12 Gidari, A.S., and Levere, R.D., J. Lab. clin. Med. *98* (1979) 872.
13 Golde, D.W., Bersch, N., and Cline, M.J., J. clin. Invest. *57* (1976) 57.
14 Goldman, A.S., Endocrinology *86* (1970) 1245.
15 Graves, P.E., and Salhanick, H.A., Endocrinology *105* (1979) 52.
16 Gregory, C.J., J. Cell Physiol. *89* (1976) 289.
17 Hackney, J.F., Gross, S.R., Aronow, L., and Pratt, W.B., Molec. Pharmac. *6* (1970) 500.
18 Hassan, M.W., Ibrahim, A., and Rieder, R.F., Proc. Soc. exp. Biol. Med. *169* (1982) 63.
19 Heath, D.S., Axelrad, A.A., McLeod, D.L., and Shreeve, M.M., Blood *47* (1976) 777.
20 Leung, P., Diss., State University of New York, Downstate Medical Center, University Microfilms International, Ann Arbor, MI, 1983.
21 Leung, P., and Gidari, A.S., Endocrinology *108* (1981) 1787.
22 Leung, P., and Gidari, A.S., Blood *60* (suppl. 1) (1982) 279.
23 Liggins, G.C., and Kennedy, P.C., J. Endocr. *40* (1968) 371.
24 Mayeux, P., Billat, C., Felix, J.M., and Jacquot, R., J. Endocr. *96* (1983) 311.
25 McLeod, D.L., Shreeve, M.M., and Axelrad, A.A., Blood *44* (1974) 517.
26 Nagel, J., and Jacquot, R., Arch. Anat. microsc. Morph. exp. *57* (1968) 99.
27 Nagel, J., and Jacquot, R., Arch. Anat. Microsc. Morph. exp. *58* (1969) 97.
28 Peschle, C., Magli, M.C., Cillo, C., Lettieri, F., Pizzella, F., Migliaccia, G., and Sasso, G.F., in: In Vitro Aspects of Erythropoiesis, p.86. Eds M.J. Murphy, C. Peschle, A.S., Gordon and E.A., Mirand. Springer-Verlag, New York 1978.
29 Rifkind, R.A., Chui, D., and Epler, H., J. Cell Physiol. *40* (1969) 343.
30 Roodman, G.D.,, Lee, J., and Gidari, A.S., Br. J. Haemat. *53* (1983) 621.
31 Schooley, J.C., Garcia, J.F., Cantor, L.N., and Haven, V.W., Ann. N.Y. Acad. Sci. *149* (1968) 266.
32 Singer, J.W., Samuels, A.I., and Adamson, J.W., J. Cell Physiol. *88* (1976) 127.
33 Snedecor, G.W., and Cochran, W.G., in: Statistical Methods, 6th ed., p.100, 265, 273. Iowa State University Press, Ames 1967.
34 Stephenson, J.R., Axelrad, A.A., McLeod, D.L., and Shreeve, M.M., Proc. natn. Acad. Sci. USA *68* (1971) 1542.
35 Tarbutt, R.G., and Cole, R.J., J. Embryol. exp. Morph. *24* (1970) 429.
36 Thiel, D.H.V., Estes, L.W., Richey, J.E., Little, J.M., and Graham, T.O., Endocrinology *107* (1980) 557.
37 Thompson, A. Jr, and Siiteri, P.K., J. biol. Chem. *249* (1974) 5373.
38 Uzgiris, V.I., Graves, P.E., and Salhanick, H.A., Biochemistry *16* (1977) 593.
39 Zanjani, E.D., and Banisadre, M., J. clin. Invest. *64* (1979) 1181.
40 Zanjani, E.D., Mann, L.I., Burlington, H., Gordon, A.S., and Wasserman, L.R., Blood *44* (1974) 285.

