Short communications

Tryptophan metabolism in characterized neurones of *Helix*

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[^{14}C]-Tryptophan perfused through the central nervous system of the snail *Helix pomatia* is taken up by both 5-hydroxytryptamine-containing (GSC) and non-5-hydroxytryptamine-containing cells. Only the GSCs have the capacity to metabolize [^{14}C]-tryptophan to form some 5-hydroxytryptophan and slightly more 5-hydroxytryptamine. Electrical stimulation of the GSCs, strong enough to elicit a firing of spikes, resulted in more 5-hydroxytryptamine being produced, though there was also a slight increase in the amount of labelled tryptophan and 5-hydroxytryptophan. Doubling the length of stimulation and the amount of [^{14}C]-tryptophan perfused through the central nervous system had no great influence on the content of radioactive substances found in the GSC. Pretreatment of snails with *p*-chlorophenylalanine, though not interfering with the uptake of tryptophan into the GSCs, almost completely prevented the formation of 5-hydroxytryptophan. As well as showing that the enzyme tryptophan-hydroxylase is only present in cells containing 5-hydroxytryptamine, these experiments demonstrate the possibility of studying the metabolism of 5-hydroxytryptamine in characterized neurones.

Though the hydroxylation of tryptophan is probably the rate limiting reaction in the formation of 5-hydroxytryptamine (Bennet & Giarman, 1965; Garattini & Valzelli, 1965; Cooper, Bloom & Roth, 1970; Page & Carlsson, 1970), the precise localization of tryptophan-hydroxylase is not yet known. A microbiochemical method (Neuhoff, 1971; Osborne, Briel & Neuhoff, 1971) was used in this study to observe the metabolism of [^{14}C]-tryptophan in two specific types of giant cells in the snail *Helix pomatia*. One type of cell (GSC) situated in each cerebral ganglion is known to contain 5-hydroxytryptamine (Cottrell & Osborne, 1970), while the other, situated in each buccal ganglion, lacks this amine (Osborne & Cottrell, 1972a). It has already been shown that only the GSCs have the capacity to synthesize [^{14}C]-5-hydroxytryptamine from the precursor [^{14}C]-5-hydroxytryptophan (Osborne, 1972a) and that both cell types contain tryptophan (Briel, Neuhoff & Osborne, 1972; Osborne & Cottrell, 1972a). In addition, the effects of electrical stimulation and *p*-chlorophenylalanine on the metabolism of tryptophan in the GSCs were studied.

Methods.—The central nervous system of individual animals was perfused with snail saline (Meng, 1960) containing [^{14}C]-tryptophan (Radiochemical Centre, Amersham, specific activity 57 mCi/mmol, 5 μCi/ml of snail saline) at a rate of 1 ml/2 hours (see Osborne, 1972b) and then rinsed in pure snail saline for 5 minutes. In some experiments the normally silent GSCs (Cottrell, 1971; Osborne & Cottrell, 1972b) were selectively stimulated via the exterior lip nerves during the perfusion procedure to produce a firing of spikes (see Osborne, 1972b). Twelve neurones (either unstimulated or stimulated GSCs or buccal cells) were transferred to 10 μl Drummond capillaries containing 3 μl 0·05 M sodium bicarbonate solution, pH 10, and then frozen in liquid nitrogen and thawed to release the cell contents. (A single cell took less than 4 min to dissect, so that it would seem unlikely that any further metabolism or redistribution of the substances occurred.) Cold acetone (3 μl) was added to each capillary which was stored in a freezer (−5° C) for 30 min and centrifuged for 15 min at 20,000 *g* to sediment the precipitated proteins. The supernatants were transferred to clean capillaries, mixed with 3 μl unlabelled dansyl-chloride in acetone (2 mg/ml) and incubated in the dark at 37° C for 45 minutes. Each sample was evaporated to dryness under reduced pressure, resuspended in about 0·5 μl acetone/acetic acid (2:1 v/v) and applied to the corner of a 3×3 cm polyamide layer chromatography sheet (Carl Schleicher und Schüll, DC-Fertigfolie F 1700 Mikropolyamid). The microchromatograms were developed in the first dimension with water/formic acid (100:3, v/v) and, after drying, in the second dimension with benzene/acetic acid (9:1, v/v). Autoradiograms were prepared with Agfa Gevaert Dentus Ultra Rapid L film, exposure time 5 days.

