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Ultrastructural Changes in the Gut and the Malpighian Tubules of *Epilachna varivestis* after Application of the New Antibiotic Nikkomycin Involve an Osmoregulatory Defect[1]

ULRICH SCHLÜTER, HARTWIG HOLST, AND KURT CHR. RÖNNAU[2]

Institut für Allgemeine und Spezielle Zoologie, and Institut für Phytopathologie und Angewandte Zoologie, Justus-Liebig-Universität Giessen, Federal Republic of Germany

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The effect of the antibiotic Nikkomycin was investigated on the Malpighian tubules and the gut of fourth-instar larvae of the Mexican bean beetle, *Epilachna varivestis*. Within the Malpighian tubules, three different stages in cell alterations can be recognized. A stage of increased activity (Stage A), and two stages of dedifferentiation (Stages B and C) which are distinguishible by characteristic mitochondrial morphology. In Stage C individuals, when Malpighian tubule function stops entirely, alterations in the midgut take place, that are signs of increased activity. Measurements of hemolymph osmotic pressure showed that there is a considerable increase to a higher level which is maintained. Compared with the ultrastructural data, the regulation of osmotic pressure on a higher level may, in part, be the result of compensation for the failure of Malpighian tubule function by the midgut.

KEY WORDS: *Epilachna varivestis;* malpighian tubules; nikkomycin; antibiotic; osmoregulation; mitochondrial morphology.

INTRODUCTION

Nikkomycin (GT 25/76; AMS 0896 Bayer, Leverkusen) is a microbial metabolite which affects chitin synthesis in several fungi (Dähn et al., 1976). On this basis, Holst et al. (1978) and Zoebelein (1979) tested this substance on various insects and mites with different degrees of success. In laboratory tests, Nikkomycin proved to be very effective against larvae of mites and aphids, and less so but also effective against coleopteran larvae. The diminished action in coleoptera (and lepidoptera) larvae is said to depend on the mode of feeding in different insect groups.

Larvae fed on Nikkomycin-treated plants usually cease molting. In those that do molt, the ensuing instar is much smaller than normal. Mostly the larvae remain in their exuvia not able to free themselves. Usually they do not survive.

The mode of action of Nikkomycin in insects and mites is not yet known, but inhibition of chitin synthesis in the integumentary and other cuticular structures seems to be the primary effect (Cohen and Casida, 1980). Preliminary electron microscopical studies show defects in cuticle deposition of *Tetranychus urticae* (see Mothes, 1981) and *Epilachna varivestis* (see Schlüter, 1981). On the other hand, Holst et al. (1978) made observations on the larvae of *Epilachna varivestis* which could not to be related to disturbed cuticle deposition alone; they were also related to alterations in osmoregulation. Osmoregulation is regulated by several organs. It is now well established that the Malpighian tubules and the gut play the main part (see reviews by Maddrell, 1971; Edney 1977). The present paper shows some effects within these regions in *Epilachna varivestis* larvae treated with Nikkomycin.

[1] Supported by the Deutsche Forschungsgemeinschaft.

[2] Address reprint requests to U. Schlüter, Institut für Zoologie, Stephanstrasse 24, D-6300 Giessen, Federal Republic of Germany.

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MATERIALS AND METHODS

Larvae of *Epilachna varivestis* were obtained from a laboratory stock where they were fed on bean plants. To ensure that cuticle deposition was completed (a defect in cuticle deposition would probably hide other effects), only fourth-instar larvae which were 1 day old were used in the experiments. They were placed on young bean plants which were sprayed with a watery solution containing 0.3% of Nikkomycin. At certain intervals, individuals were separated, killed, and the gut and the Malpighian tubules were dissected out. The tissues were fixed as follows: 4 hr in 2.5% glutaraldehyde/phosphate buffer, *p*H 7.2, then washed twice with buffer, and postfixed with 2% OsO_4 buffer solution. Dehydration followed in an acetone series and embedding in Araldite (Ciba-Geigy). Ultrathin sections were stained with uranyl acetate (20 min) and lead citrate (3 min), and viewed with a Zeiss EM 9A electron microscope. For light microscopical investigations, semithin sections were stained with toluidine blue.

For measuring hemolymph osmotic pressure (OP), three groups (normally fed, fed on Nikkomycin-sprayed plants, and starved) of five individuals each were killed with chloroform at 24-hr intervals. Under liquid paraffin, the extravasated hemolymph from the legs, which had been cut off, was picked up with thin capillaries. After centrifugation, the hemolymph was transferred into liquid paraffin and subsequently measured in a nanoliter osmometer (Clifton, N.Y.).

RESULTS

Normal Alimentary Tract

The topography of the alimentary tract of *E. varivestis* is not presented here. It corresponds to that of *E. indica* as reported by Pradhan (1936). There is one peculiarity, however, that should be mentioned: the distal (false) ends of the Malpighian tubules are attached to the hindgut and are covered by an envelope, the perinephric membrane. This arrangement is usually referred to as being cryptonephric.