0014-4754/85/040498-03$1.50 + 0.20/0

Distribution patterns of mammalian-like peptide immunoreactive cells in the midgut of *Aeshna cyanea* (Insecta, Odonata)

J.C. Andriès and G. Tramu

Laboratoire de Biologie Animale et Laboratoire Associé au CNRS no 148: Endocrinologie comparée des Invertébrés, Université des Sciences et Techniques de Lille, F-59655 Villeneuve d'Ascq Cedex (France), and U.156 INSERM, Place de Verdun, F-59045 Lille Cedex (France), 26 March 1984

Summary. The distribution of cells reacting with antisera to cholecystokinin, substance P, gonadoliberin, methionine-enkephalin, and vasoactive intestinal peptide, demonstrated by the indirect immunoperoxidase method, was studied along the entire midgut of an insect, *Aeshna cyanea*. For each antiserum, the number of reacting cells increased from the middle part to the end of the midgut. Only a few cells reacted to somatoliberin, leucin-enkephalin and somatostatin antisera. In the connective sheath surrounding the midgut epithelium, nerve fibers were stained by antisera to serotonin, somatostatin, cholecystokinin, vasoactive intestinal peptide and methionine-enkephalin.
Key words. Insect midgut; mammalian-like peptides; immunohistochemistry.

In insects, by means of immunohistochemical investigations or radioimmunoassays, it has recently been demonstrated that several mammalian-like neuropeptides, especially of gastro-entero-pancreatic type (GEP) occur in the nervous system, particularly in the brain. Except for the work of Iwanaga et al.[11] and our recent preliminary study[4], no report had dealt with the presence of these substances in the insect gut. Previously we identified, by an immunoperoxidase procedure, cells reactive for mammalian cholecystokinin octapeptide (CCK-8), vasoactive intestinal polypeptide (VIP), pancreatic polypeptide (PP), substance P, methionine-enkephalin (met-enkephalin), gonadoliberin (LHRH) and human pancreatic somatoliberin (hp GRF) in the midgut of *Aeshna cyanea*. With the same method, we have now undertaken a systematic investigation of the neuropeptides through the entire midgut. The results present the complete distribution of the midgut peptide endocrine cells.

Material and methods. The postembryonic development of *Aeshna cyanea* requires eleven larval instars. Larvae of the 8th instar were used in this study.

Tissue preparation. The midguts of nine larvae were rapidly dissected and immersed in a solution of picrid-acid-paraformaldehyde (PAF)[13] for 24 h. The samples were then washed overnight in phosphate buffer 0.1 M, pH 7.2, 5% sucrose, embedded in Tissue-Tek (Miles Labs.), frozen in liquid nitrogen and sectioned on a cryostate (section thickness: 15 µm).

Antisera. Twelve antisera raised against synthetic CCK-8, VIP, substance P, met-enkephalin, LHRH, hp GRF, leucine-enkephalin (leu-enkephalin), human calcitonin, neurotensin, serotonin, somatostatin-14 and ovine corticoliberin (CRF) were used. Somatostatin antiserum was a gift from Dr Dubois (Nouzilly, France). The others were produced in our laboratory by immunization of rabbits with the amine (serotonin) or the synthetic peptide linked to albumin or thyroglobulin, and emulsified with Freund's complete adjuvant. Somatostatin[8], CCK-8[9], leu- and met-enkephalin[14], CRF[15], hp GRF[16] and serotonin[17] antisera were previously employed.

Immunohistochemical staining for sections. Each serially cut section was incubated for 24 h at 4°C with one of the twelve antisera. All antisera were applied in dilutions of 1/200. After rinsing in Coons buffer pH 7.2, the sections were incubated according to the indirect immunoperoxidase method, with a 1/50-dilution of anti-Ig rabbit for 1 h at room temperature. For visualization of the peroxidase, the tissues were allowed to react with a solution of 4-chloro-1-naphthol in 0.1 M Tris-HCl buffer (pH 7.6) containing 0.01% H_2O_2.

