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COMPARATIVE ELECTRON MICROSCOPIC AND CYTOCHEMICAL STUDIES ON THE CUTICLE AND THE HYPODERMIS OF TRICHINELLA NATIVA (BRITOV ET BOEV, 1972) AND T. PSEUDOSPIRALIS (GARKAVI, 1972)

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Dedicated to Dr. J. Šlais D.Sc. on the occasion of his 60th birthday

Abstract. Using cytochemical methods, we disclosed both in the cuticle and the hypodermis of two-day-old females of *T. nativa* and *T. pseudospiralis*, in the area of the oesophagus, these substances: Cystine-containing proteins of the SS group, carbohydrates, acidic mucopolysaccharides (AMS), acid phosphatase (ACP) and a nonspecific esterase (NE). A regularly transverse striation (striae) in the cuticle reacts strongly with the method for cystine-containing proteins, weakly for carbohydrates, and negatively for AMS. Striae are present in most parts of the cuticle of *T. nativa*. By contrast, the amorphous cuticle of *T. pseudospiralis* is not striated, it stains for carbohydrates and AMS. The cuticular surface of both *Trichinella* species is made up of a carbohydrate matrix visible only in cytochemical reactions. By contrast to the cuticle of *T. pseudospiralis*, the electron density of the cuticle of *T. nativa* is increased by transverse extensions of the outer, hypodermal membrane which contain numerous muscle tendons terminating in desmosomes. Muscle extensions together with lysosomes entering the hypodermis react for NE and cystine, but not for ACP. The number and configuration of hypodermal glands influences the ornamentation of the cuticle. A secretion released from these glands reacts for carbohydrates and proteins. Together with the carbohydrate coat known to be present on the surface of *Trichinella* species, they might bring about an immunity reaction in the host.

In order to distinguish the two *Trichinella* species, *T. nativa* Britov et Boev, 1972 and *T. pseudospiralis* Garkavi, 1972, the authors have used genetic, biological, ecological and metrical criteria. An examination of the surface structure of the male of various *Trichinella* species with the SEM (Baruš et al. 1979, Hulínská and Shaikenov 1980) yielded a number of new data. An explanation of the observation that copulation cannot take place between males and females of different *Trichinella* species was given by results of an earlier study (Hulínská and Shaikenov 1980, 1982) who disclosed differences in the configuration of copulatory organs, and in the fine structure of sperms in the sexual organ of the female. At present, there is a considerable lack of both cytochemical and structural criteria to be used for distinguishing among the individual species of the genus *Trichinella* with a transmission electron microscope (TEM). The present study has been undertaken for the purpose of distinguishing young females of *T. nativa* from those of *T. pseudospiralis* on the basis of the structure and the cytochemistry of the cuticle and the hypodermis in the area of the oesophagus, and to compare the results with earlier descriptions of the structure of *T. spiralis* (Despommier and Müller 1970, Bruce 1970, Kozek 1971, Senuita 1978).

MATERIALS AND METHODS

Females of *T. nativa* and *T. pseudospiralis*, aged two days, were obtained from the intestine of mice experimentally infected with the two *Trichinella* species at the Zoological Institute, Academy of Sciences of the Kaz. SSR. The techniques and methods employed were essentially similar to those described in an earlier paper (Hulinská and Shaikenov 1981). A comparative study was made using a series of transverse and longitudinal sections through the oesophagus area of nine females each of the two species. These were fixed, embedded and stained with identical methods. Cytochemical reactions: Luft's (1971) method for acidic mucopolysaccharides — fixation of worms in a 0.1 M Ma-cacodylate buffered, 3 % (v/v) solution of glutaraldehyde and 500 p.p.m ruthenium red added to the buffer, rinsing and postfixing in 2 % OSO_4 and 5 p.p.m ruthenium red; the method suggested by Monga et al. (1972) for sulfated mucopolysaccharides for a staining of ultrathin sections with 3,3-diaminobenzidine (DAB) binding sulfated groups in an acid pH, oxidation with OsO_4, rinsing of ultrathin sections in 5 % boric acid, staining with 1 % DAB in 5 % boric acid for 30 min, rinsing in boric acid and flotation with 2 % OsO_4 for 20 min; PATCO test — periodic acid-thiocarbohydrazide-OsO_4 (Lumsden et al. 1970) for carbohydrates; TSC-test — thiosemicarbazide-silver proteinate (Thiery 1967) for glycogen; the method suggested by Swift (1973) for cystine-containing proteins using silver methenamine after washing sections in 0.1 N HCl. Results were controlled with the periodic-acid oxidation-silver methenamine test for carbohydrates (Rambourg 1967) and the periodic acid-chromic acid-silver methenamine (PA-CrA-S) test (Rambourg et al. 1969) for diglycols and glycoprotein; the method suggested by Barka and Anderson (1962) for the acid phosphatase — incubation of sections (50 μm) in a medium containing NA β glycerophosphate (pH 5) in a 0.2 M tris-maleate buffer, and 0.2 % lead citrate recommended by Reynolds (1963) to replace lead nitrate. Control tests in a medium without a substrate disclosed several staining artifacts, e.g., those caused by Pb ions on the surface of the cuticle; a method suggested by Holt and Hicks (1966) for nonspecific esterase (NE), using indoxyl esters with FRP. Incubation of sections for 1 hr in a medium consisting of 0.4 ml hexazonium pararosaniline and 3 ml 0.2 M sodium citrate to which 1 ml indoxyl acetate was added at pH 6 and complemented with the cacodylate buffer, washing in the buffer, placed for one hr into 2 % OsO_4, dehydrated and embedded by centrifugation in Epon 812. The absence of indoxyl acetate in the medium was confirmed by the control. Ultrathin sections were cut with glass knifes of a Reichert Ultramicrotome, and examined with the electron microscope JEM 100 B.

