg++ is practically ineffective. These salts are added to the 0.32 M sucrose suspension. The hypertonic sucrose up to 1.2 M causes not so remarkable liberation, so that the effect of hypertonicity due to the salt concentration up to 200 mM would be unnecessary to take into account. As shown in FIG. 7, the effect of K+ ion is nearly proportional to logarithm of its concentration between 20-160 mM, a fact which is somewhat similar to the depolarizing effect of its ion. On the other hand, the divalent ion such as Ca++ and Mg++ is in general of protein-precipitating nature and their ineffectiveness seems not so unreasonable. As for the effect of these ions, the 5-HT-containing particle of brain appear to be different from the nor-adrenaline containing granules of the splenic nerve investigated by V. Euler and Lisjak (1961a).

The action of reserpine to liberate neuronal 5-HT is well known (cf. Shore, Silver and Brodie, 1955) and it seems somewhat curious to us that reserpine is not so effective in liberating the 5-HT in the heavy granules as well as light ones, only about 40% of the particulate 5-HT was liberated even after...
4 hours. This might be attributed, however, to the lower temperature (4°C) we employed.

The uptake of 5-HT by cellular elements was also well-known since the experiments on blood platelet by HUMPHRAY and TOH (1954) and HARDISTY and STACY (1955). But our observation demonstrates that the 5-HT-containing particles show hardly any power of taking up 5-HT from the suspension media. It would be also attributed to the lower temperature (4°C) and lower 5-HT concentration in the suspending media. At 4°C, no appreciable spontaneous loss of the particulate 5-HT was noted in 30 min (Table 2), and so we could draw hardly any conclusion as to whether or not the added 5-HT could impede the spontaneous loss of the granule-bound 5-HT as nor-adrenaline does on the adrenergic nerve granules (EULER and LISHAIKO, 1961b). The observations at 37°C are now going on in our laboratory and the preliminary results showed that the addition of much higher amount of 5-HT not only impedes the release of 5-HT, but also causes incorporation of 5-HT (about 150-300%). Hence the uptake of 5-HT by subcellular particles should be discussed in detail in the later paper. At present, however, they might be said not to be so remarkable at 4°C and at the low concentration of added 5-HT.

Finally, an enzyme or enzymes of inactivating 5-HT found in the Ms fraction should be mentioned. The activity of our Ms fraction to destroy 5-HT is lost after boiling and inhibited by adding iproniazid, so that it would be of enzymatic nature. A finding that such an activity could be found only in the Ms fraction, however, should not be considered to prove that only the Ms fraction contains the 5-HT inactivating enzyme. The amount of particulate suspension subjected to incubation should be taken into account. The monoamine oxidase activity in the brain mitochondria as well as its inhibition after iproniazid administration were already reported by GEY and PLETSCHER (1961). In our laboratory, activity to oxidize 5-HT and p-phenylendiamine was manometrically detected in the mitochondrial and microsomal fraction of rabbits (unpublished). Therefore, the above-stated results are attributable in the 5-HT inactivating power of each fraction, the Ms fraction being the highest. In this connexion, it seems worthy to note here that copper is proved in the Ms fraction (PORTER, et al. (1961); SHINAGAWA unpublished observation) and caeruloplasmin, a copper-containing protein can oxidize 5-HT as well as p-phenylendiamine BLASCHKO and LEVINE, 1960). Indeed, it was confirmed that the crude enzyme prepared from pig serum according to Blaschko's procedure inactivates the particulate 5-HT. It seems not always so unlikely that the 5-HT inactivating action of the Ms fraction is due to the presence of caeruloplasmin in this fraction. But its activity appears not to be inhibited by iproniazid. Further experiments are needed characterize the 5-HT inactivating activity of the particulate fraction of brain.
5-HT IN RABBIT BRAIN

SUMMARY

1. Employing the centrifugal fractionation method, the particulate 5-HT of rabbit brain was chiefly found in the heavier granules of crude mitochondrial fraction as well as in the microsomal particles. The higher 5-HT content of microvesicle fraction reported by Whittaker is attributable to his omitting the acetone extraction procedure, the activity observed by him being mainly that of substance P.

2. Such a particulate 5-HT was released by raising temperature, lowering pH, lowering osmotic pressure and by treating with ether, acetone or with higher concentration of KCl, while other cations such as Na⁺, Ca²⁺ and Mg²⁺ showed hardly any effect up to 200 mM. Reserpine caused loss of particulate 5-HT in about 40% for 4 hours.

3. The particulate 5-HT is susceptible to monoamine oxidase, which seems to destroy it nearly completely when incubated at 37°C.

4. The 5-HT-containing granules show hardly any uptake of 5-HT added to the suspension media at 4°C.

5. In the microsomal fraction, a 5-HT-inactivating action is proved, which disappears after boiling and is inhibited by iproniazid, but not so activated with ether as reported by Whittaker.

The expense of this work was partly defrayed by the Grant from the Ministry of Education in Japan and partly by the Grant of Hattori Hokokai Foundation. We should be grateful to Miss Junko Shinagawa and Mr. M. Imaizumi for their kind help throughout this work.

REFERENCES