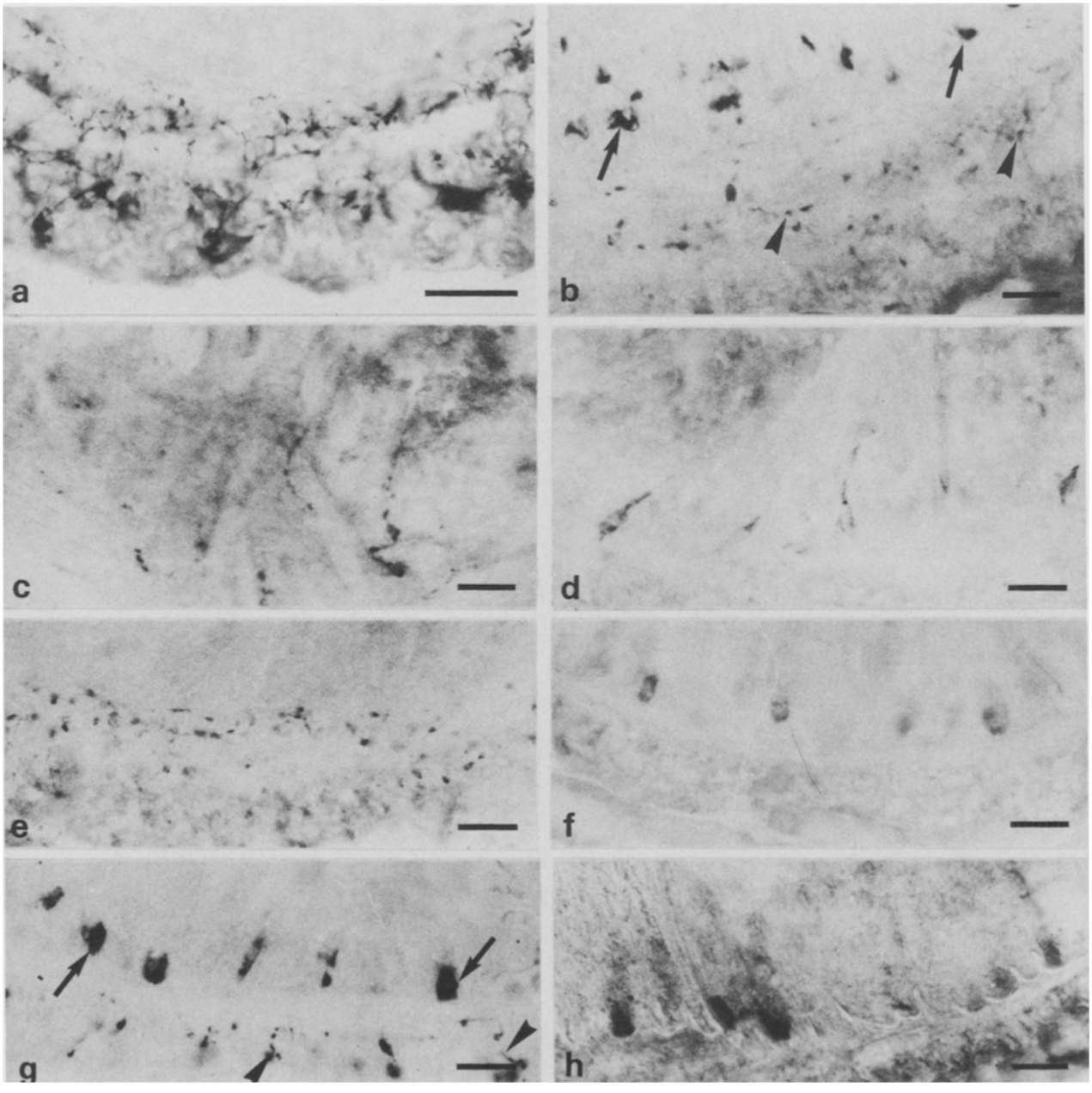

Figure 1. *a* Serotonin-like immunoreactive axons. *b* Met-enkephalin-like immunoreactive axons (arrowheads) and endocrine cells (arrows). *c* Somatostatin-like immunoreactive axons. *d* Somatostatin-like immunoreactive endocrine cells. *e* VIP-like immunoreactive axons. *f* Leu-enkephalin-like immunoreactive endocrine cells. *g* CCK 8-like immunoreactive axons (arrowheads) and endocrine cells (arrows). *h* hp GRF-like immunoreactive endocrine cells. Bar 25 μm.