RESULTS

The cuticle of a two day-old female of *T. nativa* (inspection of living specimens) has a remarkable ornamentation, it is electron dense and folded. Its thickness in longitudinal sections ranges from 0.6—0.8 μm. It is ornamented with longitudinal ridges and transverse grooves. There is considerable variation in the thickness both of the cortical layer and the amorphous, striated layer of the cuticle (Bruce 1970). The cortical layer can be subdivided into an outer, middle and inner layer. The amorphous layer of the cuticle bears a regular, transverse striation. Thin and thick striae extending into the basal lamina of the cuticle, are ill-distinguishable from extensions of the outer, hypodermal membrane. The outer, cortical layer, and mainly the inner layer react strongly for cystine-containing proteins (Plate 1, Fig. 1). In the control test (PA—CrA—S) the reaction for carbohydrates is weak, the individual layers cannot be distinguished (Plate 1, Fig. 2). The outer surface of the cuticle made up of a layer of the carbohydrate coat matrix, is about 20 nm thick. It can be distinguished only with cytochemical methods for acid mucopolysaccharides in a staining with ruthenium red (Plate I, Figs. 4, 5). Both the surface coat and striae react weakly with PATCO (Plate I, Fig. 3). The surface coat, granulated homogeneously, is evenly spread over the cuticular surface, including its ridges and grooves. It reacts for mucopolysaccharides with the DAB test (on ultrathin sections). A reaction for these substances is weak in the outer cortical layer, striae of the amorphous layer of the cuticle do not stain with this test (Plate I, Fig. 5). Neither the inner cortical layer, the amorphous layer nor the hypodermis stain with ruthenium red, only the surface coat stains uniformly with this dye. This indicates that the polycationic ruthenium red cannot diffuse to layers below the surface coat. The cuticle does not react with the TSC test for glycogen, the surface reacts faintly (Plate I, Fig. 6). Transverse striae of the amorphous layer react strongly for cystine-containing proteins (Plate I, Fig. 1), negatively for acidic mucopolysaccharides (AMS). Electron dense bodies in the basal layer are seen in a reaction for glycoprotein.

The cuticle of *T. pseudospiralis* is less folded than that of *T. nativa*, and translucent. Its thickness is 0.8—0.9 μm. The structure of the cortical layer is identical to that of *T. nativa* (both are triple-layered). The amorphous layer of the cuticle which is not striated is divided into an apical and a basal part by a thin, electron dense substance. Both the outer and the inner cortical layers of the cuticle react for cystine-containing proteins (Plate II, Fig. 4) but their reaction is less strong than that in *T. nativa.* Using ruthenium red for acidic mucopolysaccharides, the surface coat reacts strongly, both the inner cortical layer and the amorphous layer of the cuticle react weakly (Plate II, Fig. 6). Intense staining with the dye is seen in granules of the hypodermis and particularly in those of the stichosome (Plate II, Fig. 6). Both the amorphous layer and the cortical layer of the cuticle react with DAB, the surface stains intensely (Plate II, Fig. 5). A faintly opaque staining of the cuticle in the test for NE and ACP is an artifact caused by Pb ions as disclosed by the control (Plate III, Figs. 1, 3). The fact that ruthenium red stains all layers of the cuticle of *T. pseudospiralis* and also the hypodermis indicates that the cuticle of *T. nativa* is considerably less permeable than that of *T. pseudospiralis.* The test for glycoproteins shows a strong reaction of the surface coat and the outer cortical layer, a weaker reaction of the inner cortical layer. Striae have not been observed in the amorphous layer of the cuticle (Plate III, Fig. 4). In longitudinal sections, the inner cortical layer stains intensely for cystine-containing proteins, the thin layer inside the amorphous matrix of the cuticle does not stain (Plate III, Fig. 5). The thin, intermediate, electron dense substance in the amorphous layer reacts with the PATCO test (Plate III, Fig. 6).

