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Comparative investigations of the morphology and chemical composition of the eggshells of Acanthocephala *

I. *Macracanthorhynchus hirudinaceus* (Archiacanthocephala)

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Abstract. Eggshells of *Macracanthorhynchus hirudinaceus* (Archiacanthocephala) were investigated for their fine structure as well as their chemical composition. The acanthor larvae are surrounded by four eggshells (E1–4) separated by interstices of low electron density (G1–4). As these envelopes are secreted in different sequences and are reinforced to different degrees, their appearance varies throughout development. The outermost eggshell (E1) of this species has a tripartite appearance; it contains neither chitin nor keratin. Keratin appears in E2 and E3. It was localized electron microscopically using anti-keratin and, for the first time by fluorescence microscopy with the bromobimane reaction. Keratin occurs in two forms: in the second envelope (E2) it consists of twisted struts of filaments, whereas in the innermost sublayer of the third envelope (E3) it shows a conspicuous cross-striation; in the first and second sublayers of E3, neither keratin nor a discernible structure is present. Chitin occurs in the innermost layer (E4). The interstices G1, G3 and G4 seem to contain glycoproteins, whereas interstice G3 seems to contain some type of carbohydrate. After the extraction of proteins including keratin with sodium dodecyl sulfate (SDS) and dithiothreitol (DTE) only layers E1 and E4 remained.

In Acanthocephala the oocytes are penetrated by sperm before they have left the ovarian ball. During detachment from the ovarian ball, a fertilization membrane arises. Development takes place in the body cavity and ends with the formation of a characteristic larval stage, the acanthor. During development, several sheaths of differing thickness and composition are secreted by the embryo; as they surround the acanthor, they are called acanthor shells. Comparative investigations have shown that these shells differ not only among the three systematic groups of Acanthocephala, but may differ even between genera. Marchand (1984) investigated 13 species of different systematic groups and compiled the results of previous authors. Thus, to date the fine structure of eggshells has been described for 20 species. Moreover, Marchand tried to introduce a uniform terminology to replace the differing terms used by previous authors. He first distinguished between four solid sheaths (E1–4) separated by four interstices (G1–4) filled with liquid granular material; however, he examined only those parts of the shells that could be contrasted for electron microscopy. With respect to the chemical composition of acanthocephalan eggshells, Marchand (1984) stated: "The chemical composition of the different acanthor shells has never been clearly defined." Although von Brand (1940) had previously used light microscopy and chemical analysis to show that chitin is present in the acanthor shells of *Macracanthorhynchus hirudinaceus*, in 1984 Marchand doubted that chitin would occur in these shells. The following investigations have used light microscopic techniques: Monné (1964) maintained that chitin is present in the outer sheath of *Polymorphus botulus*. Anantaraman and Ravindranath (1976) found that chitin is present in the innermost sheath of *Acanthosentis oligospinus*; the same was observed by Edmonds (1966) in eggs of *Moniliformis moniliformis*.

The differing results in the literature show that a comparative investigation using extraction and modern histochemical methods might be useful for the localization of keratin and chitin in acanthocephalan eggshells. Keratin is characterized by, among other features, the presence of numerous thiol and disulfide groups. Therefore, the bromobimane reaction was used in the present study to localize keratin by fluorescence microscopy. Keratin-containing parts show a bright blue fluorescence whose intensity depends on the density of the thiol groups. Moreover, keratin was localized immunocytochemically using anti-keratin labeled with colloidal gold. Extraction of the acanthor shells was carried out using sodium dodecyl sulfate (SDS) and subsequently, SDS+ dithiothreitol (DTE) was used to extract proteins includ-

* Dedicated to Prof. Dr. J. Eckert (Zürich) on the occasion of his 60th birthday

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ing keratin. The fine structure of the egg of *M. hirudinaceus* has not yet been described, perhaps due to technical problems concerning the availability of egg material as well as the difficulty in penetrating the egg shells with resins.

