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IN VITRO ANALYSIS OF THE INSECT NEURO-ENDOCRINE ORGANS

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Cultures of neurosecretory cells and corpus cardiacum of the cockroach *Periplaneta americana* display vigorous outgrowth of nerve fiber processes *in vitro*. Numerous cells of various types migrated out of the corpus cardiacum into the medium. Light and electron microscopic studies showed the presence of neurosecretory granules in the cell bodies and in the axonal processes. Neurosecretory cells were electrically active *in vitro* and the unit electrical activity of NS cells contrasted sharply with the bursting electrical discharges of the CC cultures. The synthesis and release of radiolabeled juvenile hormone by adult and nymphal corpora allata indicated that these glands are functionally active *in vitro*.

INTRODUCTION

The insect neuro-endocrine organs (the neurosecretory cells of the brain, the corpora cardiaca, the corpora allata and the ecdysial glands) have been extensively investigated ever since they were first discovered (reviewed by BERN & HAGADORN, 1965; DOANE, 1973; MADDRELL, 1974; WIGGLESWORTH, 1964). Only recently it became possible to study them with *in vitro* techniques because the tissue culture media used for vertebrate tissues proved to be unsuitable for insect tissues. The considerable technical difficulties encountered in dissecting out the small insect endocrine glands and in devising culture media suitable to maintain these organs *in vitro* have hampered progress in this field. We owe a great deal to the efforts made by T.D.C. GRACE, G. R.WYATT, S. S. WYATT and others (see C. VAGO, 1971; 1972) who devised culture media and techniques suitable for growing insect cells and tissues *in vitro*. With further refinements of their methods, we are now in a position to investigate whole organs, or individual tissues or cells of a variety of insects and other invertebrates *in vitro*. A chemically defined synthetic medium and tissue culture techniques were developed (CHEN & LEVI-MONTALCINI, 1969) which allowed *in vitro* studies on the embryonic nervous system of the cockroach (LEVI-MONTALCINI & CHEN, 1969). These studies were extended to the neuro-endocrine organs of nymphal and adult specimens (LEVI-MONTALCINI, 1971; LEVI-MONTALCINI & SESHAN, 1973). Fully differentiated cockroach neurosecretory cells, the corpora cardiaca and the corpora allata of nymphal and adult cockroaches were maintained in organ culture for several weeks during which period they exhibited neuronal fiber processes, cell migration and other structural and functional features (SESHAN & LEVI-MONTALCINI, 1971; SESHAN *et al.*, 1974). It is the object of this article to report in a condensed form the results of these and related investigations.

MATERIALS AND METHODS

Preparation of cultures

Nymphs and adult cockroaches (*Periplaneta americana*) of both sexes were used as donors for the neuro-endocrine organs. Brain, thoracic ganglia,

foregut and heart of 15–16 day old embryos and ovaries of nymphs and adults were dissected out and cultured together with the neuro-endocrine organs. The neurosecretory cells of the pars intercerebralis, the corpora cardiaca, the corpora allata of nymphs and adults, embryonic organs and ovarian follicles were dissected out and prepared as described elsewhere (SESHAN, 1976; SESHAN & LEVI-MONTALCINI, 1971). To minimize bacterial and fungal contamination of cultures, the tissue culture room was equipped with germicidal lamps and all dissections were performed in a sterile atmosphere. Three commercially available media were used: SCHNEIDER's *Drosophila* medium, GRACE's insect medium (Grant Island Biological Co.) and EAGLE's basal medium (Microbiological Associates). At the moment of use, SCHNEIDER's and EAGLE's media were mixed in the ratio 5:4 respectively. GRACE's medium custom made without the amino acid methionine was used exclusively for biosynthetic studies on the corpus allatum. This medium was supplemented with 1% bovine albumin (Fraction V from bovine plasma, Metrix, Division of Armour Pharmaceutical Co.), 2 microcuries/ml of (S-methyl-^{14}C)-methionine (specific activities in the range of 47 to 58 Ci/mole (New England Nuclear) added to this medium served as a precursor for the juvenile hormone produced by the corpora allata. Other precursors used were 1 mg/ml each of mevalonolactone (Sigma Chemical Co.) and homomevalono-lactone. The latter was synthesized in our laboratory. Every fourth day the incubation media were collected and fresh media added to the cultures. The collected media were extracted and the hormone purified and identified by thin layer and high pressure liquid chromatography (DAHM *et al.*, 1976).