The considerable electron density of the cuticle of *T. nativa* as compared with that of *T. pseudospiralis* is due to the presence of numerous extensions of the hypodermis in it. They are extensions of the outer, hypodermal membrane which is indistinguishable from the basal lamina of the cuticle and copies exactly the folding of the cuticle (Plate I, Fig. 1). A transverse section shows the brick-like organisation of these extensions in the cuticle (Plate II, Fig. 1). Transverse extensions of subcuticular muscle fibrils into the hypodermis are membrane-bound and accompanied by lysosomes. They react weakly for cystine and NE (Plate II, Fig. 2), and do not react for ACP (Plate II, Fig. 3) in contrast to the surrounding hypodermis. The fibrillar substance in the hypodermis consists of a system of fibrils connecting muscle extensions with the cuticle.

A minimum of folds is seen both in the cuticle and the hypodermis of *T. pseudospiralis.* Extensions of the hypodermal membrane are short, garland-shaped, and react less intensely than granules of the stichosome for diglycols (Plate III, Fig. 2). Hypodermal extensions react for SS groups of proteins (Plate III, Fig. 5), ACP (Plate III, Fig. 3) and for carbohydrates (Plate III, Fig. 6). Extensions of muscle fibres are short and scarce.

The cuticle of species of the genus *Trichinella* is perforated by numerous cuticular pores in the zone of the bacillary band in which there are hypodermal glands. These open to the exterior through cuticular pores. Lamellar extensions terminate in a condensed substance reacting for carbohydrates (Plate IV, Fig. 1) and for proteins (Plate IV, Fig. 2). The cytoplasm of gland cells contains glycogen (PATCO test) (Plate IV, Fig. 3). Masses of pores form compact circles in the individual folds of the cuticle of *T. nativa* (Plate IV, Fig. 4), while in the almost smooth cuticle of *T. pseudospiralis*, there is a considerable distance between the individual pores, and there are less glands in the lateral cord (Plate IV, Fig. 5).

DISCUSSION

Using ruthenium, mucopolysaccharides were demonstrated in the surface coat of the cuticle of two day-old females of *T. nativa* and *T. pseudospiralis*. In *T. nativa*, the polycationic dye stained the surface coat only and not the underlying layers, in *T. pseudospiralis* it penetrated the cuticle as evidenced by a staining of granules of the hypodermis and the stichosome. Timonov (1970) demonstrated in his study with the luminescent microscope that *Trichinella* species feed osmotically for the period of their development in the intestine of the host. The author maintained that in contrast to muscle larvae the cuticle of intestinal *Trichinella* species increased its permeability more than eight times, but reduced in thickness from 2.3 μm to 0.9 μm. Silakova (1972) observed food in the intestine of *Trichinella* species in the initial period of their development in the intestine of the host, because the permeability of the cuticle was not yet at its optimum and the amount of food seeping through the cuticle was insufficient. Gridasova (1969a, b) maintained that the presence of mucopolysaccharides in the cuticle and the hypodermis of intestinal *Trichinella* species should be ascribed to a high permeability of their cuticle. The DAB test for sulfated mucopolysaccharides disclosed their presence both in the cuticle and the hypodermis of *T. pseudospiralis*, while in *T. nativa*, the cortical layer of the cuticle gave a positive reaction for sulfated mucopolysaccharides but not the striae of the amorphous layer. The high resistance of the cuticle of *T. nativa* might be attributed to the dense striation of the matrix. In it, as well as in the cortical layer and in the basal lamina, we determined the presence of cystine-containing proteins. Lee (1966) maintained that the striated layer consisted of closely linked protein molecules, Jamuar (1966) suggested a crystalloid structure for this layer. In *T. nativa*, the striated layer was seen to pass through the entire matrix, and was basally connected with the fibrous cortex. A similar observation was made by Wright (1968) for *Capillaria hepatica*. The different degree of permeability of the cuticle might have been influenced by the thickness of the surface coat which Jenkins (1969) suggested to have been produced by a secretion of the parasite although he did not reject the possibility of a host reaction. We do not held Bennet's (1963) view that the surface coat is a glycocalyx lining the outer surface of the cell. It is separated from hypodermal cells by a non-cellular cuticle. However, Cherian et al. (1980) observed a different situation in microfilarians. The surface coat of *Trichinella* species contained acidic mucopolysaccharides, PAS positive carbohydrates and cystine-containing proteins demonstrable only with cytochemical methods. The hypodermis of *T. nativa* having a non-nucleate area between the cords, produced a remarkable, brick-like organized system of extensions in the basal part of the cuticle. There, the outer hypodermal membrane fused with the basal lamina and obscured its triple-layered lamina described by Wright (1968) for nematodes. We demonstrated in the hypodermis an ACP activity similar to that observed by Senuita (1978) and Ruitenberg (1972) for *T. spiralis*. The hypodermis contained proteins, carbohydrates and glycogen. A fibrillar substance in the hypodermis produced a system of fibrils fastening muscle cells to the cuticle. Distinct, fibrillar bands traversing the hypodermis of large nematode species, were found by Watson (1965) in *Ascaris lumbricoides*, Lee (1966) in *Nippostrongylus brasiliensis*, Smith (1970) in *Haemonchus placei*. Other findings are available from a paper by Anya (1976). No evidence of these bands has been obtained as yet for members of the genus *Trichinella*. Using cytochemical methods, fibrillar extensions were seen in *T. nativa* in a reaction for cystine-containing proteins and glycoproteins. These fibrils were attached to the basal lamina of the cuticle, and to membrane extensions of the hypodermis. Long muscle extensions could not be seen in the hypodermis of *T. pseudospiralis* which indicated that both muscles and the cuticle of the female of *T. nativa* possessed a better capability of contraction than those in the female of *T. pseudospiralis*. The increased contraction of *T. nativa* could hardly have been a fixation artifact because identical methods of fixation and embedding were used for both *Trichinella* species. Electron dense bodies detected in the basal part of the amorphous, cuticular layer of *T. nativa* were found to be fibrillar endings serving for attachment of the muscles to the cuticle. Kozek (1971) failed in observing dense bodies in the cuticular base of young specimens of *T. spiralis* because they might have been low in number. The description of the bacillary band made by Bruce (1970) for *T. spiralis* was identical to that made by Wright (1968) for *Capillaria hepatica*. By contrast to *T. pseudospiralis*, pores in the cuticle of *T. nativa* were lying in close proximity to one another. An explanation might be the heavy folding of the cuticle of *T. nativa*. Bruce (1970) ascribed an antigenic nature to the secretion released through cuticular pores to the surface of the worm. His suggestion was supported by a demonstration of phosphatases in the lamellar apparatus of gland cells (Senuita 1978). However, neither Senuita nor we succeeded in detecting this enzyme in the secretion. The boundary layer stretching across the pore chamber of the bacillary band contained carbohydrates and proteins. Luft (1971) found both substances in the protein- carbohydrate complex on the surface of the parasite to which numerous authors attributed an immunological function in the host (Luft 1976, Bird 1976, Whitfield 1979, Cherian et al. 1980, and others).

