J. Physiol. (1974), **242**, *pp.* 61–76
With 2 text-figures
Printed in Great Britain

CELLULAR SPECIFICITY OF SEROTONIN STORAGE AND AXONAL TRANSPORT IN IDENTIFIED NEURONES OF *APLYSIA CALIFORNICA*

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(*Received 9 January 1974*)

SUMMARY

1. [^{3}H]D,L-5-hydroxytryptophan ([^{3}H]5HTP) was injected under pressure into cell bodies of identified cholinergic and serotonergic neurones in the central nervous system of the marine mollusc, *Aplysia californica*.

2. Both serotonergic and cholinergic neurones converted [^{3}H]5HTP to [^{3}H]5-hydroxytryptamine ([^{3}H]5HT).

3. The fate of [^{3}H]5HT in the two types of neurones differed. In serotonergic cells, 5HT was present primarily in particulate form; the transmitter readily moved from cell bodies into nerves by selective transport. In contrast, 5HT remained free in the cytoplasm of the cholinergic neurone, and was not transported from the cell body.

4. Treatment of *Aplysia* with reserpine decreased the proportion of [^{3}H]5HT associated with particulate material, and also decreased the amount of [^{3}H]5HT recovered.

5. Serotonergic neurones possess specific mechanisms for the storage and axonal transport of 5HT which are absent in cholinergic cells.

INTRODUCTION

A neurone is thought to release a characteristic transmitter substance at each of its synaptic terminals. The nature of the substance released is determined by several differentiated cellular properties. High-affinity uptake mechanisms (Shaskan & Snyder, 1970; Kuhar, Roth & Aghajanian, 1972; Snyder, Young, Bennett & Mulder, 1973), together with specific enzymic pathways (Kravitz, Molinoff & Hall, 1965; Giller & Schwartz, 1971; Barker, Herbert, Hildebrand & Kravitz, 1972), produce high intracellular concentrations of some transmitters. Specificity of release cannot be determined solely by a high intracellular concentration, since some putative transmitters (glutamate, glycine, and other amino acids) are

ubiquitous small molecules. For example, concentrations of glutamate have been shown to be similar in axons of excitatory and inhibitory lobster motoneurones (Kravitz *et al.* 1965), yet only excitatory neurones are believed to release glutamate (Gerschenfeld, 1973). Serotonin, however, has a limited distribution in nervous tissue. Using pressure injection to introduce radioactive compounds directly into cell bodies of neurones in the marine mollusc, *Aplysia californica*, Eisenstadt, Goldman, Kandel, Koester, Koike & Schwartz (1973) have shown that several identified cells convert choline to acetylcholine and that others convert tryptophan to serotonin. Synthesis of both transmitter substances did not occur in the same neurone. Thus, at least one component of the biosynthetic apparatus for each transmitter is a unique cellular characteristic. The first enzyme in the biosynthetic pathway for serotonin, tryptophan hydroxylase, determines whether a cell is serotonergic. Aromatic amino acid decarboxylase, the other enzyme in the pathway, is not restricted to cells which release serotonin, but is also involved in the biosynthesis of dopamine and norepinephrine (Iversen, 1967). Furthermore, a number of aromatic amino acids are substrates for this enzyme, which is distributed widely throughout nervous and non-nervous tissue (Iversen, 1967). Decarboxylases in neurones releasing the various aromatic amine transmitters may not be identical however (Sims, Davis & Bloom, 1973).

In order to determine whether there are specific characteristics which belong to a serotonergic neurone other than the presence of tryptophan hydroxylase, we have circumvented the enzyme by injecting its product, 5-hydroxytryptophan, into R2, the giant cholinergic neurone of the abdominal ganglion. Although the cholinergic cell synthesized significant amounts of serotonin, the alien transmitter remained free in the cytoplasm, and was neither stored in particles nor transported along the axon of the cell. In contrast, newly synthesized 5HT was incorporated in the serotonergic neurones into a particulate form, and was readily transported into axons. Thus, in addition to a specific biosynthetic enzyme, a neurone contains a characteristic apparatus for storage and transport of its transmitter substance.

