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THE ASSOCIATION OF OCTOPAMINE WITH SPECIFIC NEURONES ALONG LOBSTER NERVE TRUNKS

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SUMMARY

1. Octopamine and its synthetic enzyme, tyramine β-hydroxylase (TBH), are found in high concentrations at two points along second thoracic nerve roots in lobsters. The first is in the proximal section of the second root between the ventral nerve cord and the bifurcation of the root into medial (to flexor muscles) and lateral (to extensors) branches. The second region of high concentration is within a well known crustacean neurosecretory system, the pericardial organ, located close to the ends of the lateral branches of the roots.

2. With several different staining procedures, small clusters of nerve cell bodies are found within the connective tissue sheath in the proximal regions of the second roots. No cell bodies are seen in the pericardial organ regions. Cell bodies are variable in number and position between corresponding roots in the same animal and homologous roots among different animals. The average numbers of cell bodies, however, correlate well with TBH and octopamine content, and with the synthesis of octopamine in these same regions of roots.

3. Small clusters of root cell bodies dissected from preparations have greater than 500-fold higher activities of TBH than isolated efferent excitatory and inhibitory or afferent sensory axons.

4. Along with octopamine, the preferential synthesis of acetylcholine and serotonin is also seen in proximal segments of roots. Acetylcholine synthesis in these regions may represent transmitter synthesized in the nerve terminals innervating the root cells. The role of serotonin in these regions

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is not understood at this time but the amounts of endogenous serotonin found are only a tenth of the amounts of octopamine present.

5. Dopamine is not synthesized from tyrosine in second thoracic roots. However, if DOPA or dopamine are used as precursor compounds, then noradrenaline, which is usually not found in lobsters, can be accumulated in proximal segments of roots.

6. Phenolamines are converted to two further metabolites by lobster tissues. The compounds are unidentified and are named fast and slow product on the basis of their migration on electrophoresis at acid pH. Some partial characterization of slow product reveals that it is a mixture of compounds that can be converted on mild acid hydrolysis to fast product and the parent phenolamine.

7. The several lines of evidence presented suggest that nerve cells found in the proximal segments of the second thoracic roots contain and can synthesize octopamine. Since not all the cells in any single root have been analysed for octopamine or TBH, however, the possibility that one or more of the cells contain physiologically interesting substances other than octopamine is not eliminated.

INTRODUCTION

Octopamine is the phenol analogue of noradrenaline. It was isclated from Octopus nervous tissue over 20 years ago (Erspamer & Boretti, 1951), but relatively little was known of its normal physiological role until recently. More than 10 years ago, it was suggested that octopamine and its precursor compound, tyramine, might play key roles in the hypotensive effects of monoamine oxidase inhibitors (see Kakimoto & Armstrong, 1962; Day & Rand, 1963; Kopin, Fischer, Musacchio, Horst & Weise, 1965). Both these substances led to the release of noradrenaline from sympathetic nerve terminals and the replacement of endogenous stores of noradrenaline with octopamine. The accumulated octopamine, in turn, could be released with nerve stimulation (see Kopin *et al.* 1965). Since octopamine had relatively little physiological action when compared to noradrenaline it was considered a 'false neurochemical transmitter'. Later studies demonstrated the natural occurrence and rapid turn-over of octopamine in sympathetically innervated tissues and the release of endogenous octopamine with nerve stimulation (Molinoff & Axelrod, 1969, 1972). These results prompted the suggestion that octopamine might be considered a 'cotransmitter' with noradrenaline (Molinoff & Axelrod, 1972). In mammalian systems it is difficult to determine whether unique octopamine cells exist or whether octopamine is found only in neurones that utilize noradrenaline as their transmitter substance. There is no histochemical procedure to visualize octopamine cells such as the fluorescence method

used for the detection of catecholamine containing neurones. Moreover, octopamine and noradrenaline metabolism cannot readily be separated in sympathetically innervated tissues, probably due to the low substrate specificity of the enzyme dopamine-β-hydroxylase for phenols and catechols.

Invertebrate preparations have proved to be richer sources of octopamine than mammalian tissues. Octopamine is found in nervous tissues from Octopus (Erspamer & Boretti, 1951; Juorio & Molinoff, 1974), *Aplysia* (Saavedra, Brownstein, Carpenter & Axelrod, 1974), *Helix* (Walker, Ramage & Woodruff, 1972) and *Homarus* (Barker, Molinoff & Kravitz, 1972) and octopamine synthesis has been demonstrated in *Periplaneta* (Robertson & Steele, 1973*a*) and *Romalea* and *Schistocerca* (Hoyle & Barker, 1975). Of particular importance is the demonstration in several systems of the presence of significant amounts of octopamine in the absence of detectable levels of noradrenaline (Saavedra *et al.* 1974; Barker *et al.* 1972). These findings suggest that unique octopamine containing neurones exist in invertebrate preparations and that useful information about the role of this substance might be obtained from studies with these systems.

The present two papers concern the role of octopamine in the lobster nervous system. In the first paper we demonstrate the association of octopamine with cells found in the connective tissue sheaths of nerve roots emerging from the thoracic regions of the lobster ventral nerve cord. In the second paper we describe the characteristic neurosecretory appearance of the cells and demonstrate the release of octopamine from two sites associated with the cells (Evans, Kravitz & Talamo, 1976). Preliminary reports of this work already have been published (Wallace, Talamo, Evans & Kravitz, 1974; Evans, Talamo & Kravitz, 1975).

METHODS

Tissues

Lobsters (*Homarus americanus*) weighing 0·5 kg were obtained from local dealers and kept in recirculating artificial sea water at 12–15 °C. The thoracic region of the ventral nerve cord is dissected in the following way. After removing the abdomen and appendages, the exoskeleton is cut along the top of the gill chambers on both sides and across behind the eyes. The extensions of the abdominal extensor musculature, the heart, the hepatopancreas, and part of the gut are removed along with the dorsal portion of the carapace. The remainder of the thorax is pinned on a layer of Sylgard 184 (Dow Corning) in a dish containing lobster saline: 462 mM-NaCl, 16 mM-KCl, 26 mM-$CaCl_2$, 8 mM-$MgCl_2$, 11 mM glucose, 10 mM Tris, 10 mM maleic acid, adjusted to pH 7·4 with NaOH, maintained at approximately 10° C. The extensions of the abdominal flexor musculature are carefully removed by cutting the insertions of the muscles along the body wall and the nerve bundles (the medial branches of the second roots) running into the flexors from below. A small posterior

portion of the nerve cord is exposed along the ventral mid line. The remainder lies in a canal formed by the floor of the thoracic body cavity and the underlying exoskeleton. The medial branches of the second roots are freed along the mid line where they exit from this canal. The lateral branches of the second roots from each ganglion are conspicuous nerve bundles running circumferentially and slightly caudally along the wall of the body cavity. They are usually cut at the dorsal rim of the body cavity, where they penetrate the pericardial sinus that overlies the extensor musculature and freed back to the point where they exit from the ventral canal (see Text-fig. 6, in Evans *et al.* 1976, for diagram showing location of second roots).