СРАВНИТЕЛЬНОЕ ЭЛЕКТРОННОМИКРОСКОПИЧЕСКОЕ И ЦИТОХИМИЧЕСКОЕ ИЗУЧЕНИЕ КУТИКУЛЫ И ГИПОДЕРМЫ *TRICHINELLA NATIVA* (BRITOV ET BOEV, 1972) И *T. PSEUDOSPIRALIS* (GARKAVI, 1972)

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Резюме. При помощи цитохимических методов в кутикуле и гиподерме самок *T. nativa* и *T. pseudospiralis* в возрасте 2 дней обнаружены следующие вещества в области пищевода: цистин-содержащие белки SS-группы, углеводы, кислые мукополисахариды (КМС), кислая фосфатаза (КФ) и неспецифическая эстераза (НЭ). Регулярное поперечное исчерчение кутикулы дает сильную положительную реакцию на цистин-содержащие белки, слабую на углеводы и отрицательную на КМС. Исчерчение имеется почти на всех частях кутикулы *T. nativa*, тогда как у аморфной кутикулы *T. pseudospiralis* дающей положительную реакцию на углеводы и КМС исчерчение отсутствует. Внешняя поверхность кутикулы обоих видов *Trichinella* состоит из слоя углеводной матрицы, видимой только в цитохимических реакциях. В отличие от кутикулы *T. pseudospiralis*, плотность кутикулы *T. nativa* повышается поперечными отростками внешней гиподермальной мембраны, содержащей многочисленные места прикрепления мышцы, кончающие в десмосомах. Мышечные отростки вместе с лизосомами дают положительную реакцию на НЭ и цистин, а отрицательную на КФ. Количество и конфигурация гиподермальных желез в области пищевода оказывает влияние на орнаментацию кутикулы. Выделяемый этими железами секрет дает положительную реакцию на углеводы и белки и вместе с гликокаликсом, доказанным на поверхности трихинел, может вызывать иммунную реакцию хозяина.

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EXPLANATIONS OF LETTERINGS IN THE FIGURES

A	outer cortical layer
B	inner cortical layer
C	surface coat
D	desmosomes
E	fibrous muscle tendons
F	muscle fibrils
G	glycogen
H	hypodermis
CH	granules of the stichosome
I	mitochondria
J	membrane
K	dense strip in the matrix
L	lysosomes
M	matrix of the cuticle
N	lamellary apparatus of gland cells
O	secretion in layer bounding the pore chamber
P	basal lamina
Q	muscle extensions
R	ribosomes
S	striae
T	extensions of the hypodermis

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A STUDY ON THE MORPHOLOGY OF EARLY LARVAL STAGES OF TOXOCARA CATI (SCHRANK, 1788)

Till the present time, a great majority of papers dealing with larval toxocarosis have designated as causative agent of this disease the 2nd-stage larvae of dog or cat nematodes *Toxocara canis* or *T. cati,* the development of which does not continue in paratenic hosts. These papers are mostly based on morphological studies of several authors (mainly of Sprent J. F. A., Parasitology 46: 54—78, 1956, Parasitology 48: 184—209, 1958; Nichols R. L., J. Parasitol. 42: 349—362, 1956 and others), who regard as lst-stage larva the larva in the mature egg; the larva released from the swallowed egg moults from the cuticular sheath and turns to 2nd-stage larva.

