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Fisiologia. — *Morphological and ionic features of the nerve cord in* Bombyx mori *larvae* (*). Nota di GIANLUIGI MONTICELLI, MIRELLA SIMONETTA, BARBARA GIORDANA e FRANCA SACCHI, presentata (**) dal Corrisp. V. CAPRARO.

RIASSUNTO. — È stata studiata l'ultrastruttura dei gangli della catena ventrale della larva di *Bombyx mori* e, sulla base di dati elettrofisiologici e di composizione ionica del tessuto, si propone un modello di distribuzione del sodio e del potassio nei tre compartimenti identificabili dalle osservazioni morfologiche, ambiente intracellulare, volume extracellulare ed emolinfa. Poichè il rapporto Na/K nel fluido extracellulare è molto diverso da quello nell'emolinfa (5.7 e 0.04 rispettivamente), si suggerisce la presenza di meccanismi di regolazione localizzati a livello delle barriere che separano i neuroni dall'emolinfa.

INTRODUCTION

The presence of a well developed blood-brain barrier in the central nervous system of insects has been established [1–3]. Physiological data strongly suggest that this barrier plays a specific rôle in the regulation of the ionic neuronal environment. This regulation is particularly significant in those phytophagous insects, such as the larvae of Lepidoptera, that possess a haemolymph of specialized ion concentration with an extremely low Na/K ratio [4].

On the basis of ultrastructure, electrophysiological experiments and ionic concentration determinations, we suggest in the present work a model for sodium and potassium distribution in neuron cells, neuronal microenvironment and haemolymph which correlates functional activity with morphological aspects.

MATERIALS AND METHODS

Experiments were performed on *Bombyx mori* larvae in their last instar.

For electron microscopy the ventral nerve cords in the dissected animals were flooded with ice-cold fixative, then excised, sliced into short portions and placed in fresh fixative solution at 4 °C for 3 h. The fixing solution consisted of 1.5 % glutaraldehyde and 1 % formaldehyde in 22 mM phosphate buffer, pH 7.0, with 200 mM sucrose added. Tissues were postfixed with

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1 % OsO_4 in 50 mM phosphate buffer, pH 7.0. Dehydration in an ethanol series followed, and, after treatment with propylene oxide, the specimens were embedded in Epon 812/Araldite mixture and sectioned using an LKB Ultrotome III. The sections were stained with Reynold's lead citrate solution and uranyl acetate and examined using a Siemens Elmiskop 1/A electron microscope.

For electrophysiological experiments the animals were pinned ventral surface down to a Sylgard 184 layer in a glass chamber. The dorsal integument was opened by a medial longitudinal incision, the gut removed and the exposed ventral nerve cord washed with a standard solution of the following composition (mM): 1.7 NaCl, 44 $MgSO_4$, 9 $CaCl_2$, 18 K_2SO_4, 157.3 sucrose, 1.1 KH_2PO_4, 4.3 K_2HPO_4, pH 6.9±0.1 (20 °C), osmolarity 290±2.9 mOsM, the same as the hemolymph [5]. In order to aid the microelectrode penetration the tissue covering the ventral nerve cord was enzymatically softened by exposure to Pronase, a proteolytic enzyme, in standard solution (0.35 mg/ml) for about 40 min. Na and K concentrations were varied substituting the cations by equal amounts of sucrose, in order to keep the osmolarity constant. Intracellular recordings were obtained using glass microelectrodes filled with 3 M KCl and with electrical resistances of 10–20 MΩ. The electrical circuit was of the conventional type. Experiments were performed at room temperature (23–25 °C). In any single experiment the temperature did not vary more than 0.5 °C.

To determine ionic concentrations about 15 nerve cords were incubated " in situ " in standard solution for 40 min. The cords were then excised, col lected, slid onto a glass slide to remove excess saline and placed in calibrated tubes filled with 0.2 ml of 0.6 N $HClO_4$ and weighed to obtain fresh tissue values. One drop of H_2O_2 was added to the nerve cords, the tissues were boiled for 10 min adding distilled water, reweighed and centrifuged for 30 min (7000 rpm). The supernatants were then diluted and assayed for Na and K by means of a Corning Phlame Photometer 430. Dry weights were obtained from nerve cords (samples of 10–20 cords) put in a calibrated tube filled with 0.2 ml of distilled water, weighed to obtain fresh tissue values and then dried for 36 h at 104 °C.