METHODS

Aplysia californica, weighing between 70 and 120 g, were supplied by Dr R. C. Fay (Pacific Biomarine Corp., Venice, Cal.) and were kept at 15° C in aerated aquaria of Instant Ocean (Aquarian Systems Inc., Eastlake, Ohio). Ganglia were removed from the animal and pinned in a chamber designed for intracellular recording and stimulation of nerve trunks (Frazier, Kandel, Kupfermann, Waziri & Coggeshall, 1967). For impalements of RB cells (Frazier *et al.* 1967) and the giant metacerebral neurones (GCN), the connective tissue sheaths overlying the abdominal and cerebral ganglia were removed surgically. About twenty similar pigmented neurones, averaging 150 μm in diameter, form the RB cluster which is located in the right caudal

quarter of the abdominal ganglion. The two pigmented GCNs, lying symmetrically at the rostral edge of the dorsal surface of the cerebral ganglion, are the largest neurones in the ganglion, averaging 320 μm in diameter. R2, the largest neurone in the abdominal ganglion, was readily impaled through the sheath; it was also more easily damaged than were the smaller cells by removal of the sheath. Size, location, and electrophysiological properties were used to identify R2 and RB cells (see below and Fig. 1) (Frazier *et al.* 1967; Eisenstadt *et al.* 1973) and GCN (Weinreich, McCaman, McCaman & Vaughn, 1973).

Injections of [^3H]L-tryptophan and [^3H]D,L-5-hydroxytryptophan (5HTP)

Preparation of radioactive compounds and the technique of intracellular injection have been described (Eisenstadt *et al.* 1973). [^{3}H]L-tryptophan (5·6 c/m-mole, New England Nuclear Co.) and [^{3}H]D,L-5HTP (5·8 c/m-mole, Amersham-Searle) were concentrated, stored in about 1 μl. water at $-76°$ C, and assayed every few months by high-voltage electrophoresis or paper chromatography (Giller & Schwartz, 1971).

Ganglia were bathed in artificial sea-water supplemented with amino acids and vitamins at 22° C (room temperature) (Eisenstadt *et al.* 1973). For injections of [^{3}H]5HTP, 20 μM unlabelled L-5HTP was added to the sea water to dilute out [^{3}H]5HTP which escaped from the neurones after injection. After injections, ganglia were perfused continuously with fresh solution at a rate of about 15 ml./hr.

RB cells are spontaneously active neurones, with action potentials (mean and S.E. of mean) of 81 ± 1 mV ($n = 29$) and resting potentials of -46 ± 1 mV ($n = 29$). GCNs were silent, with antidromic action potentials of 75 ± 2 mV ($n = 23$) and resting potentials of -57 ± 2 mV ($n = 23$). R2 is also silent, with antidromic action potentials of 91 ± 2 mV ($n = 34$) and a resting potential of -54 ± 1 mV ($n = 34$). These values were obtained with the double-barrelled electrodes used for injection. Those for R2 are not different from values previously reported (Walker & Brown, 1970; Morales & Chalazonitis, 1970). A few cells with action potentials and a resting potential less than 75 % of the normal mean values were not injected. We did not use cells which failed to meet these criteria during or after injection. It was easier to damage the small RB cells than the larger GCNs or R2 during the injection, but fewer than one in eight RB cells were discarded.

We injected up to 3 nl. concentrated radioactive solution into R2, a volume corresponding to about 5 % of the cell volume (66 nl.; Giller & Schwartz, 1971). GCNs, whose cell body volumes are $14·4 \pm 0·8$ nl. ($n = 7$), were usually injected with less than 2 nl. RBs, whose cell body volumes are $3·2 \pm 0·4$ ($n = 14$), were injected with up to 0·5 nl.

Studies of axonal transport

At the end of the incubation period, injected cell bodies were dissected out of ganglia: R2 in sea water; RBs and GCNs under cold 70 % ethylene glycol in sea water (Giller & Schwartz, 1971). The nerves to be analysed were cut as they emerged from the ganglia. The locations of these cells and the distribution of their axons is illustrated in Fig. 1. R2 sends its major axon into the right connective, and a small branch into the branchial nerve (Frazier *et al.* 1967). The appearance of radioactivity in the right connective 6 hr after injection was measured to indicate axonal transport. Cells of the RB cluster vary in their axon distributions in the branchial, genital, pericardial, and siphon nerves: a member of this cluster can send axons into one or more of these nerves. The axon distribution of each injected RB cell was determined by intracellular recording of antidromic action potentials in response to nerve stimulation. We analysed all nerves containing axons of an injected neurone. Each of the two GCNs sends an axon into the ipsilateral connective to the buccal

ganglion (Weinreich *et al.* 1973); each cell also sends an axon into the ipsilateral lateral lip nerve. This distribution was demonstrated by recording antidromic potentials in the cell in response to stimulation of the lip nerve and by tracing the processes of the cell after intrasomatic injection of $CoCl_2$ (Pitman, Tweedle & Cohen, 1971). In each experiment, the buccal ganglion was left attached to the cerebral ganglion by the connectives, and the lip nerve was cut as it entered the lip musculature, usually at a distance of 9–15 mm. Transport from an injected GCN was determined by analysing radioactivity both in the lip nerve and in the buccal connective and ipsilateral buccal hemiganglion.