However, Araujo (Rev. Inst. Med. trop. Sao Paulo 14: 83—90, 1972) demonstrated in an experimental work that there are two well discernible cuticular sheaths on the body surface of *Toxocara canis* larvae (as well as of *Ascaris lumbricoides* and *A. suum* larvae) artificially released from eggs at +25 °C on days 15—22 of maturation. This indicates that the larva underwent two developmental stages in the mature egg. The author documented these observations by photomicrographs of larvae with sheaths especially well visible at both ends of body.

We have performed a similar experiment with the larvae of *Toxocara cati* (Schrank, 1788). The eggs were released from uteri of mature females of *T. cati* obtained at the dissection of cats. They matured in the medium of 0.1 N H_2SO_4 at +20 °C. On day 15 of maturation the eggs were repeatedly washed with water and fresh microscopic preparations in distilled water were prepared from several tens of eggs. The larvae were then gently pressed out on a cover glass while being observed in the microscope at the magnification of 100×.

Immediately after the release only one cuticular sheath was well visible at the body ends of all larvae at the magnification of 400×. Within about 30 min, however, the second, outer cuticle started to separate. Both cuticular sheaths seem to be tightly connected inside the egg and they separate from one another only after the release of the larva from the

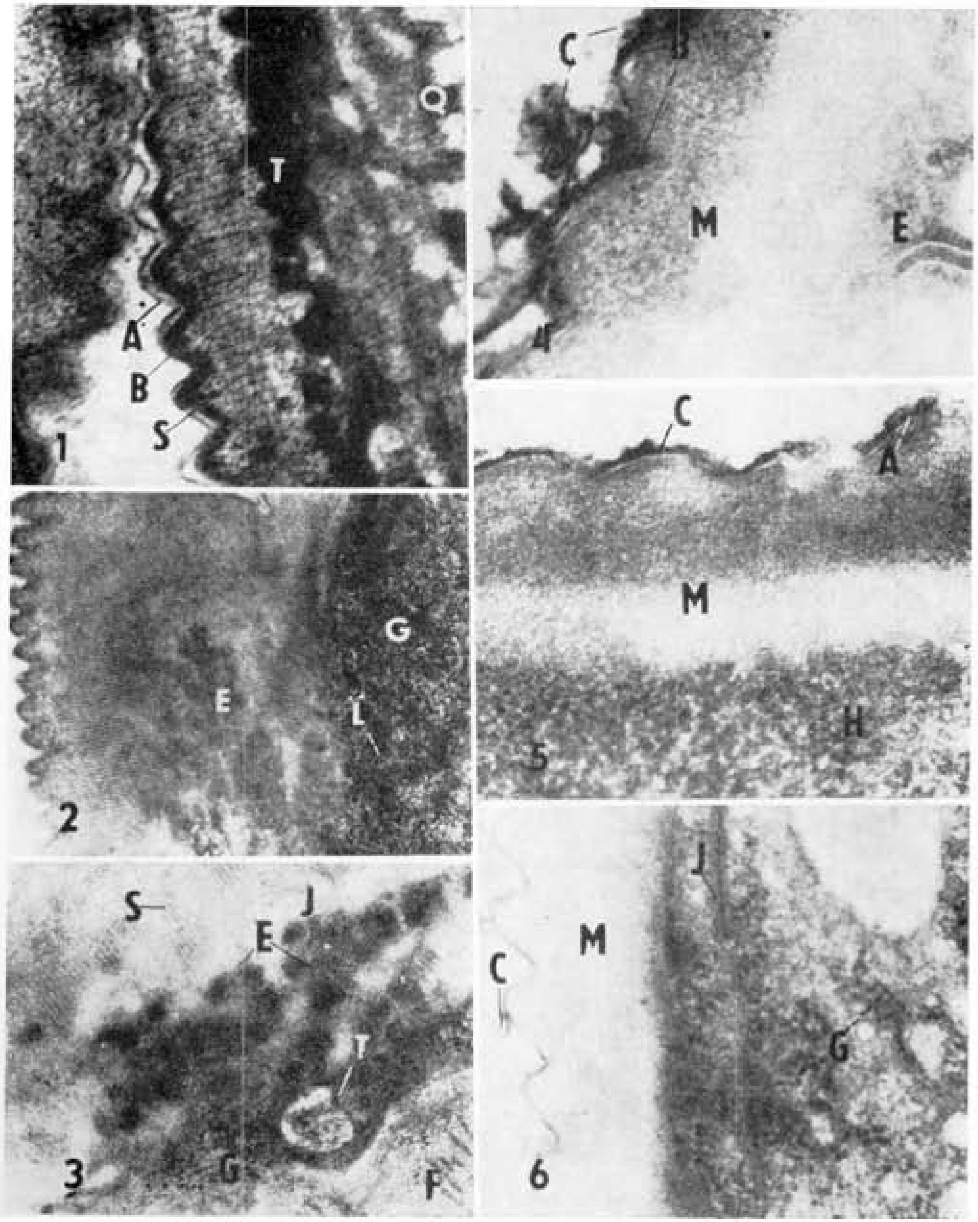

Fig. 1. Cortical layers of *T. nativa*; transverse striae of the amorphous matrix of the cuticle, and brick-like system of extensions of the hypodermis (HCl — SM-borax) (×40,000). **Fig. 2.** By contrast to the hypodermis, the reaction for carbohydrates is weak in the cortical layer and in striae of the cuticular matrix (Pa—CrA—S) (×35,000). **Fig. 3.** Cuticular matrix of *T. nativa* with fibrillar muscle tendons on the hypodermal membrane which react for carbohydrates. (PATCO) (×45 000).
Fig. 4. Outer carbohydrate coat on the surface of the cuticle of *T. nativa* containing AMS (ruthenium red) (×100,000). **Fig. 5.** The surface coat, cortical layer and the hypodermis contain sulfated mucopolysaccharides. Striae of the basal matrix do not stain (DAB—H_2O_2) (×75,000). **Fig. 6.** Weak staining for glycogen in the surface coat, no staining in the cuticle (Thiéry) (×65,000).