The skeleton forming the roof of the canal is cut along the mid line and the canal forced open, exposing the entire ventral cord. The branches of the second roots are carefully pulled through the floor of the thoracic cavity into the canal. The ventral cord is then removed by cutting the remaining roots from each ganglion, most of which branch and extend ventrally into the appendages.

The thoracic region of the ventral cord consists of six ganglia; the suboesophageal and thoracic ganglia 1–5 (Pl. 1). The ganglia are linked by connectives containing axons running from ganglion to ganglion or through the entire length of the nerve cord. Two pairs of roots leave ganglia T1–T5: thick first roots, cut close to the ganglia in Pl. 1, that branch extensively and send processes into the musculature of the body wall and appendages; thin second roots that leave connectives a short distance posterior to each ganglion and bifurcate a few millimetres from the nerve cord. Long lateral branches of the second roots are pinned above the shorter ramifying medial branches in Pl. 1. In some dissections the lateral branch is followed into the pericardial sinus to obtain the pericardial organ structure (see Evans *et al.* 1976). The proximal region of second roots referred to in Results extends from the nerve cord to 2 mm beyond the bifurcation into medial and lateral branches. The suboesophageal ganglion has many nerve roots associated with it. In this paper, only two of these are discussed, an anterior (*A*) and a posterior (*B*) root, both leaving the nerve cord posterior to the ganglion and homologous with the second roots of ganglia T1–T5.

Assays

Octopamine. Octopamine is measured by a modification of the enzymatic method of Molinoff, Landsberg & Axelrod (1969). The principle of the assay is to measure the amount of radioactive N-methylated octopamine formed from endogenous octopamine and [*methyl*-^{3}H]S-adenosylmethionine. Incubation mixtures contain: the enzyme phenylethanolamine-*N*-methyl transferase prepared from bovine adrenal glands, [*methyl*-^{3}H]S-adenosylmethionine (New England Nuclear, 12·6 c/m-mole), 0·1 M Tris buffer, pH 8·6, and tissue sample or standard DL-octopamine in final incubation volumes of 300 μl. Incubations are at 37° C for 30 or 60 min (depending on the enzyme activity). Reactions are ended by the addition of 0·5 ml. 0·5 M borate buffer at pH 10. A 6 ml. portion of toluene–isoamyl alcohol (3:2) is added, the tubes are shaken and the organic phase collected and washed once with borate buffer. Samples are extracted from the organic phase with 0·1 N-HCl. The HCl extracts are dried, taken up in a small volume of 0·1 N-HCl and counted in a scintillation spectrometer. Routine checks on the identity of the reaction products are performed by chromatography on thin layer silica gel plates (Eastman) in several solvents (see Molinoff *et al.* 1969).

Quantitation of reaction products. In a series of experiments we measured radioactive product formation with DL-octopamine and compared it with product formed with authentic samples of D(−) and L (+) octopamine. These were kindly supplied by Dr M. D. Armstrong. We observed that the D(−) isomer consistently gave

40–50 % higher values of octopamine than the L(+) isomer. The DL mixed isomers used in our standards invariably gave the sum of the individual isomers. It is not known which is the natural isomer of octopamine. If it is similar to noradrenaline, then the D(−) isomer should be the natural one and we actually should make an appropriate reduction in all our reported values. We have not done so in these studies since we have not determined the configuration of the naturally occurring isomer and the absolute levels are not essential to any of the arguments we present.

Tyramine β-hydroxylase (TBH). Enzyme activity is assayed by measuring the release of tritiated water from [1, 2-^{3}H]tyramine when tyramine is converted to octopamine. The procedure is described in detail in Wallace (1976). Briefly, tissues are rinsed in calcium and magnesium-free saline and homogenized with a ground glass homogenizer in 10–15 vol. 10 mM sodium phosphate, pH 7·4, containing catalase, 200 u./ml. Tissue debris is sedimented by low speed centrifugation and the supernatant fluid is collected. The homogenization and centrifugation steps are repeated with half the initial volume of buffer with catalase and the second extract is added to the first for assay.

Reaction mixtures contain in final concentrations: 0·05 mM-[1, 2-^{3}H]tyramine (1 c/m-mole), 100 mM potassium phosphate buffer, pH 6·6, 195 u. catalase (Boehringer), 30 mM potassium ascorbate, 0·5 % cutscum (Fisher), sufficient $CuSO_4$ or or N-ethylmaleimide to give maximal stimulation (separately determined for each tissue), and enzyme in a final volume of 12 μl. Incubation is carried out at room temperature (22° C) for 20–60 min without shaking.

The reaction is stopped by dilution with 0·5 ml. water and the entire mixture transferred to a 2 × 0·5 cm column of BioRad AG50 W-X2 (H^+ form, 100–200 mesh) cation exchange resin. The assay tube and column are rinsed with an additional 1·5 ml. water. Both tyramine and octopamine bind to the column. The eluent, containing the tritiated water formed during the assay, is collected directly in a scintillation vial for counting.

Incubations with radioactive precursors

Second thoracic roots attached to or separated from ganglia and connectives are incubated with radioactive precursors either in lobster saline (see above) or in modified L-15 culture medium (see Hildebrand, Barker, Herbert & Kravitz, 1971). The formation of octopamine and its metabolites or the synthesis of other presumed transmitter compounds is measured in the following way. Tissues are placed in small wells in Sylgard coated Petri dishes in 0·8–1·0 ml. incubation fluid containing 10–50 μc of radioactive precursor compound. The radioactive precursors used in these experiments included [*methyl*-^{14}C]choline, 61 mc/mole; [*methylene*-^{14}C]L-tryptophan, 58 mc/m-mole; [U-^{3}H]L-tyrosine, 49 c/m-mole purchased from Amersham: [U-^{14}C]L-glutamate, 260 mc/m-mole; [G-^{3}H]tyramine, 5 c/m-mole; [2-^{3}H]DL-octopamine, 4·7 c/m-mole; [G-^{3}H]dopamine, 8·6 c/m-mole, [G-^{3}H]DOPA, 11·5 c/m-mole purchased from New England Nuclear Corp. When phenolamine or catecholamine synthesis is examined, 2·8 mM ascorbate is added as well. All incubation mixtures contain antibiotics (penicillin-G, 1000 u/ml. and streptomycin sulphate, 0·5 mg/ml.). Incubations are at 12° C with shaking, usually for 18 hr. At the end of the incubation period, tissues are washed briefly in media without isotope. Tissues are placed in 20–50 μl. formate–acetate buffer pH 1·9 (0·47 M formic acid:1·4 M acetic acid) and frozen and thawed several times to extract soluble substances. The radioactive precursors and products in the extracts are separated by means of high voltage electrophoresis at pH 1·9 (Hildebrand *et al.* 1971). Electropherograms are scanned in a Packard Model 7201 Radiochromatogram Scanner to localize the radioactive peaks. Quantitative determinations are made by cutting