Histological and other techniques

i. GOMORI's chrome hematoxylin phloxin (GOMORI, 1941); aldehyde fuchsin and victoria blue (DOGRA & TANDON, 1964) to demonstrate neurosecretory activity in histological preparations. ii. CAJAL-DECASTRO silver staining technique for the demonstration of nerve fibers (LEVI-MONTALCINI & CHEN, 1969). iii. Inverted and differential interference contrast microscopy to study and photograph living cultures. iv. Electron microscopy for ultrastructural studies (SESHAN & LEVI-MONTALCINI, 1971). v. Electrophysiology to detect bioelectrical phenomena in living cultures (SESHAN *et al.*, 1974).

RESULTS

Neurosecretory (NS) cells, corpus cardiacum (CC) and corpus allatum (CA) were cultured in the presence of embryonic organs (brain, thoracic ganglia, foregut and heart) or nymphal and adult ovarian follicles. These organs were positioned at a distance of approximately 1–2 mm from the neuro-endocrine organs. Previous studies had shown that these tissues increase the amount of nerve fiber outgrowth and survival of the embryonic nervous system (CHEN & LEVI-MONTALCINI, 1969; 1970; LEVI-MONTALCINI & CHEN, 1969). Our observations agree with these findings; in the absence of the above-mentioned organs, the neuro-endocrine explants show degenerative changes.

In vitro analysis of neurosecretory cells

Toward the end of the first week of incubation *in vitro*, nerve fiber processes emerge from one or more sectors of the NS explant which usually consisted of a large aggregate, or a small cluster or individual cells. During the second and third week, the outgrowing fibers lengthen further, assume a tubular appearance and give origin to many side branches (Fig. 1). When several NS explants are cultured together, in the same vessel, adjoining NS groups or individual cells establish contact to one another with nerve fiber processes. Meanwhile, many of the smaller nerve fibers originating from large NS clusters may coalesce to form axonal bundles and merge with similar fiber bundles originating from embryonic explants. With the establishment of neuro-fibrillar contact between NS and embryonic explants, e.g., foregut and heart, the latter organs begin to exhibit contractility. Whether the motility of the foregut and heart is of myogenic or neurogenic origin was not ascertained in these studies.

NS granules were evident in many cultured NS cells after staining with GOMORI's and other techniques. Axonal processes contained only few such granules. Ultrastructural studies showed numerous electron-