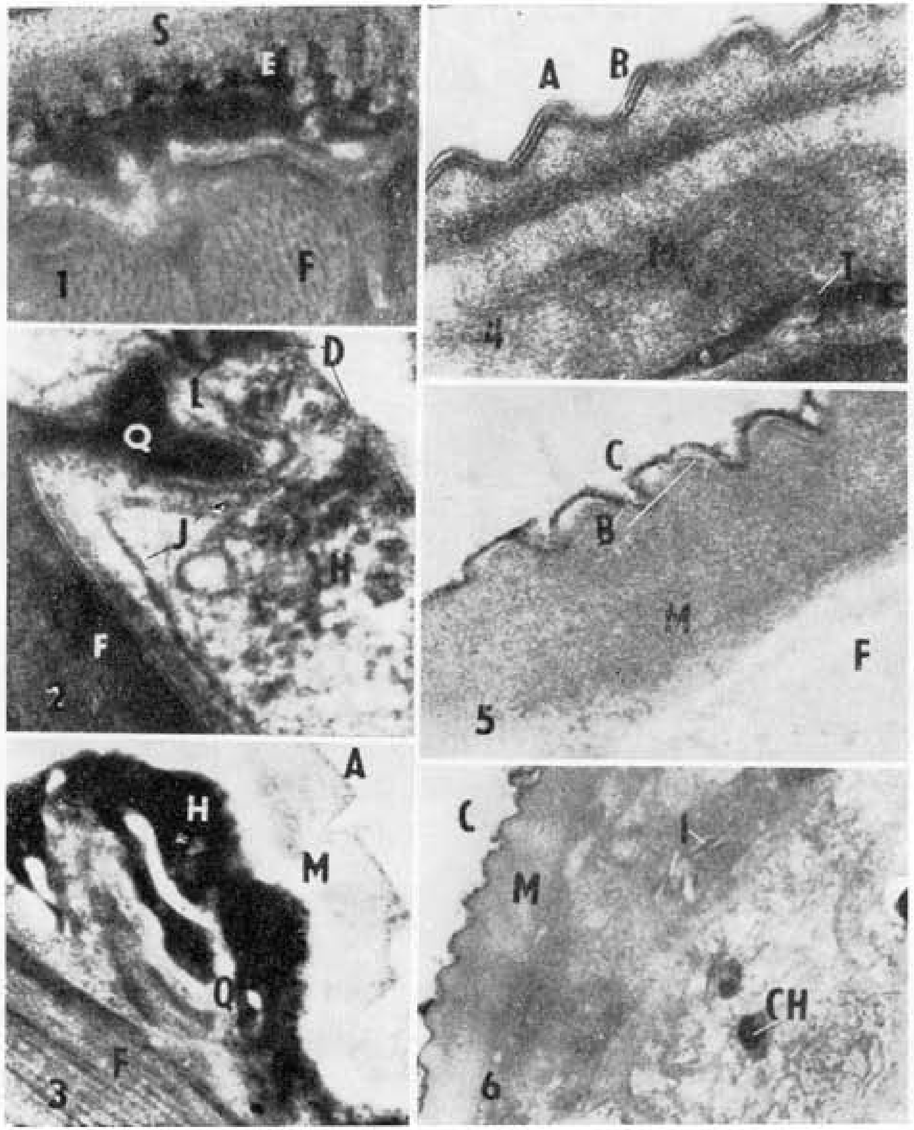

Fig. 1. Transverse section through the cuticle of *T. nativa* showing brick-like extensions of the hypodermis and fibrillar extensions in the base of the cuticle together with their termination among striae. Note two types of myofilaments in the muscles (PA—CrA—S) (×42,000). **Fig. 2.** Membrane-bound muscle extensions in the hypodermis. Granules in the hypodermis, muscle fibres and structures resembling lysosomes stain for NE (indoxyl-acetate, LC) (×78,000). **Fig. 3.** Extension of muscles and fibrils in the hypodermis in folds between grooves do not stain for ACP, granules in the hypodermis stain (B-glycerophosphate + lead citrate) (×60,000). **Fig. 4.** Cuticle of *T. pseudospiralis* (oesophagus area). The cortical layer reacts for cystine, the amorphous layer less strongly. Striae not observed (HCl—SM—borax) (×80 000). **Fig. 5.** Acidic mucosubstances in the surface coat and the cuticular matrix (DAB + OsO_4), (×70,000). **Fig. 6.** Confirmation of the permeability of the cuticle of *T. pseudospiralis*. The dye passes through the cuticle and stains granules of the hypodermis and the stichosome (ruthenium red). (×28,000).