Escape of injected radioactivity from neurones

Labelled amino acids (Eisenstadt *et al.* 1973) and sugars (Ambron, Goldman, Thompson & Schwartz, 1974) escape from neurones after injection. A small fraction of this radioactivity is taken up by surrounding tissue, but is utilized far less efficiently than the injected material. Six hours after injection of [^{3}H]5HTP, we found about 35 % of the radioactivity in the perfusate; after [^{3}H]tryptophan injections, 70 % of the injected radioactivity escaped in 6 hr (Eisenstadt *et al.* 1973). Escape was greatest immediately after injection, but continued more slowly throughout the period of perfusion. A small amount of radioactivity which escaped from the injected neurone was taken up within the ganglion, but was much less efficiently converted to 5HT than injected material remaining within serotonergic cells. To assess general uptake of radioactivity which had escaped into the perfusate, we analysed nerves which did not contain an axon of the injected cell. For injections of R2 and RB cells, we analysed the left connective. When we injected one GCN, we analysed the contralateral lateral lip nerve and buccal connective, which contain axons of the other GCN. Uptake into these nerves was always insignificant (0·02–0·6 % of the radioactivity remaining in the injected neurone).

Treatment of Aplysia *with reserpine*

Reserpine (Serpasil®, a gift of the Ciba-Geigy Corp., Summit, N.J.), was suspended in supplemented artificial sea-water containing 15 % polysorbate (Tween-80, USP). We injected 2 mg reserpine in about 0·3 ml. through the foot directly into the hemocoel each morning for either 2 or 3 successive days. These animals were kept in isolated aquaria, under the same conditions as untreated animals.

Subcellular fractionation

Ganglia containing neurones injected with [^{3}H]5HTP were perfused continuously for 1 hr. R2 cell bodies were dissected from ganglia but RB and metacerebral cells were not. The cell bodies were homogenized with an unlabelled minced cerebral ganglion in a Potter–Elvejhem tissue grinder (Kontes Glass Co., Vineland, N.J.) in 0·8 ml. of an iso-osmotic buffer (0·2 M sucrose, 0·3 M-NaCl, 10 mM Tris HCl, pH 7·6) (Israël, Gautron & Lesbats, 1970) by five strokes of a loose-fitting Teflon pestle (clearance 0·25 mm). Ganglia containing injected RB cells or GCNs were homogenized without carrier nervous tissue. The homogenate was centrifuged at 1000 *g* for 10 min. The resulting pellet was homogenized again in 0·6 ml. buffer by three strokes of a tight-fitting Teflon pestle (clearance 0·13 mm) and centrifuged at 1000 *g*. The supernatants were combined with a 1000 *g* supernatant of homogenized nervous tissue (equivalent to one ganglion) and subjected to differential centrifugation at 9000 *g* and then 105,000 *g*. Before the high-speed centrifugation, additional carrier homogenate was added. To extract radioactive 5HT, pellets and high-speed supernatants

were deproteinized with 0·2 M perchloric acid after the addition of 50 n-mole of unlabelled serotonin creatinine sulphate (Schwarz/Mann, Orangeburg, N.Y.). Perchlorate was subsequently removed after neutralization with KOH.

Analytical methods

We separated 5HT from 5HTP, tryptophan, and other labelled metabolites by chromatography on columns of Amberlite CG-50, by high-voltage paper electrophoresis at pH 4·7, and by descending paper chromatography in *n*-propanol – 0·06 M-NaCl in concentrated NH_4OH, 5:1 (Eisenstadt *et al.* 1973). Paper chromatography was used to separate tryptophan from 5HTP. Radioactivity in protein was measured by liquid scintillation after precipitation with cold 5 % trichloroacetic acid on glass fibre pads (Schwartz, Castellucci & Kandel, 1971). Radioactivity in extracts of cells injected with [^{3}H]choline was analysed by high-voltage paper electrophoresis at pH 4·7 (Giller & Schwartz, 1971). In experiments using [^{3}H]tryptophan the incorporation of tryptophan into protein was measured, and the synthesis of 5HT was normalized to incorporation (Eisenstadt *et al.* 1973).

Values in tables are mean ± S.E. of the mean.