electropherograms into 1 cm sections, placing each section into a vial containing 0·2–0·5 ml. 0·01 N-HCl for extraction for 1 hr and, after adding Aquasol (New England Nuclear), determining the radioactivity in a scintillation spectrometer.

Analytical procedures

Octopamine. The initial step in all characterization experiments is high voltage electrophoresis at pH 1·9 on either standard (40 in.) or long (72 in.) papers. Radioactive product co-migrating with authentic octopamine is eluted from papers with electrophoresis buffer and either a second dimension of electrophoresis (borate buffer at pH 6·2) or paper chromatography is performed. For paper chromatographic identification of octopamine three solvents are used: (*a*) methyl ethyl ketone:propionic acid–water (200:65:55, R_f = 0·41), (*b*) sec-butanol–pyridine–acetic acid–water (300:5:20:50, R_f = 0·26), and (*c*) *n*-butanol saturated with 1N-HCl (R_f = 0·22).

Noradrenaline and dopamine. After electrophoresis at pH 1·9, paper chromatography is performed in solvent *b* above (noradrenaline, R_f = 0·16; dopamine, R_f = 0·23). Radioactive spots are eluted and quantitated as described above.

Phenolamine metabolites. Two groups of products of phenolamine metabolism are seen after electrophoresis at pH 1·9. These are characterized and named in accordance with their migration on the electrophoresis strips. The compounds have not been identified and the radioactive peaks seen each may contain more than one metabolite. Slow product runs near the origin and fast product runs close to noradrenaline on electropherograms (see Text-fig. 1, Results). Products, eluted from papers with water or 0·1 N-HCl, run to their former positions when electrophoresed a second time. Slow products are hydrolysed by heating for 10 min at 100° C in 1 N-HCl. After hydrolysis samples are dried under a stream of N_2, then taken up and dried down in turn in 1N-HCOOH, 0·01 N-HCOOH and H_2O. Samples are then dissolved in formate–acetate buffer, pH 1·9 for electrophoresis.

Protein determination. Proteins were measured by the procedure of Lowry, Rosebrough, Farr & Randall (1951).

Histological procedures

Cobalt chloride. Ganglia with attached second roots are pinned out in lobster saline in one of two wells in a Sylgard covered Petri dish (60 mm diam). The cut distal ends of the lateral branches of the second roots are passed through a drop of oil or a Vaseline seal and dipped into a 0·1 M solution of cobalt chloride in the second well (Pitman, Tweedle & Cohen, 1973). The ends of the nerves are cut again to ensure that they have not sealed up and the tissue is incubated at 5° C for 18–48 hr. During this period cutting of the ends of the roots is occasionally repeated. After incubation the ganglia and roots are removed, rinsed with saline, and treated with a 1% solution of ammonium sulphide in lobster saline for 30 min to precipitate intracellular cobalt. The tissue is rinsed in saline and fixed for 2–18 hr in ice cold 4% formaldehyde–1% glutaraldehyde, in a buffer containing 0·1 M sodium phosphate, pH 7·4, 517 mM sodium chloride, 90 mM sucrose, and 0·17 mM calcium chloride (Orkand & Kravitz, 1971). The fixed tissue is rinsed in a solution of 0·1 M sodium phosphate, pH 7·4, containing 517 mM sodium chloride, 0·17 mM calcium chloride, and 292 mM sucrose, dehydrated in ethanol, cleared in propylene oxide, and embedded in Epon 812.

Neutral red. Second thoracic roots are incubated for 20 min at room temperature in a 0·1 mg/ml. solution of neutral red (Stuart, Hudspeth & Hall, 1974) in lobster saline. To avoid small crystals of dye that adhere to the tissue, neutral red solutions are filtered immediately before use (Whatman no. 1). After staining, tissues are rinsed briefly in saline and examined as a whole mount on a glass slide.

Zinc iodide–osmium. In an attempt to specifically stain nerve terminals in the proximal region of the second roots, tissues were incubated in a solution of zinc iodide-osmium in a modification of the procedure described by Akert & Sandri (1968). The modification involves a tenfold reduction in the amount of osmium tetroxide added and the addition of 20% (v/v) of a 36·8% solution of formaldehyde. Under these conditions staining progresses more gradually and cells and their processes are seen. Second roots are incubated in this modified solution for 1 hr, dehydrated in graded ethanols, cleared in xylene and mounted in Lustrex on a glass slide.

RESULTS

Regional distribution studies

Our attempts to find octopamine containing neurones began by measuring the levels of endogenous octopamine and the activity of the enzyme that synthesizes it, TBH, in small regions of the lobster nervous system. The results are listed in Table 1. In general, low levels of octopamine and TBH activity are found throughout the lobster ventral nerve cord but none are detectable in exoskeletal or heart muscles. In certain regions, very high levels of octopamine and TBH activity are seen (values in bold type, Table 1). These regions are the proximal portions of the second roots of thoracic ganglia, and the trunks of the pericardial organs near the ends of the lateral branches of these roots (see Pl. 1, Methods, Text-fig. 1 and Evans *et al.* 1976). The data shown in Table 1 suggest that there are far greater differences in enzyme activity than in endogenous octopamine content. For example, in thoracic ganglion 1, the proximal segments of second roots show sixtyfold higher levels of enzyme activity and only sevenfold higher amounts of octopamine than the corresponding ganglia. The octopamine data, however, are presented as total amounts in a section of tissue and do not take into account the difference in weights of tissue. We did not routinely weigh tissues. In order to help evaluate the significance of differences between ganglia and various parts of roots in this and later experiments, however, sample protein measurements may be useful. The protein contents of typical ganglia are: T_1 = 48 μg, T_2 = 39 μg, T_3 = 39 μg, T_4 = 39 μg, T_5 = 36 μg. The proximal segments of second roots are about half the length of the lateral segments (see Pl. 1, Text-fig. 1) but they contain about the same amount of protein. Some examples are: T_1 prox = 13 μg (0·8 cm), T_1 lat = 12 μg (1·8 cm); T_2 prox = 18 μg (0·6 cm), T_2 lat = 12 μg (1·2 cm); T_3 prox = 14 μg (0·4 cm), T_3 lat = 17 μg (1·7 cm). Medial root segments are more variable in length and weight but the amounts of tissue used are at least as great as in proximal segments. When one takes into account such protein differences and calculates octopamine levels per mg protein, the ratios of endogenous octopamine between proximal segments of roots and ganglia come more into line with the enzyme differences noted above (e.g. ratios of proximal