Results.—Examination of the autoradiograms revealed that both the buccal cells (Fig. 1A) and GSCs (Fig. 1B and 1C) incorporate radioactive tryptophan. Included in the figure is an autoradiogram of substances (to which standard amounts of tryptophan, 5-hydroxytryptamine and 5-hydroxytryptophan have been added) in the GSC which react with [^{14}C]-dansyl-chloride (Fig. 1D) to show the chromatographic positions of tryptophan, 5-hydroxytryptamine and 5-hydroxytryptophan. It should be pointed out that in the conditions used for dansylation in this study (pH of sodium bicarbonate 10, reaction time 30 min), 5-hydroxytryptamine and 5-hydroxytryptophan form *N*- and di-dansyl-derivatives, with the *N*- form predominating (Neuhoff & Weise, 1970; Osborne, 1973b).

It is evident that although both the buccal cells (Fig. 1A) and GSCs (Fig. 1B and 1C) contain radioactive tryptophan, only the the GSCs metabolize the amino acid to form 5-hydroxytryptamine and 5-hydroxytryptophan. Examination of the autoradiograms from five different experiments showed that the GSCs incorporate slightly more radioactivity from the tryptophan into 5-hydroxytryptamine, than into 5-hydroxytryptophan (Fig. 1B). In three separate experiments electrical stimulation of the GSCs resulted in a slight increase in the radioactivity associated with all the substances (tryptophan, 5-hydroxytryptophan and 5-hydroxytryptamine), but was greatest in the case of 5-hydroxytryptamine (Fig. 1C). In two experiments where the cells were stimulated for a period twice as long as in the previous experiments, while

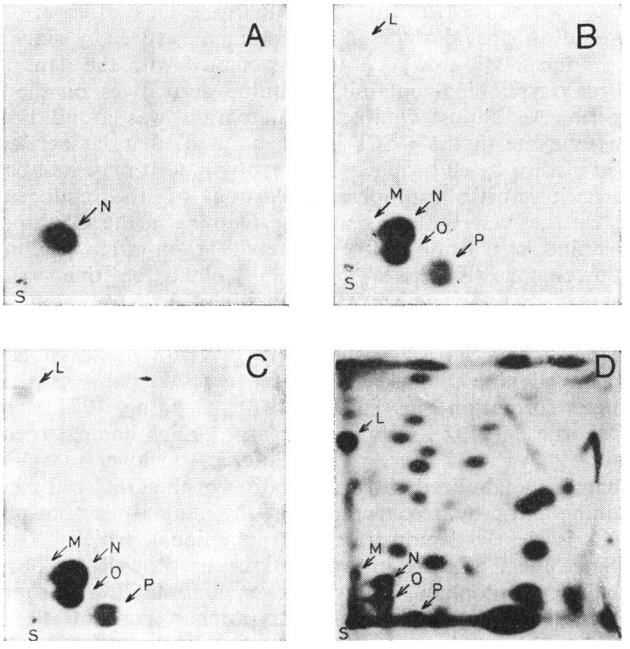

FIG. 1. Autoradiograms of microchromatograms from extracts of buccal cells (Fig. 1A), GSCs (Fig. 1B) and stimulated GSCs (Fig. 1C) from snails which were perfused with [^{14}C]-tryptophan, and cell extracts reacted with unlabelled dansyl-chloride. Figure 1D is an autoradiogram of substances (to which standard amounts of tryptophan, 5-hydroxytryptamine and 5-hydroxytryptophan have been added) in the GSC which react with [^{14}C]-dansyl-chloride. The original size of a single chromatogram measured 3×3 cm, and was developed in the 1st dimension (horizontal direction) with water/formic acid (100:3, v/v) and in the 2nd dimension (vertical direction) with benzene/acetic acid (9:1, v/v). It can be seen that both the buccal cells (Fig. 1A) and GSCs (Fig. 1B) incorporate tryptophan (as dansyl-tryptophan=N), but only the GSCs form 5-hydroxytryptamine (as *N*-dansyl-5-hydroxytryptamine=O and *N,O*-di-dansyl-5-hydroxytryptamine=L) and some 5-hydroxytryptophan (as *N*-dansyl-5-hydroxytryptophan=P and *N,O*-di-dansyl-5-hydroxytryptophan=M). Electrical stimulation of the GSCs (Fig. 1C) shows an increase in the 5-hydroxytryptamine content in relation to the tryptophan and 5-hydroxytryptophan concentrations . S=starting points on chromatogram.

the central nervous system was perfused with double the amount of [^{14}C]-tryptophan, a slight increase in labelled 5-hydroxytryptamine and perhaps tryptophan occurred, although the 5-hydroxytryptophan content remained constant.