Results and discussion

The histological organization of the ganglia of the ventral nerve cord of *B. mori* larvae shows distinct structural arrangements, as known for insects [6]. The outer part of the ganglion (Pl I, Fig. 1) is constituted by a fibrous connective tissue sheath, the neural lamella, and an acellular amorphous matrix embedding collagen-like fibrils. In this fibrous sheath profiles of the cuticular tube of some tracheoles are also present, with their associated cytoplasmic sheath.

The surface of the ventral nerve cord ganglia may be occasionally overlaid by small scattered patches of fat-body cells. These cells (Pl. I, Fig. 2) show large cavities, probably with extracted lipids, together with dark stained glycogen granules. In *B. mori*, as in *Manduca sexta* [1], these fat-body cells do not exhibit a close relation with the underlying neural lamella. In the stick insect (*Carausius morosus*) however, the fat-body cells form a continuous sheath on the surface of both the ganglia and the nervous connectives of the ventral nerve cord [7].

The external fibrous sheath is closely associated with an underlying cellular layer, the perineurium (Pl. I, Fig. 3). The ultrastructural features of this layer are similar to those observed in *C. morosus* and *P. americana* [8]. The perineurium cells are modified glial cells, characterized by the presence of large mitochondria and some free small granules. The most peculiar feature is the presence of elongated lateral cell walls, delimiting an extensive system of tortuous channels. These channels can separate to form extracellular spaces that show considerable expansion and eventually open at the outer margin adjacent to the fibrous sheath. Towards the inner margin of the perineurium the cell walls are held together and this area is increased by many long processes.

The remaining cellular layer, between the perineurial cells and the central ganglionic neuropile, is constituted by glial cells and in this layer the cell bodies of neurones are ensheathed. In the cytoplasm of the glial cells some small mitochondria are visible. The cells contain also microtubules and clumps of dark stained glycogen-like granules (Pl. I, Fig. 4).

In our preparations, as in *M. sexta* [1], the extracellular spaces between glial cells are negligible, while some specialized zones of contact between cell membranes are recognizable, as for the inner part of the perineurium.

The inner glial cells send a complex system of processes into the neuropile. The insect ganglionic neuropile is a well-defined region in the central part of the ganglion, and is constituted by a dense and complex network of axons and their branching processes and glial elements. In the neuropile large numbers of axon profiles are seen (Pl. I, Fig. 5). The axons in the ventral nerve cord of *B. mori* are small or medium size, and contain mitochondria and neurofibrils. The axon cell membranes are either closely apposed (Pl. II, Fig. 6), with a narrow channel in between, or separated by interposed glial elements (Pl. II Fig. 7), with interaxonal gaps and spaces between nervous and glial elements. In the glial elements fairly numerous arrays of microtubules are present. Small dilatations of the glial membranes between axons are also seen.

From ultrastructural observations in *B. mori* ventral nerve cord ganglia different structural barriers and 3 different compartments can therefore be identified: neurons with their branching processes, extracellular volume and haemolymph. From electrophysiological experiments and ionic determinations it is possible to calculate Na and K distribution in neurons and the

neuronal microenvironment. Extracellular potassium concentration $[K_s]$ as a function of extracellular space volume V_s (as % of total tissue volume) is given by:

$$(1) \qquad [K_s] = 100 \frac{\bar{c}_K - [K_i]}{V_s} + [K_i]$$

where $\bar{c}_K$ is the potassium concentration in the tissue water. $\bar{c}_K$ has been determined and its value is 227.2 ± 10.8 mmol/l nerve cord water (15 expts). $[K_i]$ can be estimated on the basis of electrophysiological measurements at different K concentrations in the perfusion fluids, since by the Nernst equation [9] the internal and external concentrations should be equal at 0 mV potential difference. The extrapolated value of $[K_i]$ is 278.6 mmol/l cell water. Equation 1 can be written for the sodium ion too. Sodium concentration in the nerve cord ($\bar{c}_{Na}$) has been measured and its value is 43.3 ± 5.5 mmol/l tissue water (15 expts), a very high value indeed. Intracellular sodium concentration $[Na_i]$ has been determined as follows from electrophysiological data. Assuming the rôle of chloride ions in the electrical potential to be negligible in ventral nerve cord of *B. mori*, the Hodgkin and Katz [10] equation, after Goldman [11] is:

$$(2) \qquad V = \frac{RT}{F} \ln \frac{[K_0] + P_{Na}/P_K [Na_0]}{[K_i] + P_{Na}/P_K [Na_i]}$$

where V is the electrical potential between extracellular space and the outer solution and R , T , F have their conventional meaning, P_K and P_{Na} are the relative permeabilities to potassium and sodium ions and $[X_{i,0}]$ are the concentrations in the cell (i) and in the perfusing solution (o).