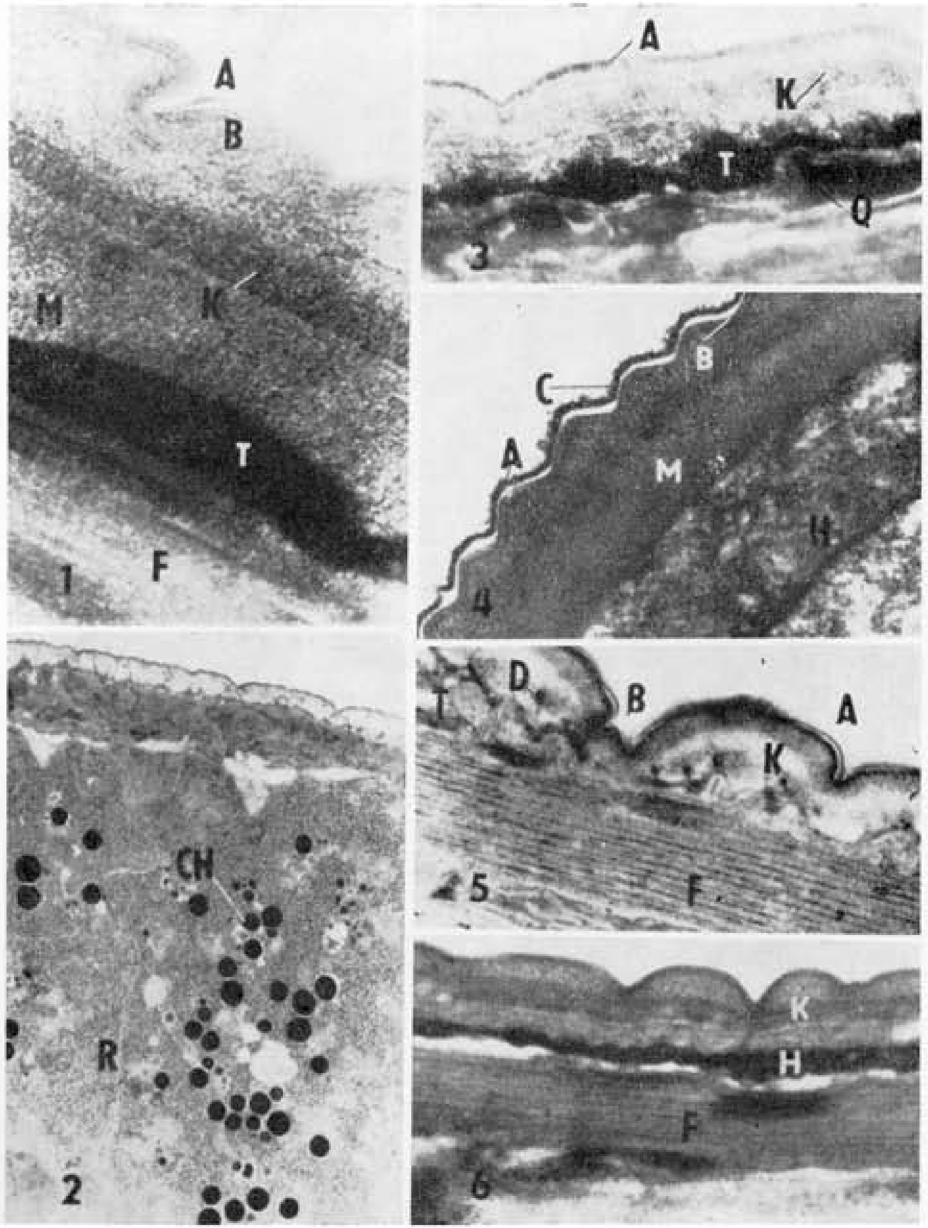

Fig. 1. *T. pseudospiralis:* a tangential section through a muscle extension and an extension of the hypodermis staining for NE (indoxyl-acetate, Pb) (×75,000). **Fig. 2.** Tangential section through the stichosome of *T. pseudospiralis* showing a reaction for diglycols in granules (PA—CrA—S) (×10,000). **Fig. 3.** Transverse section through the cuticle of *T. pseudospiralis*, ACP seen in hypodermal extensions (B-glycerophosphate, lead nitrate) (×30,000). **Fig. 4.** Surface coat, cortical layer, amorphous matrix of the cuticle of *T. pseudospiralis*, and hypodermis stain for glycoproteins (PTA) (×45,000). **Fig. 5.** Longitudinal section through the cuticle of *T. pseudospiralis* showing proteins in the inner, cortical layer and in extensions of fibrils into the base of the cuticle. The basal, cuticular matrix does not stain. (HCl—SM—borax) (×28,000). **Fig. 6.