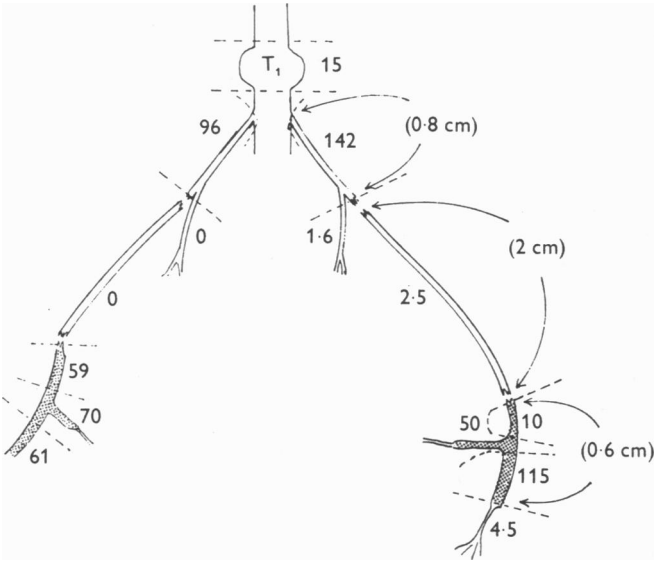
TABLE 1. Octopamine content and tyramine β-hydroxylase activity in segments of the lobster nervous system

Tissue	Octopamine (p-mole)	TBH activity (p-mole/min. mg protein) (4 expts.)
Cerebral ganglion	22 ± 3 (5)	
connective	3·3 ± 1·2 (5)	
Suboesophageal ganglion	8 ± 4·0 (3)	34 ± 3·4
Root *A* prox	**30 ± 6·4 (5)**	**497 ± 60**
lat	1·3 ± 1·7 (7)	57 ± 8·5
Root *B* prox	**84 ± 6·9 (15)**	**1937 ± 331**
lat	7·0 ± 1·7 (12)	106 ± 19·5
Connective	16 ± 8·0 (3)	29 ± 7·9
Thoracic 1 ganglion	11 ± 2·8 (4)	30 ± 3·5
Root 1		13 ± 4·6
Root 2 prox	**78 ± 8·8 (15)**	**2051 ± 401**
med	2·5 ± 0·7 (9)	53 ± 28
lat	3·2 ± 0·8 (10)	60 ± 8·5
Connective	10 ± 3·3 (4)	36 ± 5·9
Thoracic 2 ganglion	8 ± 2·9 (4)	27 ± 3·4
Root 1		13 ± 4·1
Root 2 prox	**96 ± 6·1 (16)**	**1165 ± 180**
med	3·3 ± 1·0 (9)	65 ± 17
lat	7·5 ± 3·8 (13)	36 ± 7·5
Connective	7 ± 1·7 (3)	27 ± 5·6
Thoracic 3 ganglion	15 ± 6·2 (5)	29 ± 5·8
Root 1		6 ± 1
Root 2 prox	**92 ± 13·4 (17)**	**959 ± 224**
med	3·7 ± 2·3 (9)	169 ± 36
lat	4·1 ± 1·9 (12)	18 ± 14
Connective	6 ± 1·2 (4)	20 ± 3·9
Thoracic 4 ganglion	6·8 ± 2·1 (5)	29 ± 2
Root 2 prox	**37 ± 9·6 (15)**	**485 ± 90**
med	**14·4 ± 4·1 (9)**	**383 ± 72**
lat	2·9 ± 1·0 (11)	7 ± 6·4
Connective	5 ± 1·1 (3)	11 ± 3·6
Thoracic 5 ganglion	16 ± 9·6 (3)	23 ± 2·9
Root 2 prox	11·0 ± 3·5 (9)	11 ± 3·5
lat	2 ± 2 (5)	6 ± 3·5
Connective	16 ± 6·5 (3)	20 ± 2·4
*Abdominal 1	1·8 (3)	
*Abdominal 2–5	1·4 (3)	
*Abdominal 6	2·4 (3)	
Pericardial organ T_1	**134 ± 19·8 (8)**	
T_2	**145 ± 15·8 (13)**	
T_3	**41 ± 5·0 (9)**	
Muscle (walking leg)	n.d.	
Muscle (heart)	n.d.	

* Data from Barker *et al.* (1971), recalculated to give total in p-mole.

n.d. = not detectable. The lower limit of detection of octopamine is about 1 p-mole. Results are expressed as the mean ± S.E. of the mean with the number of samples in parentheses.

segment/ganglion are $T_1 = 26$, $T_2 = 25$, $T_3 = 17$). A closer examination of the distribution of octopamine along a pair of second roots from a first thoracic ganglion is shown in Text-fig. 1. In this experiment the pericardial organ regions (stippled on the Text-figure and easily seen in the preparation by their bluish appearance) have been subdivided to show local concentrations of octopamine.



Text-fig. 1. Distribution of endogenous octopamine along a second thoracic root from a first thoracic ganglion. The relative lengths of sections of root are indicated in parentheses on the right side of the Figure. Octopamine is measured as described in Methods and values are p-mole octopamine in each section. The dotted lines signify the divisions between sections. The stippled area is the region of the pericardial organ.

Finding octopamine concentrated in the pericardial organ section of roots was not surprising since these structures are well known neurosecretory regions (see Evans *et al.* 1976, for discussion). On the other hand it was unusual to find octopamine concentrated in the proximal segments of the second thoracic roots. As far as we knew, the only neuronal elements in these segments were axons bringing information into or out of the ventral nerve cord.