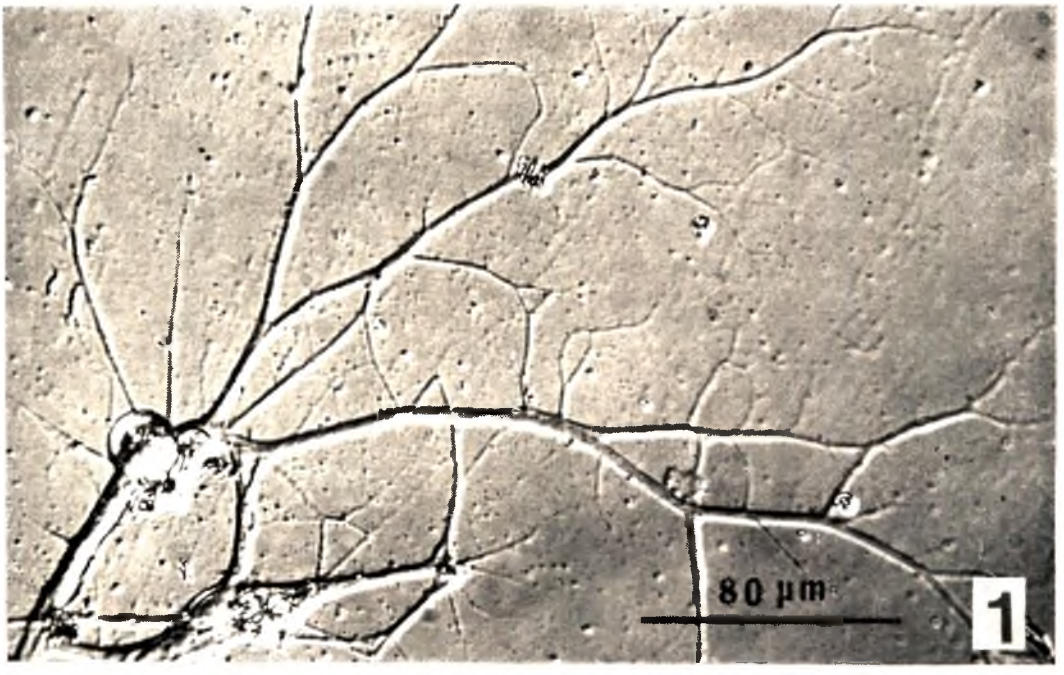


FIG. 1. Large axons and collateral fibers branch out from a small cluster of NS cell of a 7th instar nymph cultured *in vitro* for 33 days. One NS cell body is shown in this microphoto. Other NS cells (not shown) are in the adjacent frame.

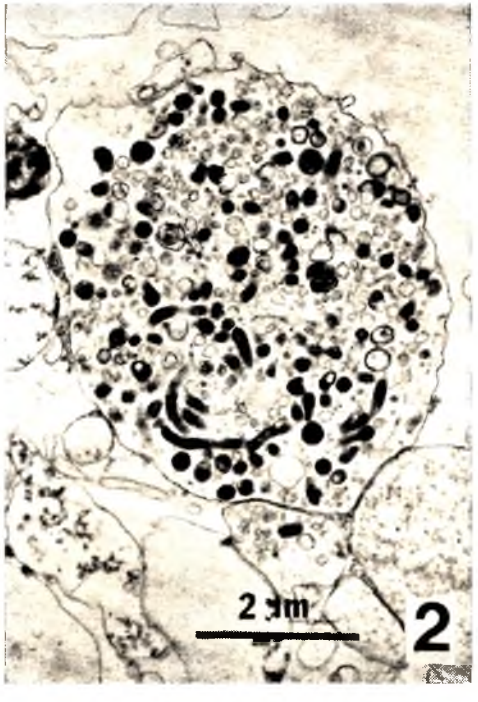


FIG. 2. An electron micrograph of axons growing out of an NS cluster of a 6th instar nymph maintained *in vitro* for 4 weeks. Note the numerous vesicles in the axon filled with varying gradations of electron dense material.