Although the normal structure is not the subject of this paper, the characteristic cell types of each tissue will be described briefly. The Malpighian tubules (Fig. 1) and the hindgut consist of transport epithelia. The main characteristics are abundant masses of mitochondria in the cytoplasm. Glycogen is always amply present. The apical surface of the Malpighian tubule cells forms microvilli, each of which contains a mitochondrion. The basal surface is irregularly infolded forming a basal labyrinth (bL). The hindgut cells are covered by a thin cuticle and the apical and basal surfaces are invaginated. The midgut epithelial cells are columnar. Apically, a brush border is seen which never contains mitochondria. Numerous small mitochondria are distributed over the whole cell with slight concentration in the basal and the apical region. Besides a lot of inclusions, e.g., mineral concretions, lipid droplets, glycogen, rough endoplasmic reticulum is frequently seen.

Effects of Nikkomycin Treatment

Malpighian tubules and midgut. Stage A. First, the cryptonephric tubule cells show no clear distinction to those of normally fed larvae. However, in some cases a change in mitochondrial position can be observed. More of them are found in the subapical region at the base of the microvilli. After 40–60 hr, this effect is frequently seen. Mitochondria are no longer found in the basal and medial region of the tubule cells. The remaining mitochondria show two peculiarities: (1) the matrix is quite dense and (2) the increased number of cristae have only narrow spaces (Fig. 2). Rough ER cisternae and aggregations of glycogen are present in negligible portions. At this time no striking changes take place in the midgut epithelium.

Stage B. After about 70 hr, most of the mitochondria in the cryptonephric tubule cells have increased in size (they are about