Anatomical observations: finding the root cells. To explore this unusual localization further we dipped the cut ends of the lateral branches of the second thoracic roots into a solution of cobalt chloride (see Methods). The next day roots were treated with $(NH_4)_2S$ to precipitate cobalt sulphide, the tissues were fixed and cleared, and the proximal segments of roots were

examined. A variable number of long spindle-shaped cells are seen in the proximal segments of all second roots (see Pl. 2*A*). The cells are approximately 100–150 μm long and 40–50 μm wide and often one can see a thin axon heading towards the end of the root that had been dipped in the cobalt chloride. The neutral red staining method of Stuart *et al.* (1974) is very effective in staining the root cells. Two examples of roots stained with this method are shown in Pl. 2*B*1, *B*2. Cells vary in position and number between the symmetrically arranged pairs of roots from the same animal and also in the analogous roots from different animals. Occasional cells are found along the medial or lateral branches of roots at considerable distances from the main groups of cells clustered at the bifurcations of the

TABLE 2. Counts of cells on proximal regions of second roots

Segment	Neutral red mean ± S.E. of mean (*n*)	Zinc iodide–osmium mean ± S.E. of mean (*n*)
Suboesophageal		
A	2·8 ± 1·3 (8)	2·7 ± 0·2 (6)
B	6·0 ± 4·0 (7)	6·2 ± 0·8 (6)
Thoracic 1	9·7 ± 3·2 (3)	11·7 ± 1·1 (6)
Thoracic 2	11·6 ± 1·5 (8)	12·3 ± 0·3 (6)
Thoracic 3	11·2 ± 1·3 (6)	11·0 ± 0·4 (6)
Thoracic 4	7·3 ± 4·2 (8)	6·3 ± 2·0 (6)
Thoracic 5	2·6 ± 2·1 (8)	1·5 ± 0·8 (6)

roots. Cell counts were made on tissues stained with neutral red and with the non-selective zinc iodide-osmium staining procedures (see Methods). The results with both methods agree closely, suggesting that neutral red stains all the cells in the second roots. The distribution of cell bodies is in the form of a bell-shaped curve with a plateau at T_1, T_2 and T_3 of about twelve cells per root (Table 2). We do not see significant numbers of cells staining darkly with neutral red in ganglia themselves or in other roots leaving ganglia. Often with either neutral red or the zinc iodide-osmium staining methods, one notes significant numbers of branches associated with the cells and a general granularity of the region around cell bodies (Pl. 2*C*) (see Evans *et al.* 1976).

Association of the root cells with octopamine

A rough correspondence can be seen between the number of root cells (Table 2) and the amounts of endogenous octopamine and TBH activity in the proximal segments of roots (Table 1). To quantitate this relationship we compared the average number of cells per root with TBH activity, endogenous octopamine, and the synthesis of octopamine from radioactive

tyrosine and tyramine. The coefficients of correlation between each of these parameters of octopamine metabolism and cell number are as follows: TBH activity = 0·77 ($P < 0·001$), endogenous octopamine = 0·59 ($P < 0·001$), synthesis from [^{3}H]tyramine = 0·40 ($P < 0·02$), synthesis from [^{14}C]tyrosine = 0·35 ($P < 0·02$).

TABLE 3. TBH activity in small clusters of root cells and in lengths of identified axons

T_1 Root cells	cpm	*E and I axons*	cpm
6 cells	1830	8 cm	0*
6 cells	1870	9 cm	0
6 cells	1690	*Sensory axons*	
2 cells	340	1·3 cm	0*
		4·5 cm	0
		5·0 cm	0

Root cell bodies are dissected from proximal segments of roots using fine forceps. *E* (excitatory), *I* (inhibitory) and sensory axon lengths are obtained as described previously (Barker *et al.* 1972). The axons are approximately 40 μm in diameter. Incubations for TBH are carried out by the technique described in Methods (see Wallace, 1976). * No activity above background (260 cpm in this experiment) is detected in any of the axon types used. However, one can set a lower limit of activity detectable in the assay (double background), and, taking into account the calculated volumes of the tissues, set a minimal ratio of greater than 500:1 for the TBH activity in root cells compared to the three other neurone types.

Next, TBH activity was measured in small groups of cell bodies dissected from roots, and compared to that seen in lengths of sensory axons and in single excitatory and inhibitory axons to exoskeletal muscles. The three kinds of axons used are the only neurone types (with the exception of the root cells) known to be present in the second thoracic roots. The results show that small groups of cells have measurable TBH activity while the other kinds of axons tested have no detectable activity (Table 3). A minimum estimate of the ratio of TBH activity between the root cells and other kinds of neurones, comparing equal volumes of tissue, shows that root cells have greater than 500-fold higher TBH activity.

Using the data of Table 3 we can estimate the percentage of total enzyme activity in a root attributable to the dissected cell bodies. A single cell body produces an average of 270 cpm of ^3H_2O in the 60 min incubation period of this experiment. This calculates out to 0·023 p-mole tyramine converted to octopamine per min per cell body. The twelve cell bodies of an average root therefore would produce 0·28 p-mole/min. From the enzyme data of Table 1 and the approximate weights of roots, the TBH activity in a single root is between 15 and 30 p-mole of octopamine formed per minute. Thus 1–2% of the enzyme activity in a root would be

associated with the cell bodies. This seems to be a reasonable figure when one considers that much of the enzyme in a root is probably in the endings of the root cells in the vicinity of the cell bodies (see Evans *et al.* 1976).

Synthesis of other transmitter candidates in regions of second thoracic roots

We next explored the possibility that transmitter candidates other than octopamine might be synthesized preferentially in the proximal regions of second thoracic roots. Roots from thoracic ganglia 1–3 were incubated overnight with radioactive precursor compounds (choline, glutamate, tyrosine, or trytophan) and then sectioned into proximal, medial and lateral regions. The regions were examined for the synthesis of likely transmitter candidates using the screening procedure of Hildebrand *et al.* (1971). There is considerable variability in the experimental results. In general, however, acetylcholine (ACh) and serotonin (5HT) are the only compounds besides octopamine synthesized preferentially in proximal segments. Between 0·05 and 0·1 p-mole ACh are synthesized per hour in proximal root regions. When proximal and lateral segments are compared, ratios of 1·6–5·5 times as much ACh is made in proximal segments. Under similar experimental conditions, the amounts of 5HT formed in proximal regions range from 0·25 to 0·5 p-mole/hr and little or no synthesis is seen in lateral segments. Since the synthetic rates for 5HT approached those for octopamine (unpublished observations) the levels of endogenous 5HT were measured as well. From 10 to 13 p-mole 5HT (about one tenth the octopamine levels, see Table 1) are found in the proximal regions (M. Livingstone, unpublished observations). Of the other transmitter candidates, small amounts of glutamate and GABA are found or synthesized equally in all segments of roots.