RESULTS

Because aromatic amino acid decarboxylase, which catalyses the conversion of 5HTP to 5HT, has been found in extracts of many identified neurones of *Aplysia*, including the GCNs and R2 (Weinreich, Dewhurst & McCaman, 1972), we expected that both serotonergic and other cells would convert injected [^{3}H]5HTP to [^{3}H]5HT.

Serotonin synthesis from [^{3}*H*]D,L-*5HTP in serotonergic neurones*

We analysed neurones 6 hr after injection, since by that time a significant proportion of the [^{3}H]5HT had moved into axons of the injected cells (described below). After 6 hr we found 0·04–1·57 p-mole [^{3}H]5HT in RB cells, which corresponded to 18·6 ± 2·0 % (n = 5) of the total neuronal radioactivity. The conversion rate of [^{3}H]L-5HTP to [^{3}H]5HT is probably 40 %, since the D-isomer is not likely to be a substrate for the decarboxylase. In GCNs we found 0·9–7·0 p-mole [^{3}H]5HT, corresponding to 24·7 ± 1·9 % (n = 6) of the total neuronal radioactivity.

The amount of radioactive transmitter found in the serotonergic neurones depended upon the amount of [^{3}H]5HTP injected (Fig. 2). Recovery of [^{3}H]5HT was directly proportional to total radioactivity in the neurone up to about 30 p-mole in GCNs and 6 p-mole in RB cells. Because of the difference in volume between the GCNs and RB cells, these amounts corresponded to an estimated intracellular concentration of 2 mM in both types of neurones. Above this value, greater concentrations did not result in proportionately greater increases in [^{3}H]5HT, suggesting that some component involved in the metabolism of 5HT was saturated.

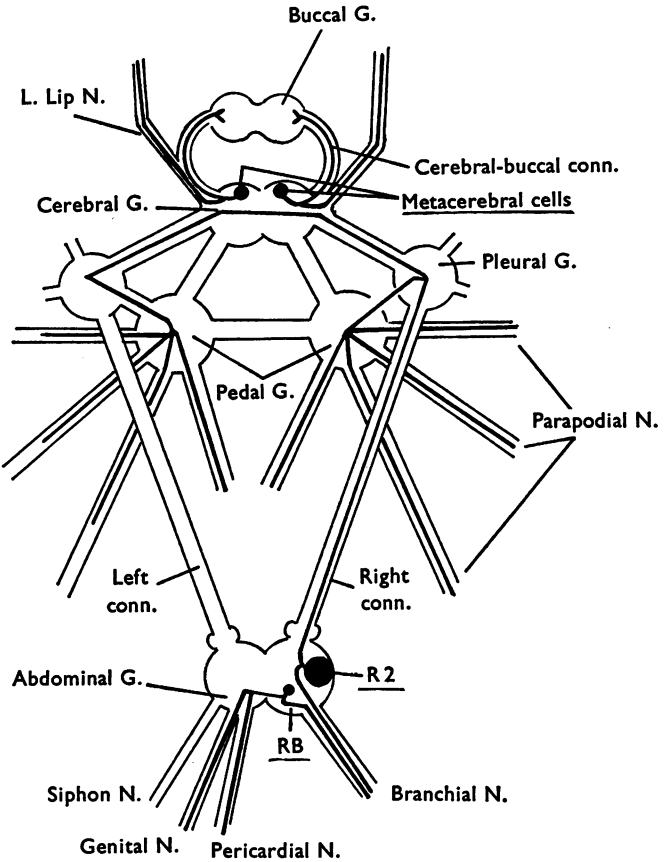

Fig. 1. Diagram of the central nervous system of *Aplysia* showing the location and axon distributions of R2 (Hughes & Tauc, 1961) and a member of the RB group of cells in the abdominal ganglion (G.), and of the pair of metacerebral cells in the cerebral ganglion. The lateral lip nerve probably corresponds to cerebral nerve 5 (*N. lateralis*) in other species of *Aplysia* (Bullock & Horridge, 1965).

Serotonin synthesis from [^3H]D,L-*5HTP in a cholinergic neurone*

R2 is larger than the serotonergic cells, and we could therefore inject more radioactivity. R2 synthesized significant amounts of [^{3}H]5HT from injected [^{3}H]5HTP, although its apparent rate of synthesis was lower than in the serotonergic cells. Six hours after injection, 0·3–0·5 p-mole [^{3}H]5HT were found in the neurone. This corresponded to $1{\cdot}4 \pm 0{\cdot}1$ % ($n = 4$) of the total neuronal radioactivity.