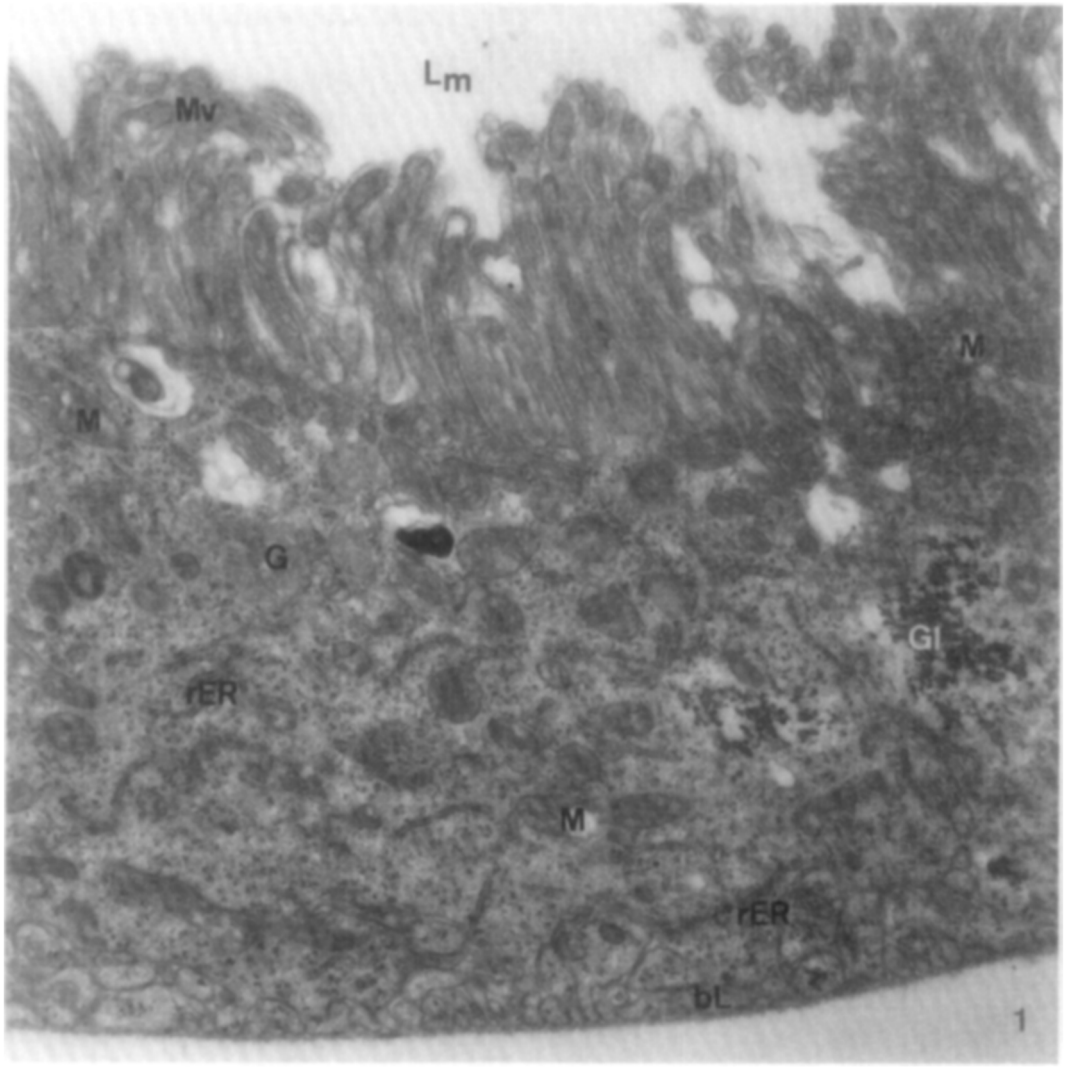

FIG. 1. Cross section through the distal part of a Malpighian tubule. bL, Basal labyrinth; G, golgi dictyosome; Gl, glycogen; L_m, lumen; M, mitochondrion; Mv, microvilli; rER, rough endoplasmic reticulum. ×17,730.

five or six times larger than normal). Most have retracted from the microvillous border. At the same time decomposition of the matrix and of the cristae has occurred (Fig. 3). The cells of the proximal tubule section are normally engaged in producing a secretion. Apocrine secreted material is frequently seen in the tubule lumen, but this is not the case in treated animals (Fig. 4). The epithelial cells seem to be rather inactive. Secretion cannot be established within these cells, but vacuolization and shrinking of the cytoplasm may indicate an impending breakdown. In the midgut epithelial cells, the most striking feature at this time is a partial swelling of the ER cisternae. Some, but not all, mitochondria show a matrix as dense as in the Stage A Malpighian tubules.

Stage C. At this stage (about 90–120 hr after the first feeding on Nikkomycin-treated leaf material) the breakdown of the cryptonephric tubule cells take place. Remains of the "giant" mitochondria are incorporated by cytolysosome-like bodies,