With tyrosine as precursor no catecholamines are synthesized by any segments of the second roots. The catechol compounds, DOPA and dopamine, however, are found to be good precursors of noradrenaline in proximal regions of roots (Table 4). With dopamine the principal product accumulating is noradrenaline and relatively little free dopamine is found in the tissues. The 'slow' and 'fast' products reported in Table 4 are unidentified metabolites of dopamine. They may correspond to the octopamine metabolites described below, but they have not been characterized further. With DOPA, large numbers of counts of the precursor compound are found in the tissue at the end of the incubation period but still one sees a preferential synthesis of noradrenaline in proximal segments of the roots.

Phenolamine metabolites

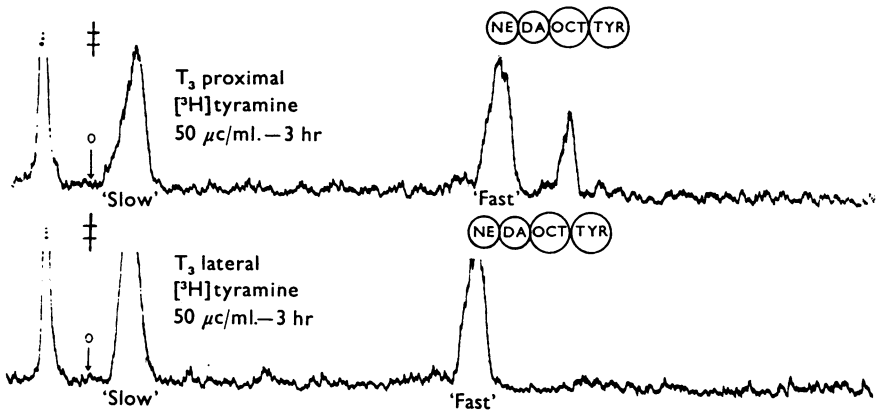
When [^{3}H]tyramine is incubated with second thoracic roots two principal categories of radioactive metabolites are seen. [^{3}H]octopamine is

found only in the proximal segments of roots (Text-fig. 2*A*), while two other radioactive peaks are seen on electropherograms of all sections of the root (Text-fig. 2*B*). One of these two peaks migrates to the amine

TABLE 4. DOPA and dopamine metabolism in second roots

		Products (c.p.m.)				
Precursor	Tissue	Slow	DOPA	Fast	NA	DA
Dopamine	T_1 prox	2180			7090	880
	T_1 med-lat				130	235
	T_2 prox	4330		245	10570	680
	T_2 med-lat	9730		1920	720	140
DOPA	T_1 prox	2750	42025	1180	11540	1910
	T_1 med-lat	830	32600	745	605	250
	T_2 prox	2765	63940	625	8150	1735
	T_2 med-lat	3405	90190	970	640	280

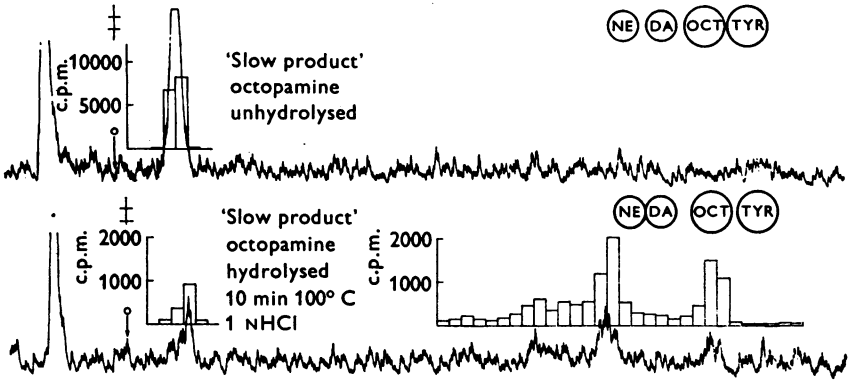
Results are expressed as c.p.m. in the various products. The noradrenaline (NA) column shows the greatest difference when comparing proximal with medial or lateral regions of roots. There is also a preferential accumulation and synthesis of dopamine (DA) in proximal segments. 'Slow' and 'fast' products may correspond to the phenolamine metabolites described in Results but they have not been characterized further.



Text-fig. 2. Radiochromatogram scans of high voltage electropherograms showing preferential accumulation of octopamine in proximal segments of second thoracic roots. The positions of standard compounds are indicated on the electropherogram above each radiochromatogram scan. The origin is signified by an 0 and arrow on the scan and a line with two cross lines on the electropherogram. Other abbreviations are NE = noradrenaline, DA = dopamine, OCT = octopamine and TYR = tyramine. The large peak at the left of each tracing is a radioactive marker spot used for lining up electropherograms and scans of radioactivity.

region of electropherograms and is called fast product and the second peak moves only a short distance from the origin, and is called slow product. The products are named solely on the basis of the regions of the electropherograms to which the metabolites run. Analogous metabolites are seen when other phenolamines are used as precursor compounds. Thus, with [^{3}H]octopamine as precursor, radioactive octopamine and the octopamine fast and slow products are seen in proximal regions of roots while only fast and slow products are seen in lateral root segments.

The radioactive substances found in slow or fast product regions of electropherograms are unidentified, but some partial characterization of



Text-fig. 3. Radiochromatogram scans of electropherograms showing the hydrolysis products derived from slow product. Abbreviations and general description are the same as in the legend of Text-fig. 1. The insert graphs provide quantitative data on the amounts of each substance found. They are obtained by cutting electropherograms into 1 cm segments and counting as described in Methods.

slow products has been carried out. When electrophoresis is carried out at pH 7·4 rather than the usual pH of 1·9, slow products still remain near the origin, indicating a lack of net change on these substances at both pH values. The slow product formed in each incubation consists of at least two substances. Mild acid hydrolysis (see Methods) will convert a slow product to the parent phenolamine and the fast product metabolite of that phenolamine. In Text-fig. 3 an experiment is shown in which slow product isolated from an octopamine incubation was treated in this way. In the upper trace it can be seen that unhydrolysed slow product runs as a single peak near the origin on electrophoresis. Hydrolysis converts the single peak to at least two products; one co-migrating with authentic octopamine, the other to the same position as the fast product formed from octopamine (lower trace, Text-fig. 3). On prolonged incubation slow products are released from tissues and begin to accumulate in the incubation media.

We presume that the unidentified metabolites and octopamine are made in different cell types because only the synthesis of octopamine is restricted to the proximal segments of the roots. A reasonable suggestion would be that root cells make octopamine while the other products are made in the glial or connective tissue cells found in all parts of the root. Some other preliminary evidence of differential sites of synthesis of the two types of product comes from an experiment in which sodium ions are left out of the incubation medium when tyramine is used as precursor. Quantitative measurements of fast product and octopamine synthesis are made in second thoracic roots in normal and sodium-free media. Roots on one side of a ganglion serve as controls for the experimental points obtained with the contralateral roots. In this experiment short incubation times are used (1 hr) and product accumulation in tissues usually is linear with time under these conditions. Octopamine synthesis is reduced to 27% of normal ($0{\cdot}27 \pm 0{\cdot}056$, $n = 6$) in sodium-free medium while fast product synthesis is unchanged or increased slightly ($1{\cdot}41 \pm 0{\cdot}42$, $n = 6$).