Controls. The specificity of positive staining was tested by replacing the specific antiserum with normal rabbit serum or with specific antiserum preincubated with an excess of the respective peptide (CCK-8, hp GRF, LHRH, substance P or met-enkephalin).

Estimate of the number of immunoreactive cells. For each of the nine blocks, each antiserum was applied to transverse sections taken at the same regular interval (180 μm). This procedure made it possible to determine all along the entire midgut the number of immunoreactive cells for each antiserum. When the number of immunoreactive cells was adequate, a mean value per section was obtained from which the standard deviation (vertical bars in the graphs) was calculated.

Results. The results of the control studies showed that when a non-immune serum was used in place of an immune serum, no staining was detected. Also, the absorption of CCK-8, hp GRF, LHRH and substance P antisera with their respective antigen blocked the staining reactions. A moderate number of staining cells (20–30%) were found after incubation with anti-met-enkephalin serum absorbed with an excess of met-enkephalin.

Neuronal elements. As previously reported[4], serotonin-like immunoreactivity was observed in a strongly-stained profuse innervation (fig. 1a) of the muscular layers which surround the midgut epithelium, especially in the vicinity of the hindgut. In addition, antisera to met-enkephalin (fig. 1b), somatostatin (fig. 1c), VIP (fig. 1e) and CCK-8 (fig. 1g) stained a moderate number of axonal fibers.

Endocrine cells. In the midgut, met-enkephalin (fig. 1b and 2a), somatostatin (fig. 1d), leu-enkephalin (fig. 1f), CCK-8 (fig. 1g