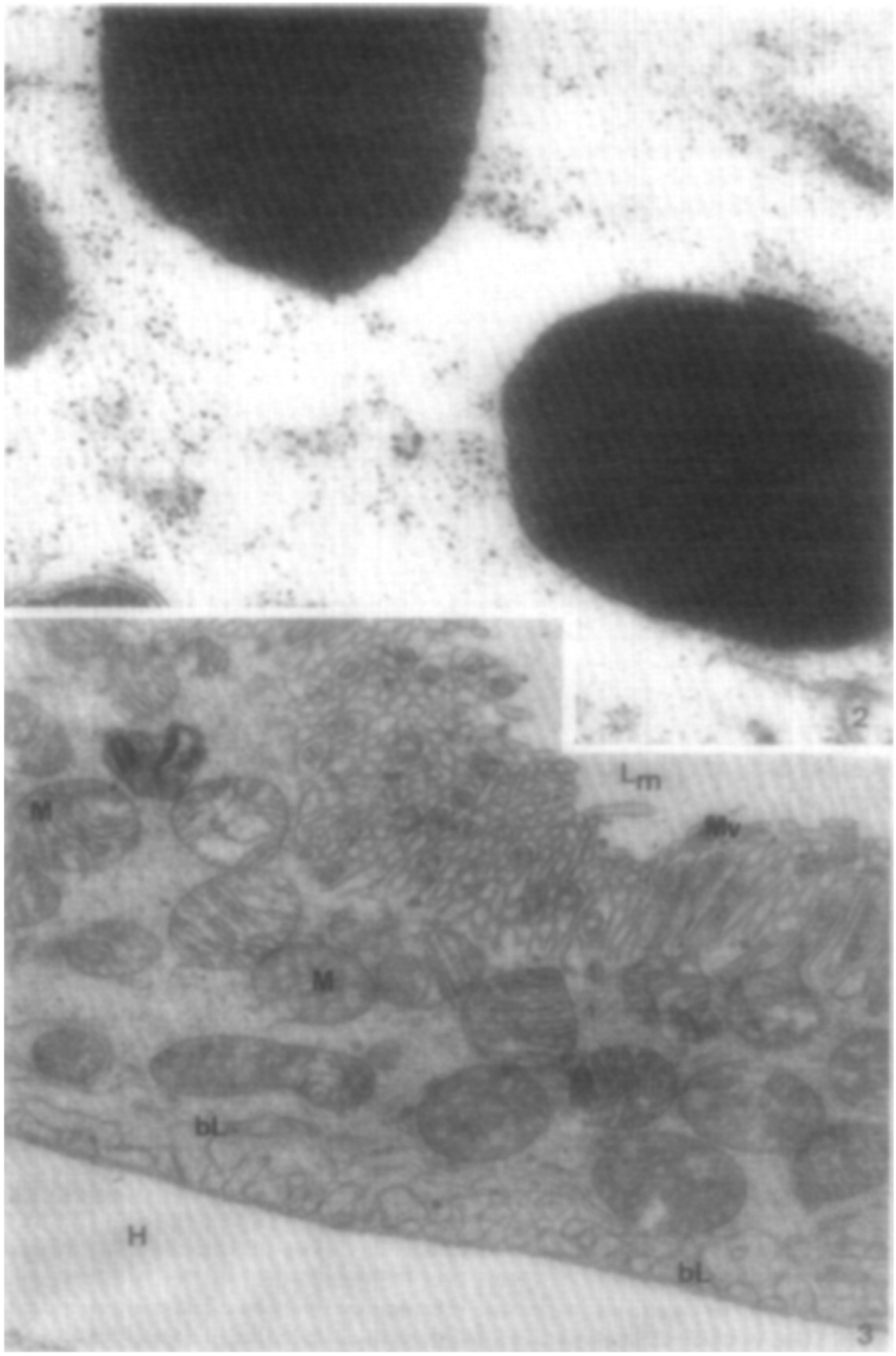

FIG. 2. Stage A distal tubule cell (about 50 hr after the first Nikkomycin treatment). The apically located mitochondria have a more dense matrix and more cristae. ×62,530.

FIG. 3. Distal tubule section of Stage B larvae (about 70–80 hr). Only few microvilli contain mitochondria. Most of the mitochondria have a lightened matrix and fewer cristae. bL, Basal labyrinth; L_m, tubule lumen. ×17,730.

which are now the most abundant organelles (Fig. 5). This process initiates the total dedifferentiation of these cells. The cells of the proximal tubule section undergo similar processes. They degenerate in two different ways. Some cells suffer a gradual dedifferentiation, starting with the elimination of cell organelles until only small shrunken mitochondria remain in the electron-lucent cytoplasm (Fig. 6, left side). Sometimes cells occur the contents of which are fully condensed to an amorphous mass. The enlarged clefts between microvilli and basal infoldings and big vacuoles point to an extreme shrinking of the cytoplasm. The mitochondria of the midgut continue to darken while they increase in volume and change their form dramatically. In Figure 7 some elongated mitochondria with club-shaped endings are visible. Those mitochondria show the tendency to be bent, cup shaped, or ring shaped. The latter show, centrally or near the periphery, cytoplasmic inclusions. However, a form which seldom appears is that with two inclusions, as shown in Figure 8. The cytoplasmic inclusions contain remains of rough endoplasmic reticulum and free ribosomes. Besides these, mitochondria with electron-dense matrix, voluminous giant mitochondria with electron-lucent matrix, and few cristae occur. The basal infoldings of the midgut cells, which are normally broad spaced only in the basal region, now extend up to the nuclear region (Fig. 9). Centrally located branches of the basal infoldings contain paracrystalline inclusions (Fig. 10). The particles are about 5 nm in size and average about 20 nm from each other.

Hindgut. In the hindgut, the alterations in cell morphology are nearly the same as in the Malpighian tubules. Initially, the mitochondrial matrices condense and finally the volume of the mitochondria is greatly enlarged (Fig. 11). Glycogen and rough endoplasmic reticulum disappear after 60–100 hr. Tests with third-instar larvae showed that the hindgut cells remain in this phase until the next molting begins. However, this molting is regularly inhibited: the cells vacuolize for the most part before apolysis is achieved. In some cases, apolysis and secretion of parts of the new epicuticle (cuticulin layer) are observed, but during this process cells usually degenerate.

Hemolymph osmotic pressure. The osmotic pressure (OP) of the hemolymph was measured during a 5-day period (a longer range was not advisable because fourth-instar larvae reach the prepupal stage after this time).

The alterations in hemolymph OP are shown in Figure 12. During the first 3 days, no change is visible in Nikkomycin-treated and normally fed individuals (about 335 mosmol), while the osmotic value of the starved individuals increases rapidly after 2 days, reaching 515 mosmol after 4 days. In treated larvae, there is a significant increase (t test) in OP after 4 days, up to 390 mosmol. This value does not change considerably during the next day. The OP of the untreated controls does not change during the period of investigation.

DISCUSSION

The present investigation shows first of all that the Malpighian tubules are affected after Nikkomycin treatment. It further shows that on the cellular level, the mitochondrial appearance and mitochondrial population undergo characteristic alterations.

Within the cryptonephric complex, the function of the Malpighian tubules is of great importance (Ramsay, 1964; Grimstone et al., 1968), while the hindgut plays a minor role (Maddrell, 1978). Ions are transported actively via the tubule cells from the hemolymph into the tubule lumen. The increased osmotic pressure exerts a long-term effect on the watery feces present in the hindgut lumen. The water is withdrawn from the hindgut lumen along an osmotic gradient into the perirectal space where it is recovered by the hemolymph. If this pathway is disturbed, as it seems to be in Nikkomycin-treated *Epilachna,* water re-