DISCUSSION

Octopamine and root cells. Several lines of evidence demonstrate a clear association of octopamine with thoracic root cells in the lobster nervous system. The highest levels of endogenous octopamine and of the activity of its synthetic enzyme, TBH, are found along the second roots of thoracic ganglia. These are thin roots that contain sensory axons from peripheral tissues and axons innervating the large fast flexor and extensor muscle systems of the abdomen that extend into the thoracic region. The proximal regions of these roots, extending from the ventral nerve cord to the bifurcations of the roots into medial (to flexors) and lateral (to extensors) branches, contain octopamine and TBH activity at levels one to several orders of magnitude higher than do the ganglia themselves or the medial and lateral branches of the roots. Farther along the lateral branches, the trunk regions of the pericardial organ system are found. This is another region high in octopamine that will be dealt with more fully in the next paper (Evans *et al.* 1976). The only morphological correlate that we can find for the high concentration of octopamine in the proximal regions of roots are the root cells themselves. The positive correlation of octopamine and TBH activity with the numbers of cells in roots supports the close association of octopamine with the cells.

Of several other transmitter candidates examined, ACh and 5HT also showed an asymmetric synthesis along second roots. While proximal segments again showed a higher synthesis of ACh compared to medial or lateral segments, the differential (2–5 ×) was much smaller than the differences in octopamine synthesis seen along the same roots. We believe these differences are significant, however, since in all cases equal or greater amounts of tissue were used when lateral and medial were compared to proximal regions. The total amounts of ACh formed were only 5–10% of the amounts of octopamine synthesized from tyramine in comparable

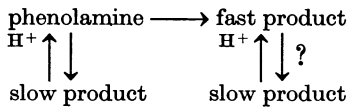
root segments. The preferential synthesis of ACh is probably important, however, and from evidence briefly described earlier (Wallace *et al.* 1974) it may represent transmitter synthesized in terminals innervating the root cells. The finding that 5-HT is concentrated and synthesized in the same regions as octopamine (M. Livingstone, unpublished) is of more direct relevance to the contents of the root cells. The levels of endogenous 5-HT are approximately one tenth those of octopamine. This raises the possibility that some of the root cells are 5-HT containing cells or that the root cells may contain both amines. These possibilities are presently under investigation.

Probably the strongest evidence that octopamine is actually contained in some of the root cells comes from the analysis of TBH activity in small groups of cell bodies. In all cases small clusters of root cell bodies dissected from roots had easily detectable levels of activity. By visual inspection the cell bodies were not contaminated by axons present in the roots but there are invariably small amounts of glial and connective tissue cell fragments associated with cells dissected in this manner. Similar contaminants, however, would be associated with the sensory and efferent excitatory and inhibitory axons used (the only other axon types known to be present in the roots), and in these latter cases no TBH activity was detected. It remains possible that contaminating terminals of other neurons could contain the octopamine, but generally, nerve terminals are not seen on or around root cell bodies on electron microscopic examination (B. Talamo, unpublished). Moreover, physiological evidence that octopamine has no effect on the cells (E. A. Kravitz, unpublished), makes it unlikely that octopamine is contained within contaminating terminals. The most reasonable conclusion is that root cells themselves contain and can synthesize octopamine from suitable precursor compounds It should be emphasized, however, that we have not individually analysed all the cells in any single root for octopamine or TBH. Therefore, the possibility that one or more of the cells contain substances other than octopamine cannot be eliminated.

Synthesis of noradrenaline from catechol precursors. Although synthesis of dopamine has been demonstrated in lobster nervous tissue (Barker *et al.* 1972), the second thoracic roots make no dopamine with either tyrosine or tyramine as precursor. This shows clearly that dopamine and octopamine are made in different cell types in lobsters. It suggests further that dopamine cells may contain a tyrosine hydroxylase enzyme and be missing TBH activity while octopamine cells will contain an inverse complement of enzymes. Lobster tissues contain no detectable noradrenaline (Barker *et al.* 1972). Lobster tyramine β-hydroxylase, however, will metabolize dopamine as well as tyramine to their corresponding β-hydroxylated

derivatives (Wallace, 1976). If the lobster enzyme catalyses the first step in octopamine biosynthesis, the decarboxylation of tyrosine could also convert DOPA to dopamine. One would then be presented with the interesting possibility of forming noradrenaline as a 'false transmitter' (see Day & Rand, 1963; Kopin *et al.* 1965). This is exactly the result observed, as both DOPA and dopamine can be converted to noradrenaline in proximal segments of roots.

Metabolism of phenolamines. No monoamine oxidase is found in lobster nervous tissue (Barker *et al.* 1972). Accordingly it was of interest to observe the metabolites produced when tyramine or octopamine is incubated with lobster tissues. In almost all tissues examined two classes of metabolites are seen. These remain unidentified at present but some comments can be made about their relationships to each other. Octopamine and tyramine each can be converted to a corresponding metabolite that still has a positive charge (fast product). Both the parent amines and their fast product presumably can be converted to second, slow products, that have no net charge at pH 1·9 or 6·4. We have not isolated fast products and directly demonstrated their conversion to slow products. Nor do we know whether there are intermediate steps in the conversion of the parent amines to either of their products. We have been able to demonstrate that slow product can be converted back to fast product and the parent amine under mild acid hydrolysis conditions, whereas fast product requires far more drastic hydrolysis conditions to convert it back to the parent phenolamine (unpublished observations). Therefore we suggest that phenolamines are metabolized to a family of products that bear the following relationship to each other.



Studies are currently under way to determine the identity of these compounds. Other preliminary studies show that slow product is released by tissues on prolonged incubation and that 15 min after injection of radioactive phenolamine into animals, slow products are the main radioactive substances found in the haemolymph (unpublished observations).