The apparent P_{Na}/P_K ratio can be calculated by recording the intracellular potentials in standard conditions and in nerve cords exposed to a solution with a ten-fold rise of sodium concentration. The differences in the electrical potential (ΔV) between normal (Na = 1.7 mM) and test solution (Na = 17 mM) were utilized in the following equation:

$$(3) \qquad \Delta V = \frac{RT}{F} \ln \frac{[K_0] + P_{Na}/P_K [Na_0]_2}{[K_0] + P_{Na}/P_K [Na_0]_1}$$

where $[Na_0]_1$ and $[Na_0]_2$ are the external sodium concentrations in the two experimental conditions. This equation is directly derived from equation 2 assuming that the intracellular concentrations and the apparent relative permeability remain constant on changing sodium concentration in the perfusing solution. The mean value of $\Delta V \pm SE$ (7 exp.) for a ten-fold change in external sodium concentration is 12.14 ± 1.20 mV and the P_{Na}/P_K ratio is 2.03 ± 0.27. From equation 2 $[Na_i]$ in normal conditions can be calculated and its value is 23.1 mM.

The calculated curves for K and Na extracellular concentrations ($[K_s]$ and $[Na_s]$) as a function of the extracellular space volume V_s are reported in Fig. 1, with $\bar{c}_K$ and $\bar{c}_{Na}$ determined experimentally. Other curves have also

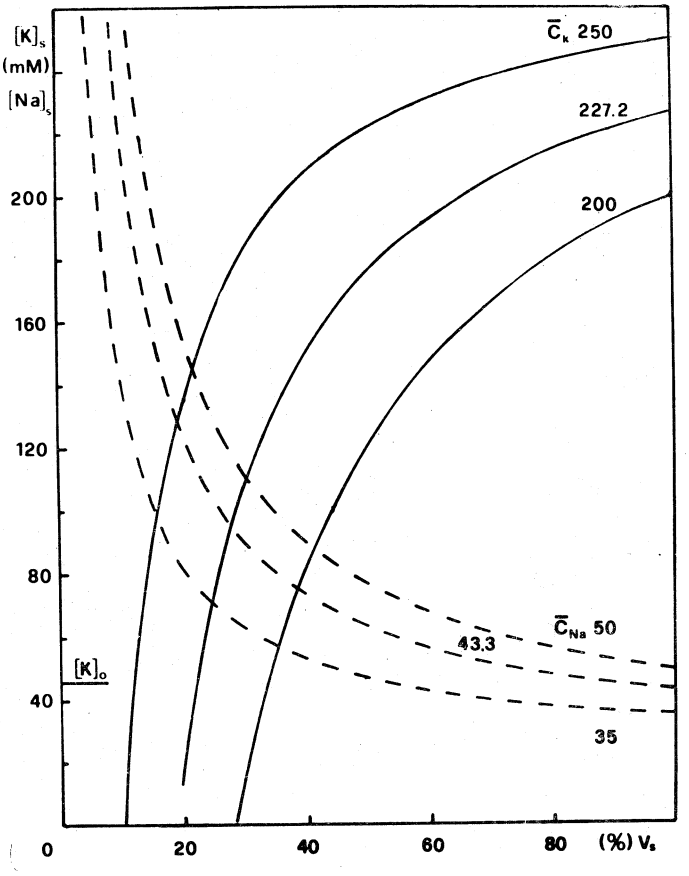

Fig. 1. – Calculated curves (from eq. 1, see text) of extracellular sodium and potassium concentrations as a function of extra-axonal space volume (V_s) for different values of Na and K contents of the nerve cord.

been traced with $\bar{c}_K$ and $\bar{c}_{Na}$ near to experimental values. It can be seen that the extracellular space volume is a very critical value for the distribution of ions in the ganglia. If we assume an extracellular space of 20 % total nerve cord water, a value determined for other insects nerve cords [12, 13], from equation 1, the concentrations of K and Na in the extracellular fluid are 21.6 and 124.2 mmol/l respectively.