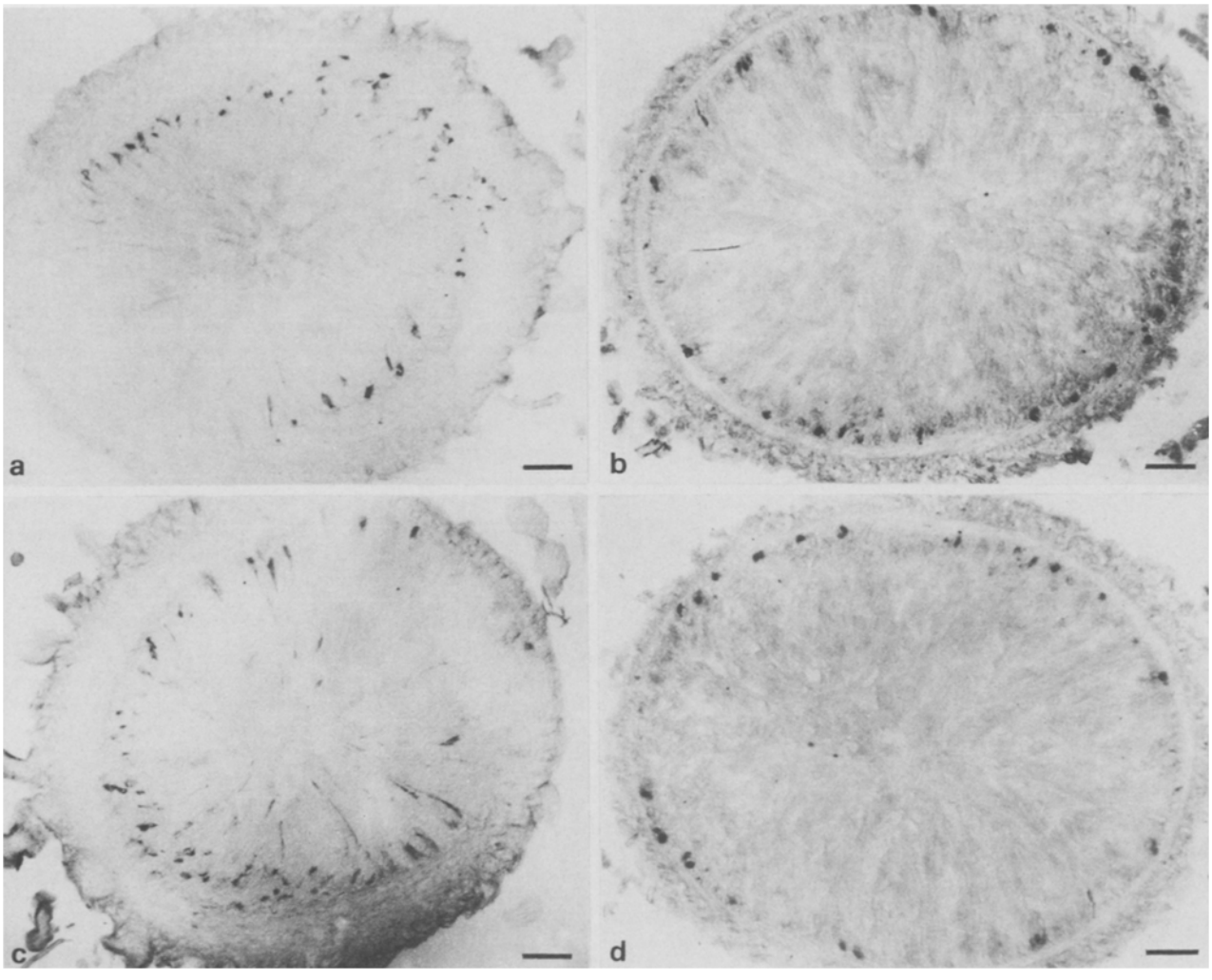

Figure 2. Transversal sections of the posterior part of the midgut showing. *a* Met-enkephalin-like immunoreactive endocrine cells. *b* Substance P-like immunoreactive endocrine cells. *c* LHRH-like immunoreactive endocrine cells. *d* CCK 8-like immunoreactive endocrine cells. Bar 60 µm.

and 2d), hp GRF (fig. 1h), substance P (fig. 2b), LHRH (fig. 2c) and VIP immunoreactive cells were detected. The small number of cells reacting for somatostatin, leu-enkephalin and hp GRF made numerical estimations difficult if not impossible. On the contrary, numerous endocrine cells stained for substance P, met-enkephalin, LHRH, CCK-8 and, to a lesser extent, for VIP. Very few if any immunoreactive cells are present in the anterior part of the midgut. Their density increases towards the posterior part to become maximal near the hingut, as illustrated in figures 3 to 7. No immunoreactive cells were detected using neurotensin, CRF, calcitonin and serotonin antisera. The shape of the cells reacting for a given peptide varied, being triangular, fusiform, bowl-shaped, etc. Some of them, especially those located near the junction of the hindgut (fig. 2c) appeared to be of the open type, showing a long slender apical process directed towards the gut lumen. Such cells were observed after incubation with CCK 8, somatostatin, LHRH, met-enkephalin and VIP antisera.