The general occurrence of octopamine neurones. Cells have been found in *Aplysia* that contain endogenous octopamine but do not contain dopamine or noradrenaline (Saavedra *et al.* 1974). The concentration of octopamine in these cells is 10^{-5}–4×10^{-4} M. In contrast if one determines the octopamine content of the lobster cells and relates it to the volume of the cell bodies, concentrations as high as 0·1 M are calculated. One should be cautious in such calculations, however, as much of the octopamine may be

contained in 'endings' of the root cells in the vicinity of cell bodies (see Evans *et al.* 1976). Physiological effects of octopamine also have been observed in *Aplysia* ganglia (Carpenter & Gaubatz, 1974). When octopamine is iontophoretically applied to neuropil regions in cerebral and other ganglia, hyperpolarizing responses are observed in cell bodies. Octopamine at high concentrations leads to the enhanced phosphorylation of specific proteins found in extracts prepared from the abdominal ganglia of *Aplysia* (Levitan & Barondes, 1974). Effects of octopamine have been reported on insect nerve cord glycogen break-down (Robertson & Steele, 1973*b*) and an octopamine sensitive adenyl cyclase is seen in homogenates of cockroach thoracic ganglia (Nathanson & Greengard, 1973). More recent studies suggest that insect dorsal unpaired medial neurones contain octopamine (Hoyle & Barker, 1975). Our studies demonstrate the likely presence of octopamine in root cells of the second thoracic roots of lobsters. In the next paper in this sequence we present morphological and physiological evidence suggesting that the cells serve a neurosecretory function (Evans *et al.* 1976).

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EXPLANATION OF PLATES

PLATE 1

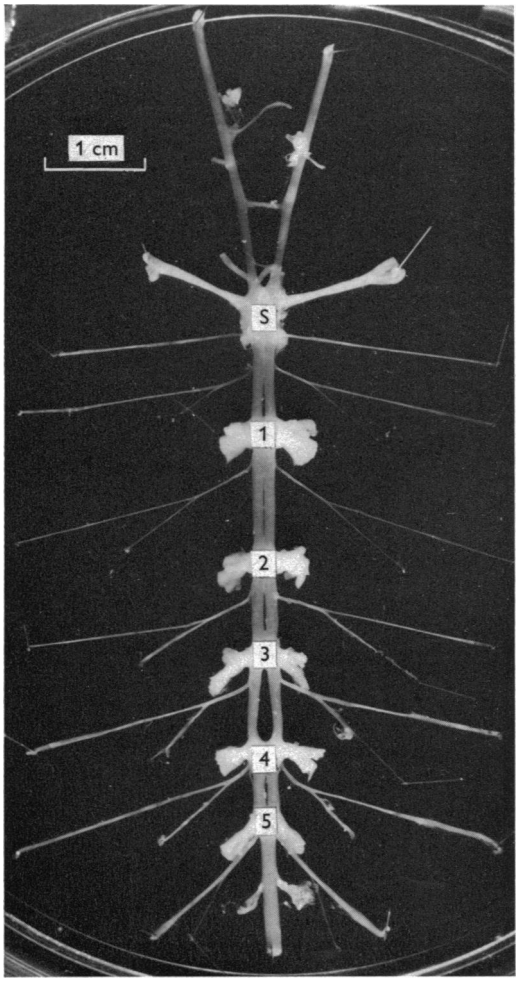
Dissection of the thoracic region of the ventral nerve cord. Ganglia are labelled, S = suboesophageal, 1–5 = thoracic ganglia 1–5. Further details of the dissection are provided in Methods.

PLATE 2

A, a cell body stained by cobalt chloride backfilling of axons. One cell body is visible with a fine process running towards the end of the root dipped in cobalt chloride. Another, more lightly stained cell body is seen below the darkly stained through axons found in the centre of the root.

*B*1, *B*2, cell bodies stained with neutral red. Both preparations are from third thoracic roots. In the lower figure one cell body is seen on a medial branch of the root. In general there is great variability in cell position.

C, branches in the immediate vicinity of cell bodies stained with zinc iodide–osmium. These cell bodies are from suboesophageal root SB, but similar results are obtained with all thoracic roots.



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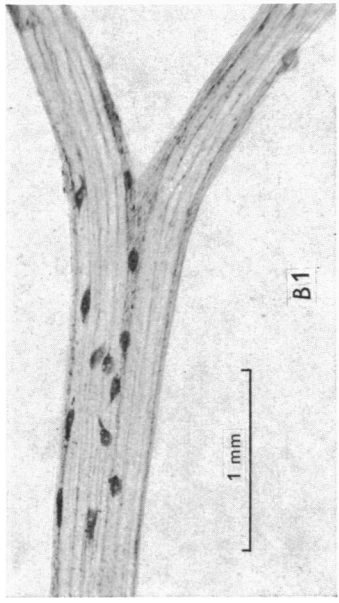
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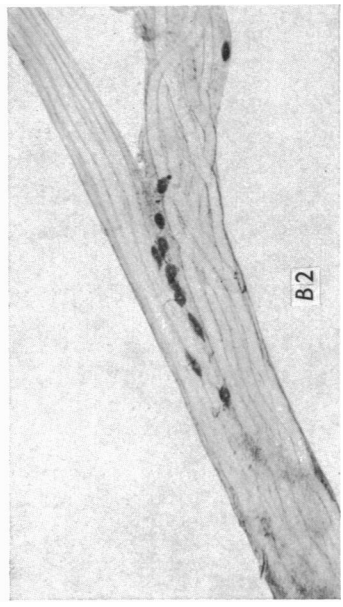
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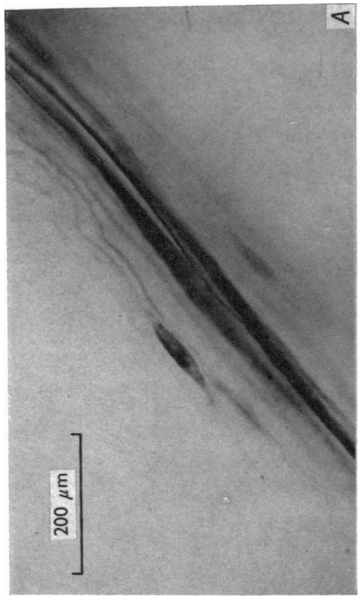
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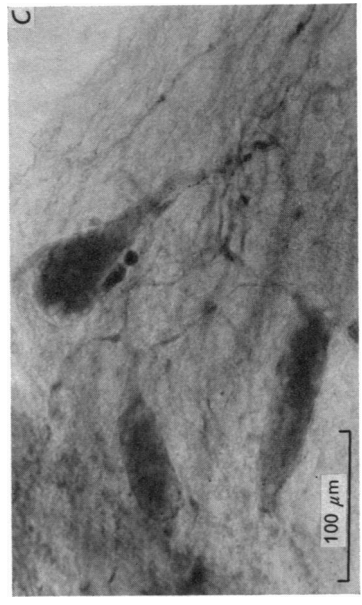
C
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