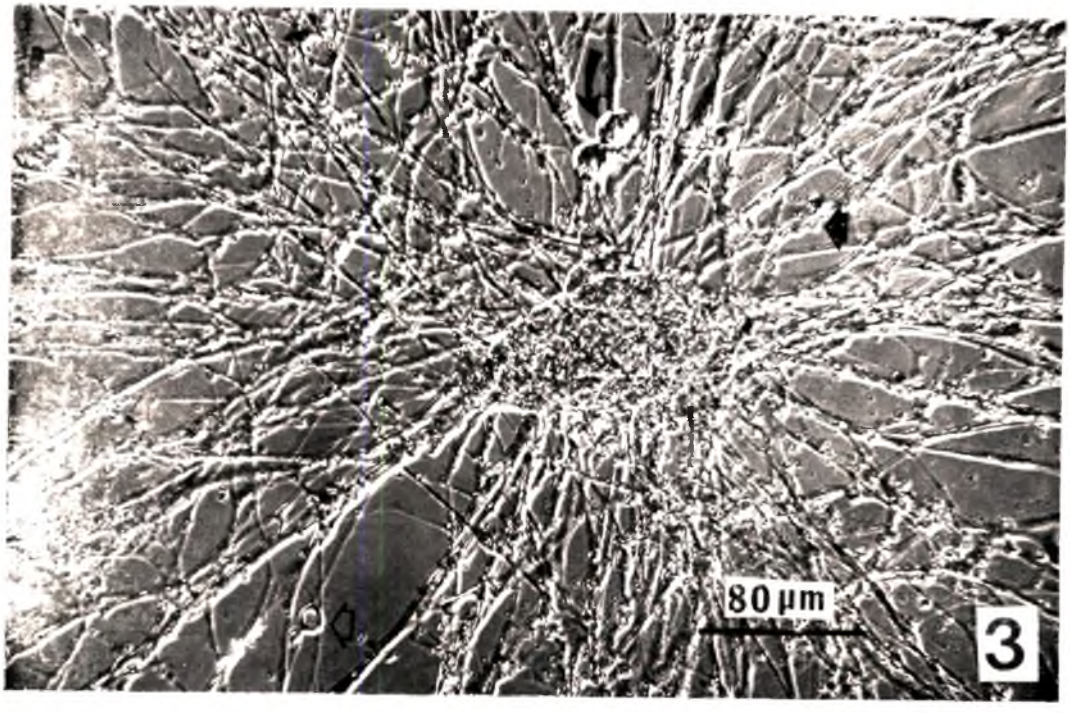
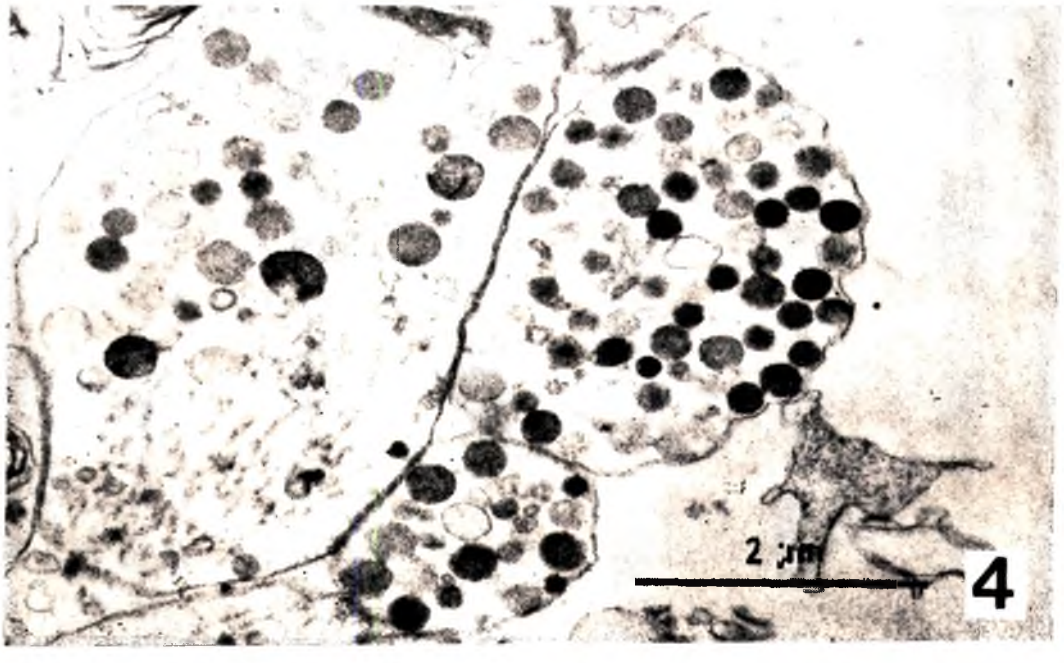


FIG. 3. Nomarski microphoto of a living culture of a segment of corpus cardiacum taken from a male adult and cultured *in vitro* for 47 days. Note the prolific outgrowth of axons, small glia-like cells (small closed arrows), large migrated neurons (long arrows), and a tracheolar cell (small open arrow).

FIG. 4. Electron micrograph of axons growing out of a CC segment of a male adult cultured *in vitro* for 47 days. Numerous electron dense granules fill the axons.