In two further experiments snails were preinjected with 0·5 mg *p*-chlorophenylalanine before perfusion of the central nervous system with labelled tryptophan. An examination of the autoradiograms showed tryptophan to be taken up by the GSC with trace amounts of 5-hydroxytryptophan formed. Electrical stimulation of the cells did not result in an increase in radioactive 5-hydroxytryptophan or produce any labelled 5-hydroxytryptamine.

Discussion.—Though studies on vertebrate tissue have shown the distribution of the tryptophan hydroxylating enzyme (tryptophan-hydroxylase) to be similar to that of 5-hydroxytryptamine (Lovenberg, 1972), this investigation provides the first direct proof of the localization of such an enzyme in 5-hydroxytryptamine-containing cells (GSCs) alone. An almost complete inhibition of this enzyme in the GSCs in snails pretreated with *p*-chlorophenylalanine would suggest that it is tryptophan-hydroxylase, as in the vertebrates, since *p*-chlorophenylalanine specifically affects the activity of this enzyme (Koe & Weissman, 1966; Jequier, Lovenberg & Sjoerdsma, 1967). The results also confirm earlier findings which have revealed that *p*-chlorophenylalanine blocks 5-hydroxytryptamine formation in snails (Cottrell & Osborne, 1970; Osborne, 1973a).

That tryptophan is metabolized to form 5-hydroxytryptamine and 5-hydroxytryptophan in the GSCs is clearly demonstrated in this study. Since the GSCs are known to convert 5-hydroxytryptophan into 5-hydroxytryptamine (Osborne, 1972a), these data provide additional proof that 5-hydroxytryptophan is the intermediary product in the synthesis of 5-hydroxytryptamine from tryptophan. However, it is surprising to find only slightly lower amounts of radioactivity associated with 5-hydroxytryptophan compared to 5-hydroxytryptamine, as the amount of endogenous 5-hydroxytryptophan in the GSC was found to be very much lower than that of 5-hydroxytryptamine (Osborne & Neuhoff, 1973; Osborne, unpublished observations). Electrical stimulation of the GSCs strong enough to produce a firing of spikes, however, clearly produces an increase in the amount of radioactive 5-hydroxytryptamine, while the content of labelled 5-hydroxytryptophan remains more or less constant. Unfortunately the radioactivity associated with the metabolites from tryptophan on the individual microchromatograms was very low, so that it was difficult to quantify accurately the increase in the 5-hydroxytryptamine content following stimulation. It may be worthwhile noting that over a 100% increase in the synthesis of 5-hydroxytryptamine from labelled tryptophan has been observed in the mid-brain region of the rat after electrical stimulation (Shields & Eccleston, 1972).

Doubling the length of stimulation and the amount of perfused tryptophan was found to increase the radioactivity associated with 5-hydroxytryptamine only slightly. This discovery requires further comment, especially since the fluorescence associated with the dansyl-5-hydroxytryptamine derivatives on the individual chromatogram was at all times only slightly less than that associated with dansyl-tryptophan. It may well be that only a proportion of the endogenous 5-hydroxytryptamine in the GSC incorporates radioactvity even after prolonged stimulation. This could be due to various factors. Firstly, 5-hydroxytryptamine is localized in a number of compartments within the GSC, including synaptic-type vesicles, lysosome-like particles (Cottrell & Osborne, 1970; Osborne, 1973a) and also the cytoplasm. Each compartment may have very different turnover rates. Secondly, stimulation of the GSC will result in an increase of the axoplasmic transport of 5-hydroxytryptamine, which occurs in snails (Osborne, Powell & Cottrell, 1971). Moreover, stimulation probably alters the tryptophan concentrations, as this amino acid is known to be reduced on stimulation of the ganglia (Osborne, Powell & Cottrell, 1972). Obviously, all these factors have to be considered if the precise rate of metabolism of 5-hydroxytryptamine within the resting or stimulated cell is to be analysed.

As with the findings of a number of other studies on identifiable molluscan cells (see Osborne & Neuhoff, 1973; Osborne, 1973b), the present experiments confirm the heterogeneity of neurones within the gastropod central nervous sys-

tem with respect to their amine content and the enzymes requisite for the production of transmitter substances. The procedure adopted in this investigation also makes it theoretically possible to study the precise turn-over rate of 5-hydroxytryptamine within a neurone in different physiological states.

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REFERENCES

BENNET, D. S. & GIARMAN, N. J. (1965). Schedule of appearance of 5-hydroxytryptamine (serotonin) and associated enzymes in the developing rat brain. *J. Neurochem.* **112**, 911–918.

BRIEL, G., NEUHOFF, V. & OSBORNE, N. N. (1972). Determination of amino acids in single identifiable nerve cells of *Helix pomatia*. *Intern, J. Neuroscience*, **2**, 129–136.