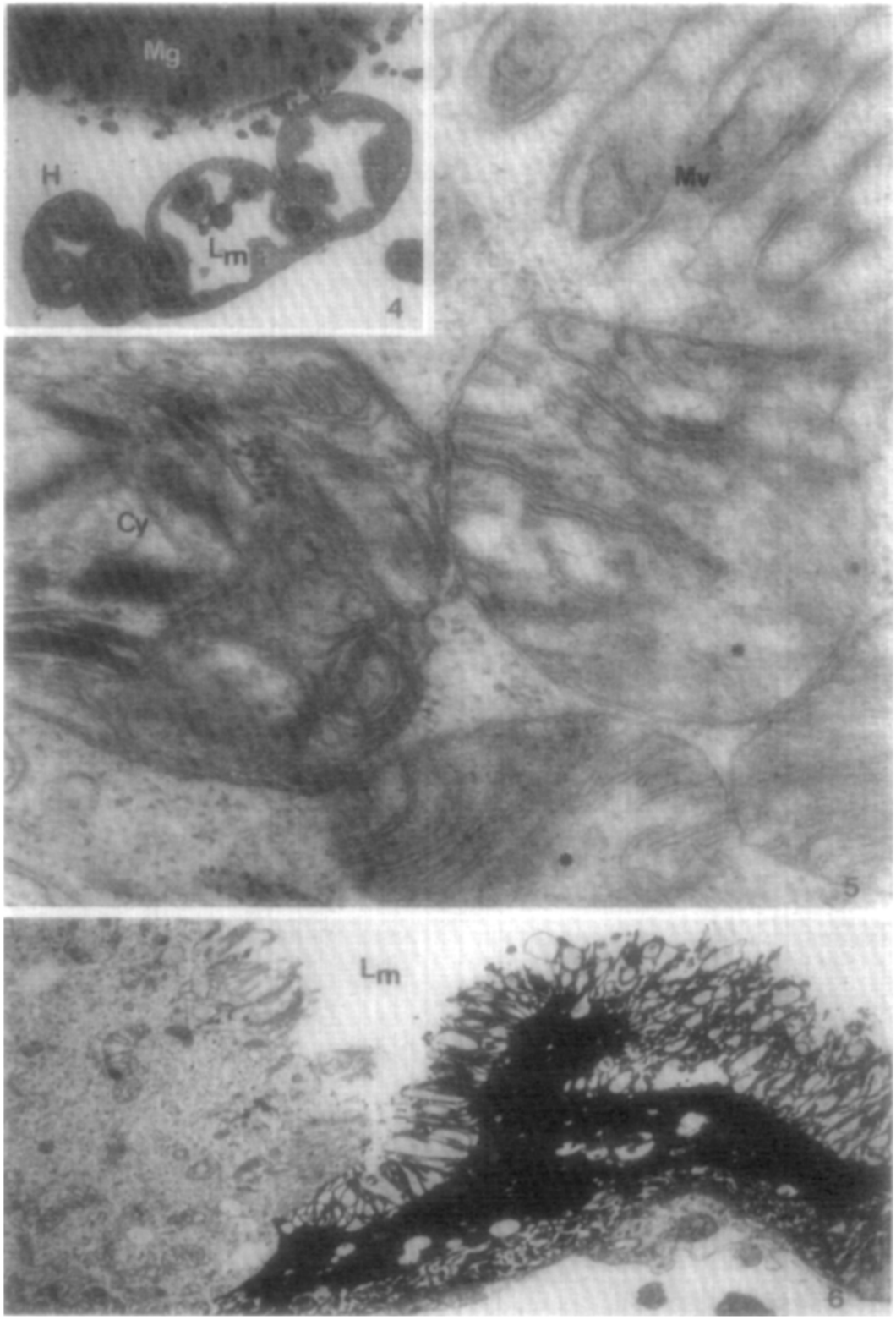

FIG. 4. Cross section through Stage B Malpighian tubules. The cells seem to be very stressed and degenerated. H, Hemocoel; L_m, tubule lumen; Mg, midgut. ×200.

FIG. 5. Stage C distal tubule section (about 100 hr after Nikkomycin treatment) with disintegrating mitochondria (*). Cy, Cytolysosome-like body; Mv, microvilli. ×62,530.

FIG. 6. Stage C proximal tubule section showing two different phases of dedifferentiation. L_m, Tubule lumen. ×4,340.