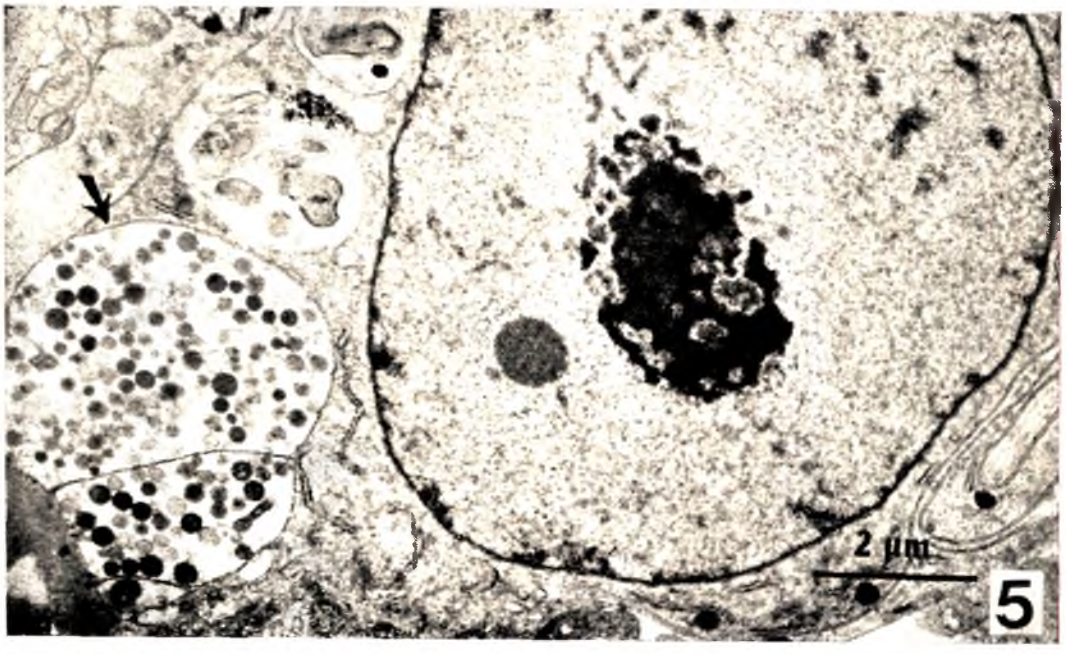
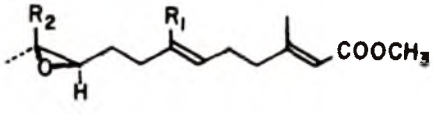


FIG. 5. Electron micrograph of cells of the corpus allatum of a 10th instar nymph maintained *in vitro* for 22 days. Cellular details and NS axons (arrow) are evident.



1	$R_1 = R_2 = -C_2H_5$	JH - I
2	$R_1 = -CH_3$, $R_2 = -C_2H_5$	JH - II
3	$R_1 = R_2 = -CH_3$	JH - III

FIG. 6. Structures of the known juvenile hormones.

dense NS granules in the cell pericarya and axons. In Fig. 2 profile of a large axon is filled with a mixture of high, medium and low electron dense granules. However, the number of NS granules in these cells were much lower than those found *in vivo*. It remains to be determined whether this decrease is due to release of NS material or to a decrease in the synthesis of NS material *in vitro* or a combination of both.

Electrophysiological techniques provided additional information on the functional state of the NS cultures. Unit electrical activity was observed in numerous NS cells. This activity was spontaneous and endogenous in nature and was comparable to similar activity *in vivo* (see discussion). Action potentials taken from cultures incubated for a few days did not differ significantly from those of older cultures. The frequency of discharge of single units ranged from 0.2/sec to 2.3/sec. The duration of individual action potentials was variable and long and lasted from 2 to 7 milliseconds (SESHAN *et al.*, 1974).

In vitro analysis of the corpus cardiacum

The extensive nerve fiber outgrowth from the whole surface of the corpus cardiacum or from small segments of the organ (Fig. 3) is a prominent feature of this gland. Inspection of a large number of cultures with inverted and interference microscopes showed that these fibers resemble those found in cultures of the embryonic nervous system and have morphological features of true nerve fibers. Their identification as true axons was confirmed by the silver technique which stains them either deep brown or black. Aside from the neuronal processes, numerous cells of different types migrate out of the explant. One type of cells found in large numbers is small and spindle-shaped and has been identified as glial cells. In Fig. 3 these cells at this magnification are hidden among the fibers (small closed arrows). A few cells larger in size with neuronal features (long arrows) suggestive of neuroglandular cells are present in the migratory zone. A third cell type (small open arrow) seldom found in CC cultures resembles the cells of the tracheal system.

In long term cultures (4–8 week old), the CC and embryonic explants present in the same culture vessel establish contact to one another with neuro-fibrillar connections. Contractility of foregut and heart explants become evident during this period and this activity persists as long as both these sets of explants are in intimate neuro-fibrillar contact with each other.

The intercellular spaces of the corpus cardiacum of 4–8 week old cultures are rich with NS granules after staining with GOMORI and others' methods and only few such granules are evident in the axons. The electron microscope provided additional evidence for the presence of numerous electron dense granules in the cell pericarya and axons which is suggestive of NS activity *in vitro* (Fig. 4).