Discussion. Previously[2], we have described two types of basal granulated cells in the midgut of *A. cyanea* which, by exocytosis, discharge their contents into the internal milieu and therefore must be considered as endocrine cells. These cells corresponded well in ultrastructure to mammalian gut endocrine cells. A study in progress shows that the variety of endocrine cells in the *A. cyanea* midgut is much greater than was anticipated[3]. This seems likely to be true for many insects. At least 10 types of ultrastructurally different endocrine cells have been identified in the midgut of the cockroach, *Blaberus craniifer*[5]. Such an ultrastructural variety agrees well with our present results in so far as we described 8 immunoreactive cell types reacting with antisera against CCK-8, VIP, somatostatin, met-enkephalin, leu-enkephalin, substance P, LHRH and GRF; a ninth cell type containing PP-like material was detected previously[4]. It may be, however, that two or more distinct peptides co-exist in the same cell, as reported in vertebrates[10, 12]. Neuromodulators related to CCK-8, VIP, somatostatin and met-enkephalin are present in a moderate number of axons innervating the midgut. Most of the nerve fibers react to serotonin-antiserum. This amine is known to act in insects as a neurotransmitter and/or a neuromodulator. Although nothing is known about its action on the musculature of the midgut, it has been established that serotonin has a striking effect on the contraction of the musculature of the foregut[6, 7] and hindgut[6]. GEP hormone-like substances resembling CCK 8, somatostatin, VIP, substance P, LHRH, hp GRF, leu- and met-enkephalin occur in insect midgut endocrine cells. This large number of mammalian-like peptides found both in insect nervous systems and midgut indicates that peptide production represents a primitive mechanism and that, due allowance being made for the

difference in complexity, the insect brain-midgut system is analogous to the brain-GEP system of vertebrates.

In insects, nothing is known either about the possible roles of these peptides, or on the origin of the endocrine cells. What has been established in this study is that, as in vertebrates, the gut has an endocrine function which, in the case of *Aeshna cyanea,* appears to be preferentially localized in its posterior part.

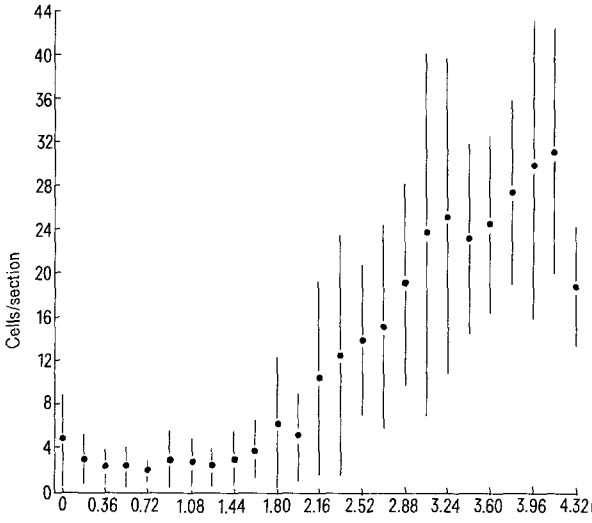

Figure 3. Distribution of CCK-like immunoreactive endocrine cells through the entire midgut.

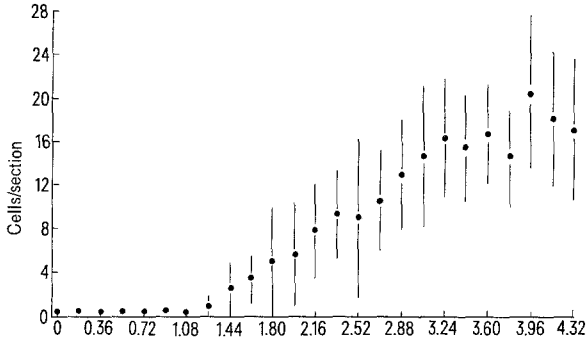

Figure 4. Distribution of substance P-like immunoreactive endocrine cells through the entire midgut.

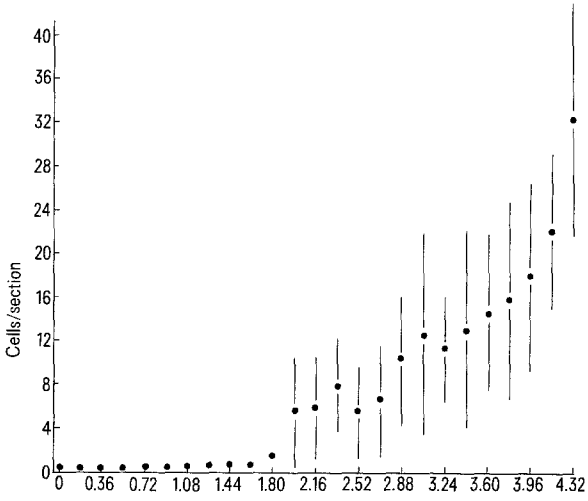

Figure 5. Distribution of LHRH-like immunoreactive endocrine cells through the entire midgut.