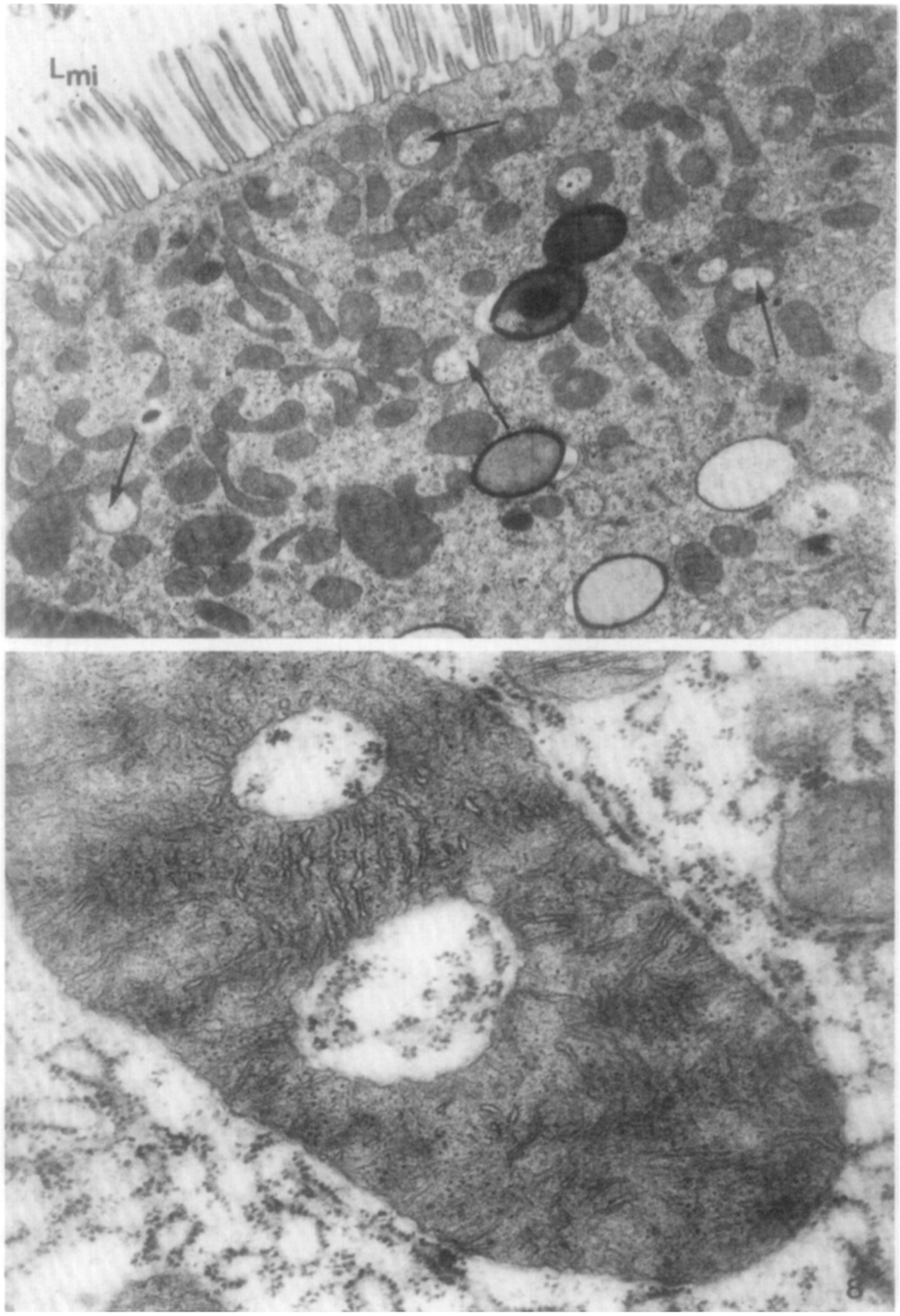

FIG. 7. Apex of Stage C midgut epithelial cell with different transformed mitochondria (about 90 hr after Nikkomycin treatment; arrows). L_{mi}, Lumen of the midgut. ×11,000.

FIG. 8. A stage A midgut mitochondrion with two enclosed plasma areas. ×62,530.