COOPER, J. R., BLOOM, F. E. & ROTH, R. H. (1970). *The biochemical basis of neuropharmacology*. Oxford University Press New York.

COTTRELL, G. A. (1971). Synaptic connections made by two serotonin-containing neurons in the snail (*Helix pomatia*) brain *Experientia*, **27**, 813–815.

COTTRELL, G. A. & OSBORNE, N. N. (1970). Subcellular localization of serotonin in a serotonin-containing neurone. *Nature, Lond.*, **225**, 470–472.

GARATTINI, S. & VALZELLI, L. (1965). *Serotonin*. Elsevier, Amsterdam.

JEQUIER, E., LOVENBERG, W. & SJOERDSMA, A. (1967). Tryptophan-hydroxylase inhibition: The mechanism by which *p*-chlorophenylalanine depletes rat brain serotonin. *Molec. Pharmacol.*, **3**, 274–278.

KOE, B. K. & WEISSMAN, A. (1966). *p*-Chlorophenylalanine: A specific depletor of brain serotonin. *J. Pharmaco. exp. Therap.*, **154** 499–516.

LOVENBERG, W. (1972). Serotonin synthesis in the brain. *5th Int. Congress on Pharmacol.* San Francisco, U.S.A. pp. 250–251. (Invited presentations).

MENG, K. (1969). Untersuchungen zur Störung der Hertztätigkeit beim *Helix pomatia*. *Zool. Fahr.*, **68**, 193–204.

NEUHOFF, V. (1971). Micromethods for protein and enzyme analysis. In: *Recent advances in Quantitative histo- and cytochemistry.*, eds. by Dubach, U. C. & Schmidt, U. pp. 109–142. Hans Huber, Bern.

NEUHOFF, V. & WEISE, M. (1970). Determination of picomole quantities of γ-aminobutyric acid (GABA) and serotonin. *Arzneim. Forsch.* (*Drug Research*), **10**, 368–372.

OSBORNE, N. N. (1972a). The *in vivo* synthesis of serotonin in an identified serotonin-containing neuron of *Helix pomatia*. *Intern. J. Neuroscience*, **3**, 215–219.

OSBORNE, N. N. (1972b). Effect of electrical stimulation on the *in vivo* metabolism of glucose and glutamic acid in an identified neuron. *Brain Research*, **41**, 237–241.

OSBORNE, N. N. (1973a). Microbiochemical and physiological studies on an identified serotonin neuron in the snail *Helix pomatia*. *Proc. IVth European Malacological Congress* (Geneva) (in press).

OSBORNE, N. N. (1973b). The analysis of amines and amino acids in microquantities of tissues. In: *Progress in Neurobiology*, eds. Kerkut, G. A. & Phillis, J. W. Pergamon Press, Oxford. Vol. 1 part 4 (in press).

OSBORNE, N. N., BRIEL, G. & NEUHOFF, V. (1971). Distribution of GABA and other amino acids in different tissues of the gastropod mollusc *Helix pomatia*, including *in vitro* experiments with ^{14}C-glucose and ^{14}C-glutamic acid. *Intern. J. Neuroscience*, **1**, 265–272.

OSBORNE, N. N. & COTTRELL, G. A. (1971). Transport of amines along the visceral nerve of *Helix pomatia*. *Z. Zellforsch.* **109**, 171–179.

OSBORNE, N. N. & COTTRELL, G. A. (1972a). Amine and amino acid microanalysis in two identified snail neurons with known characteristics. *Experientia*, **27**, 813–815.

OSBORNE, N. N. & COTTRELL, G. A. (1972b) The effect of optic tentacle removal on the transmitter content of the giant serotonin cell of *Helix aspersa*. *J. Neurochem.*, **19**, 2363–2368.

OSBORNE, N. N. & NEUHOFF, V. (1973). Neurochemical studies on characterized neurons. Naturwissenschaften. **60**, 78–87.

OSBORNE, N. N., POWELL, B. & COTTRELL, G. A. (1972). The effect of electrical stimulation on the levels of free amino acids and related compounds in the snail brain. *Brain Research*, **41**, 379–386.

PAGE, I. H. & CARLSSON, A. (1970). Serotinin. In: *Handbook of Neurochemistry*, ed. Lajtha, A. Plenum Press, New York, Vol. IV, pp. 251–261.

SHIELDS, P. J. & ECCLESTON, D. (1972). Effect of electrical stimulation of rat mid-brain on 5-hydroxytryptamine synthesis as determined by a sensitive radioisotope method. *J. Neurochem.*, **19**, 265–272.

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