Table I reports Na and K distribution in the haemolymph, the extracellular space volume and the cellular water. Since the ratio Na/K in the extracellular fluid is very different from that in the haemolymph, a local regulation must be present on the blood-brain barrier, which corrects the

unsuitability of insect blood as a neuronal environment for a conventional working of neurons. The small positive electrical potentials [14] recorded during microelectrode penetration in the tissue could be in good agreement with the different K ionic concentrations determined in the haemolymph (46.2 mM) and the extracellular system (21.6 mM).

TABLE I.

Estimated cellular and extracellular sodium and potassium concentrations in the nerve cords. Extracellular space was assumed to be 20 % of total tissue water.

Ion	Haemolymph (mmol/l)	Extracellular water (mmol/l)	Cellular water (mmol/l)
Na	1.7	124.2	23.1
K	46.2	21.6	278.6

Ultrastructural studies show that in *B. mori* no extraneural structures, as a continuous fat-body sheath, are present; the " barrier " systems, with active and passive mechanisms, may be represented by different structures such as the neural lamella, the perineurium, the glia and the extracellular systems. The regulation of Na and K ions is probably a glial-mediated process, with the perineurium involved.

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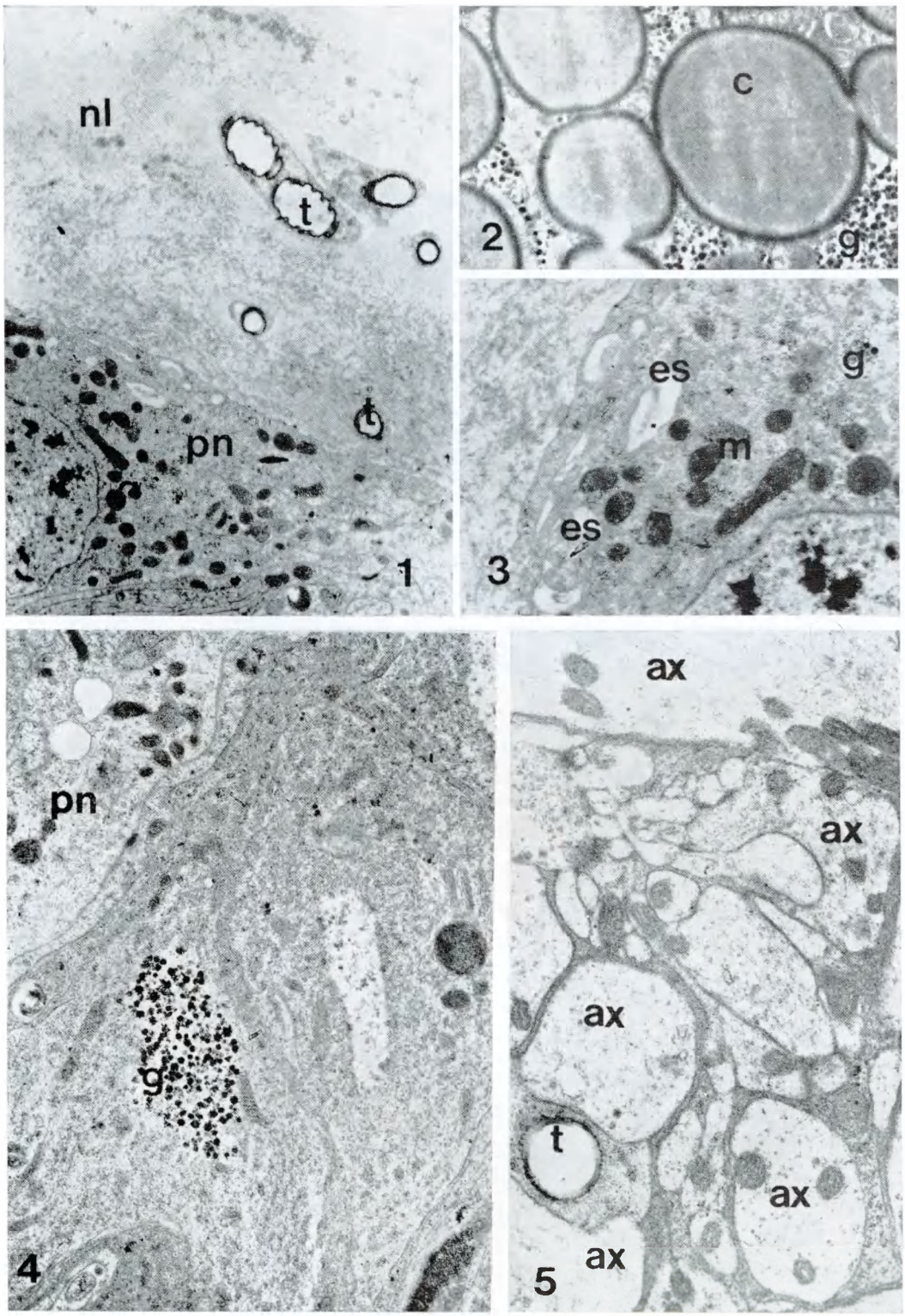
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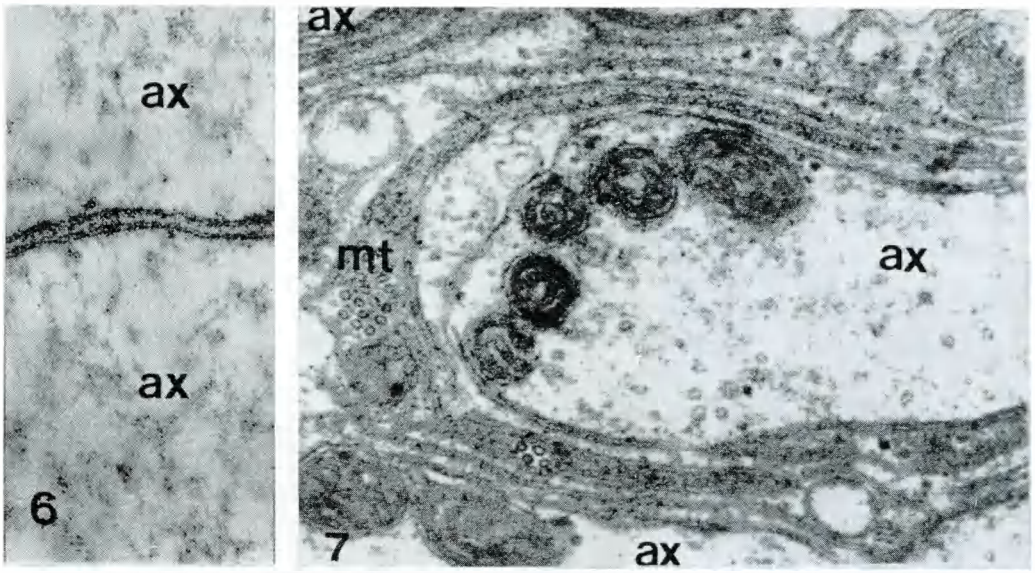
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EXPLANATIONS OF TABLES I–II