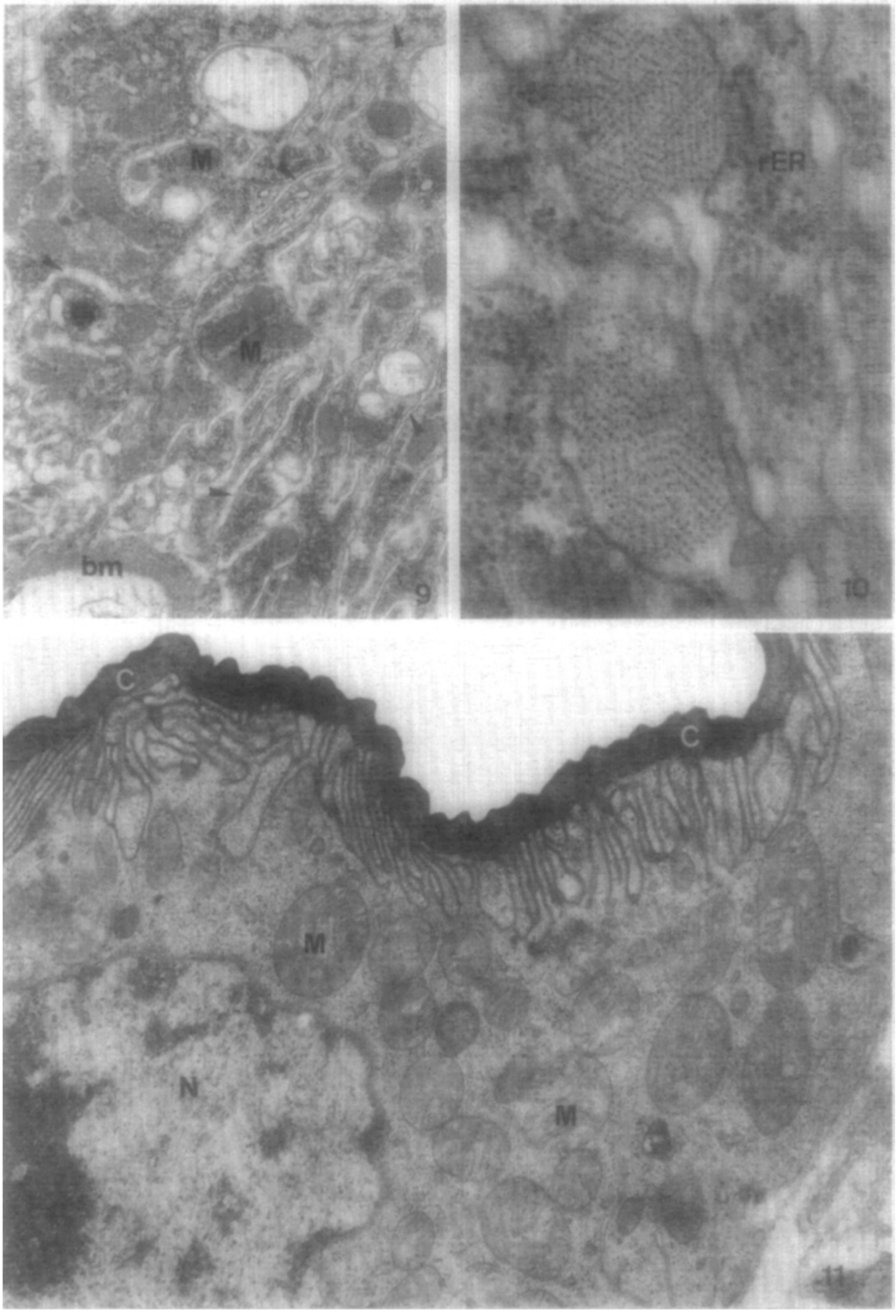

FIG. 9. The clefts of the basal labyrinth in Stage C individuals (about 90 hr after Nikkomycin treatment) are much wider than normally occurring (arrowheads). bm, Basal membrane; m, mitochondria. ×11,300.

FIG. 10. In Stage C individuals the deep basal invaginations of the midgut cells often contain paracrystalline structures. rER, Rough endoplasmic reticulum. ×82,300.

FIG. 11. Appearance of the hindgut epithelium in Stage C individuals. c, Cuticle; m, mitochondria; n, nucleus. ×21,900.