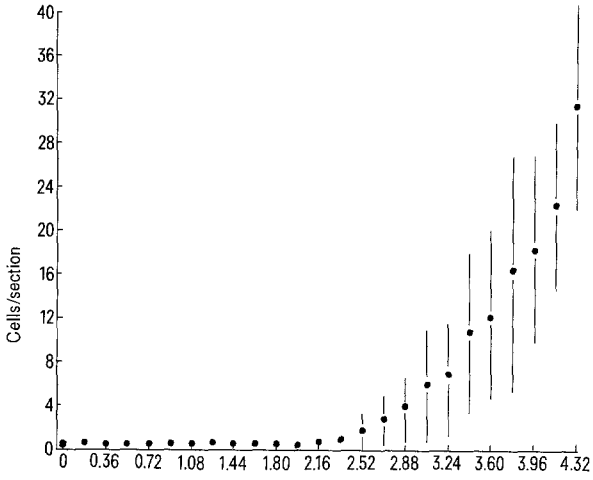

Figure 6. Distribution of met-enkephalin-like immunoreactive endocrine cells through the entire midgut.

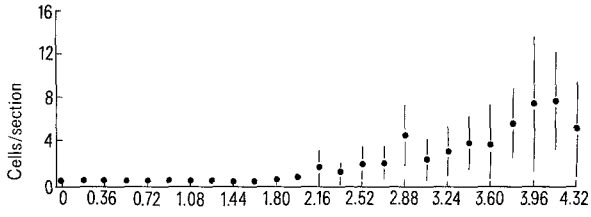

Figure 7. Distribution of VIP-like immunoreactive endocrine cells through the entire midgut.

1 The technical assistance of A. Pillez is gratefully acknowledged.

2 Andriès, J.C., Int. J. Insect Morph. Embryol. *5* (1976) 393.
3 Andriès, J.C., unpublished.
4 Andriès, J.C., and Tramu, G., C.R. Acad. Sci. Paris *299* (1984) 181.
5 Andriès, J.C., and Tramu, G., unpublished.
6 Brown, B.E., Gen. comp. Endocr. *5* (1965) 387.
7 Cook, B.J., Eraker, J., and Anderson, G.R., J. Insect Physiol. *15* (1969) 445.
8 Dubois, M.P., Proc. natn. Acad. Sci. USA *72* (1975) 1340.
9 Engelhardt, R.P., Dhainaut-Courtois, N., and Tramu, G., Cell Tissue Res. *227* (1982) 401.
10 Ito, S., Takai, K., Shibata, A., Matsubara, Y., and Yanaihara, N., Gen. comp. Endocr. *38* (1979) 238.
11 Iwanaga, T., Fujita, T., Nishiitsutsuji-Uwo, J., and Endo, Y., Biomed. Res. *2* (1981) 202.
12 Rawdon, B.B., and Andrew, A., Cell Tissue Res. *220* (1981) 279.
13 Stefanini, M., De Martino, C., and Zamboni, L., Nature *216* (1967) 173.
14 Tramu, G., and Leonardelli, J., Brain Res. *168* (1979) 457.
15 Tramu, G., Croix, D., and Pillez, A., Neuroendocrinology *37* (1983) 467.
16 Tramu, G., Beauvillain, J.C., Pillez, A., and Mazzuca, M., C.R. Acad. Sci. Paris *297* (1983) 435.
17 Tramu, G., Pillez, A., and Leonardelli, J., Cell Tissue Res. *228* (1983) 297.

0014-4754/85/040500-04$1.50 + 0.20/0