Using the same electrophysiological techniques as those used for NS cultures, one-third of the CC cultures showed bioelectric activity (SESHAN *et al.*, 1974). The firing pattern of the CC was very irregular and contrasted sharply with those of the NS cultures. The mean firing rate of 29 single units was 1.88 spikes/sec (range 0.28–4.37/sec). Many units displayed a bursting pattern of firing in which the interburst interval of individual units averaged 13 sec (range 2–25 sec). The CC units rarely fired continuously with regular inter-spike intervals as those of the NS cells.

In vitro analysis of the corpus allatum

Cultures of nymphal corpus allatum produce a few protoplasmic filaments and

small spindle-shaped cells are found associated with the filaments. It is not certain whether these cells and fibers originate from the CA or from CC tissue which extends to this organ. When CA–CC is cultured together with other tissues, the cells and nerve fibers produced by the CC envelop the CA to such an extent that it is difficult to determine whether the CA contribute to these activities. In serial sections of the cultured CA stained with hematoxylin and toluidine blue techniques, the cells were either closely packed together or in some cases they were spread apart. NS granules were sparingly distributed among the cells. At the electron microscope, NS axons filled with electron dense granules were present among the cells (Fig. 5).

The production of juvenile hormone by the corpus allatum was studied in radiotracer experiments. The known juvenile hormones are acyclic sesquiterpenes (Fig. 6). L–methionine is efficiently used as a donor of the ester methyl group *in vivo* (METZLER *et al.*, 1971; 1974) and *in vitro* (JUDY *et al.*, 1973). This procedure enabled us to detect and quantify the hormones secreted by the corpus allatum when methyl–^{14}C–methionine had been added to the culture medium.

The time course of incorporation of methyl–^{14}C–methionine into juvenile hormone indicated a high rate of JH production by adult cockroaches of both sexes (approximately 10 ng/day/CA pair). Females carrying fully developed ootheca produced nearly four to five times the amount of JH in contrast to those without ootheca during the first week of incubation. Toward the end of the second and third week, JH production declined sharply and was undetectable after the fourth week. The hormone was identified as JH III (Fig. 6). There was no evidence for the production of other juvenile hormones, e.g. JH–I or JH–II. Corpora allatta of mature nymphal specimens (10th–12th instar) of both sexes also produced JH–III exclusively, but the amounts recovered were about ten times lower than those of adult glands. The addition of mevalonolactone to CA cultures increased the JH–III yield upto ten-fold. Addition of homomevalonolactone had no effect.

DISCUSSION

The successful *in vitro* culture and long-term maintenance of the cockroach neuro-endocrine organs have shown the considerable flexibility and adaptability of these structures in a synthetic chemically defined medium. Further, the results obtained by these techniques have opened new lines of investigations which were not accessible previously. The ability of fully differentiated nymphal and adult cockroach neuro-endocrine organs to produce neuronal fiber processes and at the same time display neurosecretory granules in them at the optic and ultrastructural levels demonstrates the dual attributes of these tissues. Moreover, the capacity of the NS and CC cultures to generate bioelectric potentials *in vitro* adds physiological evidence for their functional integrity. Electrical properties of NS tissues first analyzed in vertebrate systems (BERN & YAGI, 1965) have recently been investigated in invertebrate groups and in particular in insects (MADDRELL, 1974). However, to our knowledge, no attempts have been made to test these properties in insect systems *in vitro*. Our *in vitro* techniques lend themselves to detailed investigations in this field.

The high concentration of cysteine/cystine-rich peptides in the NS cells has been utilized for biosynthetic studies (see review by MADDRELL, 1974). In these *in vivo* tracer studies type "A" NS cells are labeled preferentially in contrast to other NS or non-NS neurons. STEEL & MORRIS (1975)

using X-ray microanalytical techniques reported a more direct measurement of NS activity in *Rhodnius prolixus*. We did not find any preferential incorporation of ^{35}S–cysteine in our *in vitro* studies of the NS cells. These investigations now in progress will tell whether NS cells synthesize, transport and release specific proteins *in vitro*.