The amount of [^{3}H]5HT found in the cholinergic cell also depended

upon the amount of [^{3}H]5HTP injected (Fig. 2, inset). [^{3}H]5HT recovered after 6 hr was directly proportional to the amount of total radioactivity in R2 from 18·4 to 44·7 p-mole, the higher value corresponding to an intracellular concentration of 0·8 mM. Larger injections are possible and would presumably show saturation similar to that seen in RB cells and GCNs.

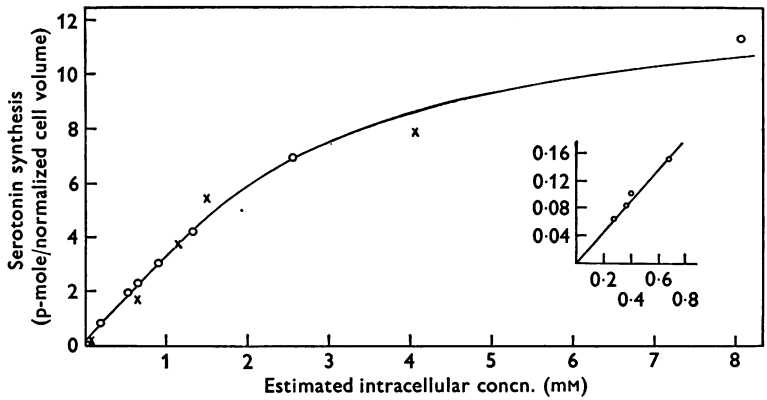

Fig. 2. Dependence of [^{3}H]5HT synthesis on the amount of radioactivity in identified neurones 6 hr after intrasomatic injection of [^{3}H]5HTP. The amounts of synthesis in GCNs (○—○) were plotted directly; values for RB (×—×) and R2 (inset) were normalized to a standard volume of 14·4 nl., which is the mean volume of a GCN. Thus, values for RB were multiplied by 4·5, those for R2 by 0·22. Under our conditions of scintillation counting, 1 p-mole of [^{3}H]5HTP corresponded to 2600 counts per minute.

[^{3}H]5HT from injected R2 cell bodies was identified by several methods. Radioactivity extracted from R2 was applied to a column of Amberlite CG-50. Material eluting together with unlabelled 5HT was lyophilysed and subjected to high-voltage electrophoresis at pH 4·7 or descending paper chromatography in *n*-propanol-ammonia. Three cells were analysed, two by electrophoresis and one by chromatography. At 6 hr, approximately half the applied radioactivity migrated with unlabelled 5HT during both electrophoresis and paper chromatography.

In R2, GCNs, and RB cells a small amount of the total cellular radioactivity ($0{\cdot}7 \pm 0{\cdot}1$ %, $n = 10$) was acid-precipitable. We have not characterized this material further.

Axonal transport of [^{3}*H*]*5HT*

In serotonergic neurones, newly synthesized 5HT was transported selectively into axons; this process was absent in the cholinergic neurone, R2. Six hours after injecting [^{3}H]5HTP into RB cells and GCNs, [^{3}H]5HT was found only in nerves containing axons of the injected cells, where it

constituted a greater proportion of the radioactivity than in the cell bodies (Table 1). A large fraction of the total neuronal [^{3}H]5HT had been transported by 6 hr; at the same time, a much smaller fraction of all other acid-soluble radioactive material was found in nerves (Table 1). We have not studied the axonal transport of [^{3}H]5HT synthesized from injected [^{3}H]tryptophan thoroughly, but in each of several experiments we also found that [^{3}H]5HT in axons of RB cells and GCNs was enriched compared to free tryptophan and to labelled protein.

TABLE 1. Axonal transport of [^{3}H]5HT 6 hr after intrasomatic injection of [^{3}H]-5HTP. Distribution and analyses of radioactivity appearing in nerves containing the axons of injected cells were obtained in several individual experiments. Whereas the radioactivity in the nerves for the other cells were characterized by ion exchange chromatography and by electrophoresis, those for R2 from the right connective were only chromatographed on Amberlite since the amount of radioactivity was insufficient for the complete analysis. Although some of the 5HT found in the ganglion fraction after injection of R2 would be localized in the intraganglionic proximal axon, most of the transmitter probably was synthesized in surrounding nervous tissue from precursor which had escaped from the injected cell body