** The band of dense substance in the cuticular matrix; a positive reaction for carbohydrates is given by the hypodermis (PATCO) (×25,000).

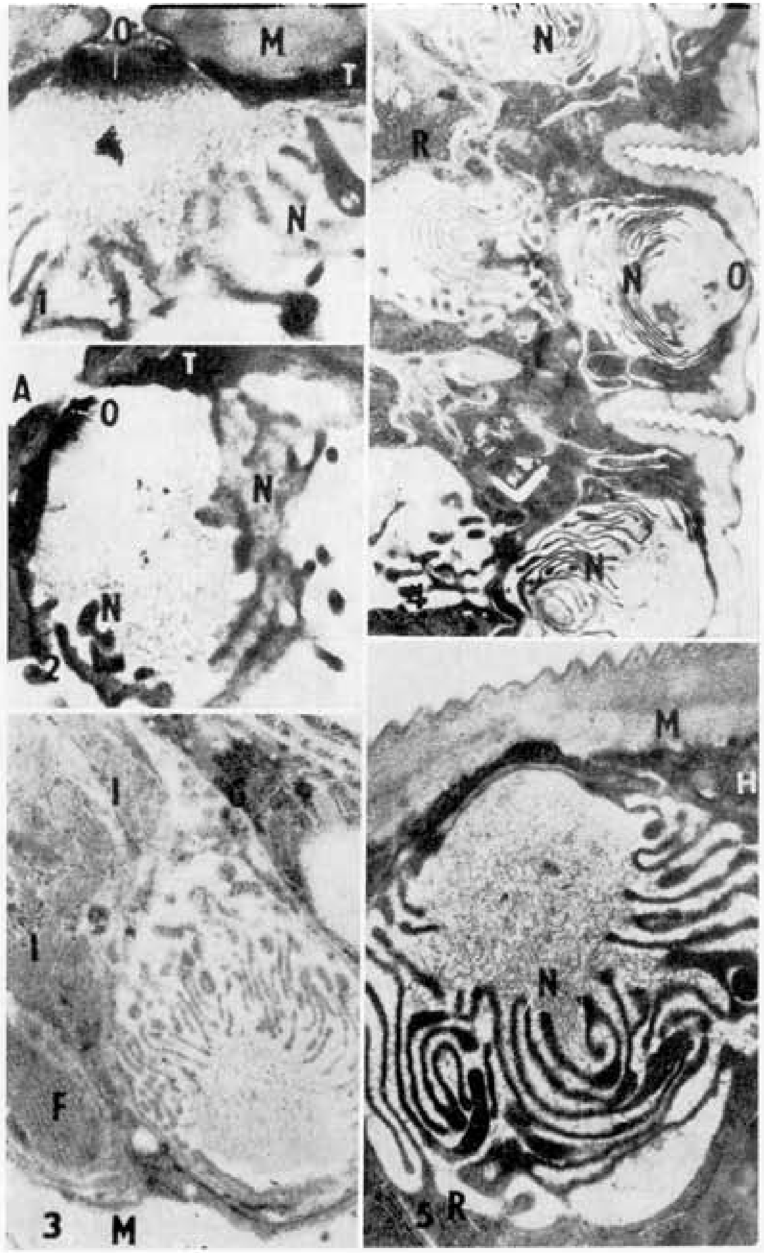

Fig. 1. *T. pseudospiralis:* The secretion concentrated in the boundary layer of the pore chamber reacts for carbohydrates (PA—CrA—S) (×30,000). **Fig. 2.** Proteins in the boundary layer of the pore chamber and in the fibrous secretion (HCl—SM—borax) (×21,000). **Fig. 3.** Glycogen in the hypodermal cell and in the base of lamellae draining the gland (PATCO) (×28,000). **Fig. 4.** Numerous glandular openings are seen in the heavily folded cuticle of *T. nativa* (oesophagus area) (UA) (×15,000). **Fig. 5.** The cuticle of *T. pseudospiralis* (oesophagus area) is not folded, glands of the lateral cord are remote from one another (UA, LC) (×30,000).