The significance of the effect of embryonic organs or ovarian follicles cultured together with NS and CC cannot be underestimated. While it is well known that the neuro-endocrine glands *in vivo* release a variety of hormones into the circulation which affect the target organs, the reciprocal effects of target organs upon the neuro-endocrine system are not clearly understood, at least in insects. Our observations that neuro-endocrine organs atrophy *in vitro* in the absence of target tissues raises the question whether the embryonic and ovarian tissues release any specific agents into the medium which enhance the survival of the neuro-endocrine organs. The release of a 'foregut' factor by cultured segments of embryonic foregut promoting the long-term survival of dissociated embryonic nerve cells has been suggested in earlier studies (CHEN & LEVI-MONTALCINI, 1970). Equally important is the question whether the neuro-endocrine organs synthesize and release hormones or hormone-like factors promoting further differentiation of the embryonic organs and ovarian follicles with which they establish neuro-fibrillar connections.

The capacity of the CA of nymphs and adults of *P. americana* to produce juvenile hormone *in vitro* supplements the morphological data that these glands are in fact quite active *in vitro*. The CA of other cockroach species (*Periplaneta fuliginosa* and *Blaberus discoidalis*) *in vitro* also produce JH-III (DAHM *et al.*, 1976). The only JH found in these cultures is JH-III, which is in agreement with the results of other workers on this and other hemimetabolous species (JUDY *et al.*, 1973; MULLER *et al.*, 1974; PRATT *et al.*, 1975). JH-I and JH-II have been detected in only one orthopteran species. LANZREIN *et al.*, (1975) demonstrated the presence of all the three JH homologues in blood samples of nymphs and adults of the cockroach *Nauphoeta cinerea*. Otherwise JH-I and/or JH-II was produced by CA *in vitro* by lepidopteran insects (DAHM *et al.*, 1976; JUDY *et al.*, 1973; ROLLER & DAHM, 1968; 1970; 1974). Whether one or more of these hormones are specialized for morphogenetic or gonadotropic functions in the living insect is still a matter of controversy. Nevertheless, we hope, studies along these lines in combination with other techniques on the cockroach and other insects will help to illuminate the complex and diversified roles of the neuro-endocrine glands in the life of insects.

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REFERENCES

BERN, H. A. & I. R. HAGADORN (1965) Neurosecretion, 353–429. *in : Structure and Function in the Nervous System of Invertebrates* (ed. BULLOCK, T. H. & G.A. HORRIDGE), W. H. Freeman and Co., San Francisco and London.

BERN, H. A. & K. YAGI (1965) Electrophysiology of neurosecretory systems. *Proc. 2nd Intern. Cong. of Endocrinol., Excerpta Medica Intern. Cong. Ser.* 83, *Part* 1, Excerpta Medica, Amsterdam, pp. 577–583.

CHEN, J. S. & R. LEVI-MONTALCINI (1969) Axonal outgrowth and cell migration *in vitro* from nervous system of cockroach embryos. *Science*, **166** : 631–632.

CHEN, J. S. & R. LEVI-MONTALCINI (1970) Long term cultures of dissociated nerve cells from the embryonic nervous system of the cockroach *Periplaneta americana*. *Arch. ital. Biol.*, **108**: 503–536.

DAHM, K. H., G. BHASKARAN, M. G. PETER, P. D. SHIRK, K. R. SESHAN & H. ROLLER (1976) On the identity of the juvenile hormone in insects. *in: Juvenile Hormones* (ed. GILBERT, L. I., Plenum Press, New York (In Press).

DOANE, W. W. (1973) Role of hormones in insect development, 291–497. *in : Developmental Systems : Insects*, Vol. 2 (ed. COUNCE, S. J. & C. H. WADDINGTON), Academic Press, London.

DOGRA, G. S. & B. K. TANDON (1964) Adaptation of certain histological techniques for *in situ* demonstration of the neuro-endocrine system of insects and other animals. *Quart. J. micr. Sci.*, **105** : 455–466.

GOMORI, G. (1941) Observation with differential stains in human islets of Langerhans. *Amer. J. Path.*, **17** : 395–406.

JUDY, K. J., D. A. SCHOOLEY, L. L. DUNHAM, M. S. HALL, B. J. BERGOT & J. B. SIDDALL (1973) Isolation, structure and absolute configuration of a new natural insect juvenile hormone from *Manduca sexta*. *Proc. nat. Acad. Sci. USA*, **70** : 1509–1513.

LANZREIN, B., M. HASHIMOTO, V. PARMAKOVICH, K. NAKANISHI, R. WILHELM & M. LUSCHER (1975) Identification and quantification of juvenile hormones from different developmental stages of the cockorach *Nauphoeta cinerea*. *Life Sci.*, **16** : 1271–1284.