Cell	Total radio-activity (p-mole)	Enrichment of 5HT (% radioactivity in fraction)			Movement of 5HT into nerve (% total [^{3}H]5HT in neurone)	Movement of other substances into nerve (% total)
		Cell body	Ganglion	Nerve		
GCN	9·4	18·8	21·8	66·5	36·4	6·3
	13·5	28·5	27·3	52·4	13·7	4·7
	19·3	21·2	14·3	54·1	17·7	4·8
RB	0·2	8·8	25·3	33·5	35·1	14·8
	2·0	6·3	35·2	57·4	32·4	4·9
	3·4	5·1	36·7	53·4	56·0	13·8
	4·5	8·1	35·1	50·9	53·2	16·9
	12·1	2·2	20·3	31·8	61·5	19·5
R2	18·4	1·0	4·8	1·8	21·1	13·8
	24·4	1·1	3·2	2·1	12·5	9·9
	26·1	0·7	4·3	1·1	6·3	6·9
	44·7	0·8	4·0	1·4	12·6	13·3

To explore the cellular specificity of transport, we analysed the movement of [^{3}H]5HT from the cell body into the axon of R2. Six hours after injecting R2 with [^{3}H]5HTP some [^{3}H]5HT was found in the right connective (Table 1). This transmitter was not transported selectively, however, since there was no enrichment in the connective. [^{3}H]5HT constituted about 1 % of the total radioactivity in the cell body (Table 1). The amount of radioactivity in the connective was too small to characterize by electrophoresis or chromatography. Nevertheless, only 1–2 % was

eluted from Amberlite columns together with carrier 5HT. Failure to observe axonal transport of 5HT did not result from a general absence of transport mechanisms, since R2 has been found to transport selectively both acetylcholine (Koike, Eisenstadt & Schwartz, 1972) and [^{3}H]fucose-labelled glycoproteins (Ambron, Goldman & Schwartz, 1974).

Subcellular distribution of newly synthesized serotonin

In order to obtain evidence that 5HT is incorporated into a stored form in serotonergic cells, we injected RB cells and GCNs with [^{3}H]5HTP, and subjected them to differential centrifugation. We found that 60–70 % of the newly synthesized 5HT was contained in particulate fractions, most of which was found in the 105,000 *g* pellet (Table 2). Sedimented material was greatly enriched in [^{3}H]5HT. In R2, however, [^{3}H]5HT, was recovered in the soluble fraction, and there was no enrichment in the pellet fractions (Table 2).

TABLE 2. Subcellular fractionation of identified neurones 1 hr after injection with [^{3}H]5HTP. Three ganglia were analysed individually by differential centrifugation for each cell type. A representative experiment for each cell is presented

Cell	Total radio-activity (p-mole)	Total 5HT (p-mole)	Fraction	Distribution: % total neuronal radio-activity	Distribution: % total neuronal 5HT	Enrichment of 5HT (% of radio-activity in fraction)
			A. Serotonergic neurones			
RB	4·7	1·0	9000 *g*	4·3	14·2	62·1
			105,000 *g*	9·9	47·6	90·5
			Supernatant	85·8	38·2	8·3
GCN	14·8	3·5	9000 *g*	3·2	8·9	61·4
			105,000 *g*	12·2	51·6	92·2
			Supernatant	84·6	39·5	10·2
			B. Serotonergic neurones after reserpine treatment			
GCN	11·7	0·2	105,000 *g*	2·2	16·7	10·0
			Supernatant	97·8	83·3	0·9
			C. Cholinergic neurone			
R2	34·7	0·9	9000 *g*	2·2	0·8	1·0
			105,000 *g*	1·6	1·4	2·1
			Supernatant	96·2	97·8	2·6

Effects of reserpine on subcellular distribution

Reserpine, which is thought to inhibit vesicular storage of biogenic amines (Iversen, 1967), diminishes serotonin fluorescence in several species of molluscs (Dahl, Falck, Mecklenburg, Myhrberg & Rosengren, 1966;

Cottrell & Osborne, 1969, 1970). Therefore, to strengthen the idea that the sedimentable [^{3}H]5HT was in storage granules, we injected GCNs from reserpine-treated *Aplysia* with [^{3}H]5HTP and subjected them to subcellular fractionation. We found that the proportion of [^{3}H]5HT associated with particulate material was considerably less than that in cells from untreated animals (Table 2). The amount of [^{3}H]5HT found in these cells had also diminished. Results from animals treated for 1 and 2 days showed no significant differences.