PLATE I

Fig. 1. – A transverse section of the peripheral part of a ganglion of ventral nerve cord of Bombyx m. Beneath the fibrous nerve sheath (*nl*), with some tracheole profiles (*t*), the cellular layer of perineurium (*pn*) is shown. No continuous fat-body sheath is present. ×7500.

Fig. 2. – A portion of a fat-body cell. Dark-stained glycogen granules (*g*) and large cavities are seen (*c*). ×9000.

Fig. 3. – Micrograph at higher magnification of the same field as Fig. 1. The perineurium cells contain a number of mitochondria (*m*) and granules (*g*). The tortuous intercellular clefts separate to form extracellular spaces (*es*) toward the outer margin, ×15000

Fig. 4. – Glial cells with clusters of glycogen-like granules (*g*). Membrane linking between glial elements and between glial and adjacent perineurial cells (*pn*) are seen. ×7800.

Fig. 5. – A field in the neuropile. Profiles of axon branches (*ax*) of different size and in transverse section are shown (not all are indicated). The axoplasm contains mitochondria, a large number of neurofilaments and sometimes small vesicles. A tracheole profile (*t*) is also seen. ×13000.

PLATE II

Fig. 6. – A region of close apposition of two axons profiles (*ax*). The axons contain many neurofilaments. In the interaxonal gap glial elements are excluded. ×40000.

Fig. 7. – Glial elements ensheathing axons (*ax*) and delimiting fine channels. In the glial cytoplasm arrays of microtubules (*mt*) are seen. ×54000.