LEVI-MONTALCINI, R. (1971) *In vitro* analysis of the insect nervous system. *Boll. Zool.*, **38** : 385–399.

LEVI-MONTALCINI, R. & J. S. CHEN (1969) *In vitro* studies of the insect embryonic nervous systems 277–298. *in : Cellular Dynamics of the Neuron* (ed. BARONDES, S. H.), Academic Press, New York.

LEVI-MONTALCINI, R. & K. R. SESHAN (1973) Long term cultures of embryonic and mature insect nervous and neuro-endocrine systems, 1–33. *in : Tissue Culture of the Nervous System* (ed. SATO, G.), Plenum Press, New York.

MADDRELL, S. H. P. (1974) Neurosecretion, 307–357. *in : Insect Neurobiology* (ed. TREHERNE, J. E.), North Holland / American Elsevier, Oxford, New York.

METZLER, M., K.H. DAHM, D. MEYER & H. ROLLER (1971) On the biosynthesis of juvenile hormone in the adult Cecropia moth. *Z. Natur.*, **26 B**: 1270–1276.

METZLER, M., K.H. DAHM, D. MEYER & H. ROLLER (1974) Biosynthesis and degradation of juvenile hormone, 176–181. *in : Invertebrate Endocrinology and Hormonal Heterophylly* (ed. BURDETTE, W. J.), Springer-Verlag, New York, Heidelberg, Berlin.

MULLER, P. J., P. MASNER, K. H. TRAUTMANN, M. SUCHY, & H. K. WIPF (1974) The isolation and identification of juvenile hormone from cockroach corpora allata *in vitro*. *Life Sci.*, **15** : 915–921.

PRATT, G. E., S. S. TOBE, R. J. WEAVER & J. R. FINNEY (1975) Spontaneous synthesis and release of C_{16} juvenile hormone by isolated corpora allata of female locust *Schistocerca gregaria* and female cockroach *Periplaneta americana*. *Gen. comp. Endoc.*, **26** : 478–484.

ROLLER, H. & K. H. DAHM (1968) The chemistry and biology of juvenile hormone. *Recent Progr. Hormone Res.*, **24** : 651–680.

ROLLER, H. & K. H. DAHM (1970) The identity of juvenile hormone produced by corpora allata *in vitro*. *Naturwissenschaften*, **57**: 454–455.

ROLLER, H. & K. H. DAHM (1974) Isolation of juvenile hormone in different species, 235–237. *in : Invertebrate Endocrinology and Hormonal Heterophylly* (ed. BURDETTE, W. J.), Springer-Verlag, New York, Heidelberg, Berlin.

SESHAN, K. R. (1976) Apparatus and techniques for the preparation of cockroach neuroendocrine organs for long-term maintenance *in vitro*. *Tissue Culture Assoc. Manual* (In Press).

SESHAN, K. R. & R. LEVI-MONTALCINI (1971) *In vitro* analysis of corpora cardiaca and corpora allata from nymphal and adult specimens of *Periplaneta americana*. *Arch. ital. Biol.*, **108** : 81–109.

SESHAN, K. R. & R. LEVI-MONTALCINI (1973) Neuronal properties of nymphal and adult insect neurosecretory cells *in vitro*. *Science*, **182** : 291–293.

SESHAN, K. R., R. R. PROVINE & R. LEVI-NONTALCINI (1974) Structural and electrophysiological properties of nymphal and adult insect medial neurosecretory cells: an *in vitro* analysis. *Brain Research*, **78** : 359–376.

STEEL, C. G. H. & G. P. MORRIS (1975) Evidence from X-ray microanalysis of sulphur-rich protein in insect neurosecretion : A new approach to the estimation of neurosecretory activity. *Gen. comp. Endoc.*, **26**: 517–524.

VAGO, C. (ed.) (1971) *Invertebrate tissue culture*, Vol. I, 441 pp ; Vol 2 (1972) 415 pp. Academic Press, New York and London.

WIGGLESWORTH, V. B. (1964) The hormonal regulation of growth and reproduction in insects, 247–335. *in : Advances in insect physiology* Vol. 2 (ed. BEAMENT, J. W. L., J. E. TREHERNE, & V. B. WIGGLESWORTH), Academic Press, London and New York.