Abdominal ganglia from animals treated for 2 days were incubated with 40 μM[^{3}H]L-tryptophan for 15 hr. Two values for normalized synthesis of transmitter (0·004, 0·005 p-mole [^{3}H]5HT/p-mole [^{3}H]tryptophan incorporated into protein) were significantly lower ($P < 0{\cdot}02$ by t test) than those in ganglia from untreated animals ($0{\cdot}06 \pm 0{\cdot}006$, $n = 12$). It is unlikely that the diminished amount of [^{3}H]5HT produced after treatment with reserpine resulted from a non-specific effect of the drug. The amplitudes of antidromic spikes and resting potentials in R2 and GCNs were within the normal range. The incorporation of [^{3}H]tryptophan into protein and the conversion of injected [^{3}H]choline into acetylcholine in R2 were also unaffected.

DISCUSSION

Pharmacological and electrophysiological studies support the idea that 5HT is a neurotransmitter in nervous systems of molluscs (Gerschenfeld, 1973). Little biochemical information is available on its biosynthesis, storage, and axonal transport, however. We studied the neurones belonging to the RB cluster of the abdominal ganglion and the two metacerebral cells of the cerebral ganglion of *Aplysia* because of the many indications that they are serotonergic (Weinreich *et al.* 1973; Paupardin-Tritsch & Gerschenfeld, 1973; Liebeswar, Koester & Goldman, 1973). Homologous metacerebral neurones in *Helix* (Cottrell, 1970) and *Limax* (Cottrell & Osborne, 1970) have been shown to contain 5HT.

Synthesis of 5HT in Aplysia *neurones*

Both RB cells and GCNs are able to synthesize 5HT from [^{3}H]-tryptophan injected directly into their cell bodies (Eisenstadt *et al.* 1973). Synthesis is presumably catalysed by the same two enzymic reactions that have been described in vertebrate nervous tissue: hydroxylation of tryptophan, followed by decarboxylation. Although tryptophan hydroxylase has not yet been assayed in extracts of *Aplysia* nervous tissue, we found that approximately 5 % of the radioactivity could be recovered as [^{3}H]5HTP 6 hr after injecting [^{3}H]tryptophan into RB cells. In addition, Weinreich *et al.* (1972) have found aromatic amino acid decarboxylase

activity in extracts of identified neurones, both serotonergic and non-serotonergic. The decarboxylation of injected [^{3}H]5HTP to form [^{3}H]5HT in RB cells and GCNs reported here is consistent with those *in vitro* assays.

Dependence of 5HT synthesis on amount of precursor injected

We found that the amount of [^{3}H]5HT recovered in RB cells and GCNs depended upon the amount of [^{3}H]5HTP injected (Fig. 2). Above intracellular concentrations estimated at 2 mM, the introduction of larger amounts of [^{3}H]5HTP did not result in proportionately greater increases in [^{3}H]5HT. One explanation is that the synthetic enzyme had become saturated. Saturation has also been observed for synthesis of acetylcholine from [^{3}H]choline (Eisenstadt *et al.* 1973) and incorporation of [^{3}H]fucose into glycoproteins (Ambron *et al.* 1974) when large amounts of precursor were injected into R2. These data cannot be used to determine kinetic constants of the decarboxylase, however, since they are not initial rates. In addition, the intracellular concentration of the coenzyme, pyridoxal phosphate, is not known. Another explanation for saturation is that the amount of [^{3}H]5HT recovered might be affected by the availability of intracellular storage sites. If these sites were to become filled, excess 5HT might be unprotected, and would therefore be degraded more rapidly. Whatever the mechanism underlying saturation, if the percentage conversion is used as an indication of an injected cell's synthetic capabilities, proper interpretation requires information about saturation.

Storage of newly synthesized 5HT

A large proportion of the 5HT in molluscan nervous tissue is found to be associated with particulate material after subcellular fractionation (Cottrell & Maser, 1967; Hiripi, Sálanki, Zs.-Nagy & Muskó, 1973). In single identified serotonergic neurones, the transmitter has been localized to storage granules (Rude, Coggeshall & Van Orden, 1969; Cottrell & Osborne, 1970). We found that most of the newly synthesized 5HT in RB cells and GCNs was associated with particulate fractions (Table 2). Although identification of the cellular structures containing [^{3}H]5HT must await radioautographic studies, the distribution of [^{3}H]5HT and its enrichment in the high-speed pellet suggest it is located primarily in vesicular structures. These might be storage granules or organelles from which granules are synthesized (Holtzman, Teichberg, Abrahams, Citkowitz, Crain, Kawai & Peterson, 1973). The 9000 *g* pellet would be expected to contain pinched-off nerve endings (synaptosomes) (Whittaker, 1965). Since radioactivity from the injected cell body would not be expected to

have reached nerve terminals by 1 hr it is not surprising that we found relatively little [^{3}H]5HT in this fraction.