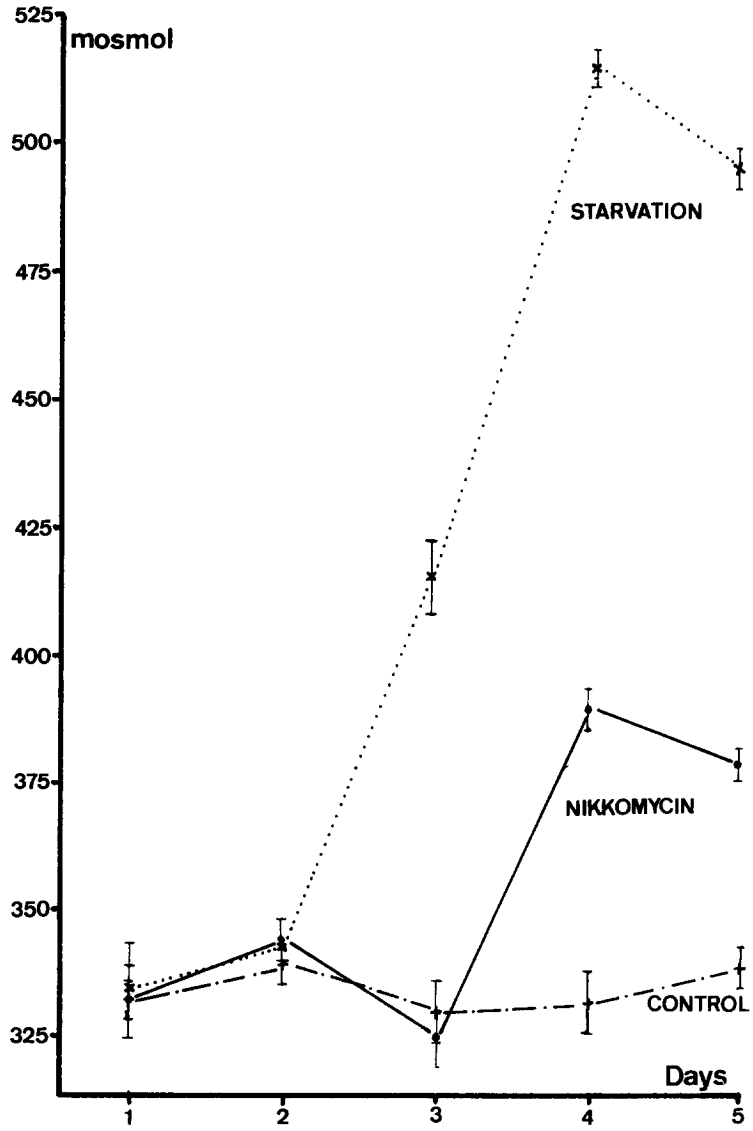

FIG. 12. Development of the osmotic pressure of the hemolymph during a 5-day period. The points represent the arithmetical means of the osmotic value of five individuals each. The bars indicate the standard deviation.

covery from the hindgut is no longer possible. The consequence must be a considerable increase of the osmotic pressure in the hemolymph, as it is in many other cases (for references see Edney, 1977). As can be seen in Figure 12, there is indeed an increase of OP after the third day, which does not change the following day. Obviously the OP is regulated at a considerably higher level. A possible explanation for this phenomenon is given by the ultrastructural investigations.

Mitochondria play a central role in all active transport processes, which are known to require a great amount of energy in the form of ATP. If the mitochondria are disturbed, as is the case in the Malpighian tubules here, the active transport of ions fails with the consequences mentioned above.

The symptoms in Stage A individuals are not pathological. Moreover, the concentrations of mitochondria in the apical region close to the cytoplasm membrane and the

condensed matrix represent, in our opinion, a high level of activity. Since energy is required mostly at the site of the active transport (this is in fact the cytoplasm membrane), mitochondria must have close contact with it. Such combinations of mitochondria and cytoplasm membranes are often found in transport-active tissues (Oschman and Wall, 1969; Wessing and Eichelberg, 1973; Schlüter 1980). The contrary is observed in inactive Malpighian tubules of the pharate pupal stage of *Calpodes ethlius* (Ryerse, 1979). In this stage, fluid secretion is switched off and the mitochondria retract from the microvilli and consequently degenerate.

As concerns the density of the matrix and the increased number of cristae, the following may be assumed: both the matrix and the cristae bear many enzyme systems required for energy production. The cristae, for example, contain the ATP-synthesizing enzymes and the enzymes of the respiratory chain. It is assumed that more enzymes are present within the Stage A Malpighian tubule mitochondria and, as a consequence of that, more potential (transport) capacity. Mitochondria like these occur in various tissues which have a high energy turnover under normal conditions (e.g., see Berridge and Gupta, 1968). However, the production of ATP under the present conditions must be confirmed by biochemical examinations. The dense mitochondrial forms of Stage A Malpighian tubules and Stage C midgut cells must be clearly separated from those found in pathological tissues, for example, after intoxication (Endo and Nishiitsutsuji-Uwo, 1980; Rydzynski and Cieciura, 1980), which show irregular puffings of the intracristal spaces.

Undoubtedly pathological is the swelling of the mitochondria in Stage B individuals. At this stage, protein synthesis has ceased for the most part (disappearance of rough ER) and material for energy production is no longer available (absence of glycogen). At the same time as this swelling occurs there is a part disintegration of the matrix and the cristae. Such transformed mitochondria are also found in the hepatopancreatic cells of *Ligia oceanica* after long starvation (Storch and Lehnert-Moritz, 1980). However, these authors propose that these effects might not be specifically caused by starvation. Afzelius and Mohri (1966) observed a reduction of cristae in mitochondria of various spermatozoa: cells which are not supplied with energy-rich material during their entire life. They assumed that mitochondria use their own substances, such as phospholipids from the cristae, as energy sources if the main glycolyzable substances of the cells are spent. This would explain the disappearance of the cristae membranes in Stage B individuals also.

An interesting feature is the occurrence of the ring- and cup-shaped mitochondria in Stage C midgut cells. Among others, Ratcliffe and King (1969) investigated the genesis of these aberrant forms in *Nasonia vitripennis*. They found them finally turned into lipid droplets after a long starvation period. But these and other authors (e.g., Ghadially, 1975) consider these forms as being very active. If this is accepted, and if the transport capacity of the midgut is raised, a possible explanation might be given as to why the OP is maintained at the increased level and does not elevate, as would be expected after the destruction of the Malpighian tubule cells. The midgut cells possibly take over part of the osmoregulation function as compensation for the disturbed Malpighian tubules (and, finally, the cryptonephric complex). The extremely widely extended basal labyrinth and the paracrystalline inclusions in the midgut would support this hypothesis. The latter might be substances which are withdrawn from the hemolymph in order to lower the OP as suggested by Maddrell (1971). At present these assumptions are clearly speculative and further investigations on the metabolic pathway of Nikkomycin are needed.

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