We found that a smaller proportion of the total cellular [^{3}H]5HT was associated with particulate material after treating the animal with reserpine, and the amount of [^{3}H]5HT recovered was also less than that in cells from untreated animals (Table 2). This decrease in recovery might have resulted from decreased synthesis, increased turnover, or both. Increased turnover is perhaps consistent with current theories on the mode of action of this drug (Iversen, 1967). If reserpine prevents uptake into granules, then unprotected 5HT might be rapidly destroyed. It is possible that most if not all of the neuronal 5HT is normally contained in granules, and that some of the [^{3}H]5HT which appeared to be free after differential centrifugation was stored initially, but escaped from granules which were damaged during fractionation.

Axonal transport of newly synthesized 5HT

Considerable evidence has been obtained that transmitter storage granules are transported along axons to synaptic regions (Dahlström, 1971). The site of synthesis of their contents was not determined. Direct evidence for transport of transmitters synthesized in the cell body has been obtained in only a few studies (Livett, Geffen & Austin, 1968; Koike *et al.* 1972; Fibiger, McGeer & Atmadja, 1973). It is likely that the transport of newly synthesized 5HT from cell bodies into axons of RB cells and GCNs is actually a measure of the movement of serotonergic granules. Movement of the transmitter appeared to be specific, since [^{3}H]5HT was transported preferentially to other acid-soluble radioactive material. We have begun to study the kinetics of transport by sectioning nerves into 1 mm slices at various times after intrasomatic injection and have found that, as the distance from the cell body increases, a greater proportion of the radioactivity in the nerve is present in the form of 5HT. In the most distal segments, 5HT constituted over 85 % of the total tritium.

The amount of neurotransmitter transported into the axon may be affected by the physiological activity of the neurone. A greater proportion of total neuronal [^{3}H]5HT moved into the axons of RB cells, which are spontaneously active neurones, than into those of GCNs, which are silent. While suggestive of a relationship between physiological activity and axonal transport, this correlation has not yet been studied in detail.

Specificity of axonal transport and storage

In addition to the presence of synthetic enzymes, there are other cellular components involved in metabolism and utilization which are specific for each transmitter. Axonal transport of a neurotransmitter is likely to be a

specific property of a neurone. For example, acetylcholine is transported selectively in R2 (Koike *et al.* 1972), and 5HT is transported selectively in RB cells and GCNs. There was no evidence for the transport of 5HT in the cholinergic cell, however. The small amount of [^{3}H]5HT found in the right connective probably moved by diffusion or by bulk axoplasmic flow.

Neurotransmitters are probably transported in vesicular form (storage granules, synaptic vesicles, or their precursors) (Dahlström, 1971; Geffen & Livett, 1971). A likely explanation for the absence of selective transport of 5HT in R2 is that the cholinergic cell did not package the alien transmitter. Cholinergic vesicles may be more labile during subcellular fractionation than serotonergic vesicles. It is therefore possible that the cholinergic vesicles of R2 do concentrate 5HT, but that their greater instability during fractionation accounts for our results. We feel this is unlikely however, since if 5HT were stored in R2, it would have been transported in the axon.

Storage granules also protect neurotransmitters against degradation. Since R2 did not store 5HT, the actual rate of synthesis in the cholinergic cell might have been greater than the one observed. Alternatively, degradative enzymes might be present in greater concentrations in serotonergic than in cholinergic neurones. The nature of these enzymes has not been characterized in molluscan nervous tissue.

We have found that R2 does not contain mechanisms for storage and transport of 5HT. In R2, therefore, 5HT cannot be described as a *false transmitter*, which would be stored and released as an analogue of a neurone's own transmitter substance (Kopin, 1968).

R2 does not normally contain detectable amounts ($< 0{\cdot}1$ p-mole) of 5HT (Weinreich, *et al.* 1973), indicating that in the animal the decarboxylase does not function in cholinergic cells to synthesize this transmitter. Presumably there might be conditions produced by therapy (Garattini & Valzelli, 1965) or by disease (Pare, 1968; Sandler, 1968; Baldessarini & Fischer, 1973) in which a cholinergic neurone might contain significant amounts of 5HTP or 5HT. Nevertheless, since a specific storage apparatus is absent, the alien transmitter would not be utilized by the neurone.

We thank Dr Jose Musacchio for his critical reading of the manuscript.

This research was supported by U.S. Public Health Service Medical Scientist Training Grant GMO-1168 to J.E.G; Career Award GM-28,125 to J.H.S.; and by NIH Research Grant NS-09955.

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