Materials and methods

Macracanthorhynchus hirudinaceus from naturally infected domestic pigs were obtained from Drs. T.T. Dunagan and D.M. Müller (Carbondale, USA) and from Dr. Zhao Bin (Taiyuan, China). Isolated eggs were fixed with 2.5% or 5% glutaraldehyde in 0.1 mol/l cacodylate buffer (pH 7.2), washed in the same buffer, dehydrated in a series of ethanol and embedded in LR White (London Resin Company). Chitin was localized with a lectin, wheat germ agglutinin (WGA) that is mainly specific for *N*-acetyl glucosamine; WGA was coupled with colloidal gold as an electron-dense marker (Peters and Latka 1986).

Gold sol with a particle diameter of 17 nm was obtained by reducing tetrachloroauric acid with sodium citrate according to the method of Horisberger (1979). WGA (Serva, Heidelberg, FRG) is a relatively small molecule with a molecular weight of less than 40 kDa; therefore, it had to be coupled with bovine serum albumin (BSA; Sigma, Munich, FRG) using glutaraldehyde according to the method of Horisberger and Rosset (1977) before it could be labeled with gold. WGA was then labeled with colloidal gold under conditions that have been described in detail by Geoghegan and Ackerman (1977), Horisberger (1979), Goodman et al. (1981) and Peters et al. (1983). The labeled gold sol was stored at 4° C. Before its use, the content of colloidal gold in each probe was checked by measuring the optical density at 520 nm (Goodman et al. 1981) to ensure that a comparable amount of gold was present in the incubation medium.

Ultrathin sections were transferred to copper grids and treated for 10 min with phosphate-buffered saline (PBS) prior to incubation with lectin-gold (Roth 1983; Fryer et al. 1983). WGA-BSA-gold was then diluted 1:50 or 1:100 (v/v) with PBS. After 10 min incubation the grids were washed in PBS and distilled water for 10 min each, stained with uranyl acetate and lead citrate, and observed with a Zeiss EM 9-S2 electron microscope.

Specificity was controlled by competition tests. The lectin-gold conjugate and either a 0.2 to 0.3 mol/l solution of *N*-acetylglucosamine or a 10 or 20 mmol/l solution of triacetyl chitotriose were mixed for 20 min prior to the incubation of sections as described above. Moreover, sections were incubated with chitinase (0.1 units/100 µl PBS, pH 6.5) for 12 or 24 h at 37° C before being incubated with WGA-gold.

Keratin was localized using a monoclonal antibody raised against bovine epidermal keratins in the mouse (Sigma, Munich, FRG). The sections were incubated for 1 h in anti-keratin diluted 1:20 or 1:30 (v/v) in PBS (pH 7.4) +0.1% BSA+0.1% Tween; subsequently they were incubated for 20 min with an anti-mouse antibody, raised in rabbits (Dakopatts, Hamburg, FRG) and then labeled with 5 nm gold (obtained from Dr. J. Schmidt, Düsseldorf) diluted 1:20 (v/v) with PBS (pH 7.6).

In addition, keratin was localized by fluorescence microscopy using the specificity of the bromobimane reaction for thiols and disulfides (Kosower et al. 1979; Gainer and Kosower 1980; Danielsohn and Nolte 1987). Fixed eggs were embedded in a methacrylate, LR White. Semithin sections were transferred to slides and incubated with different substances. A series of sections were equilibrated with PBS and then incubated for 10 min with a 0.1 mmol/l solution of bromobimane (thiolyte DB: Calbiochem, Frankfurt, FRG), in the darkroom; the incubation medium was also prepared in the absence of light. After being washed in distilled water the sections were mounted in non-fluorescing glycerol and were immediately observed and photographed on TMV 5053 Kodak film using an Olympus fluorescence microscope.

A second series of sections were pretreated for 15 min at 45° C with 50 mmol/l iodoacetamide in distilled water to trap free sulfhydryl groups and were then washed in distilled water and incubated with 0.1 mmol/l bromobimane (thiolyte DB). A third group of sections were pretreated with iodoacetamide, washed with PBS, and then treated with 50 mmol/l dithiothreitol + 1 mmol/l ethylenediamine tetraacetic acid (EDTA) in 0.01 mol/l PBS for 15 min at 45° C, washed and incubated with bromobimane (thiolyte DB) as described above. A fourth series of sections were pretreated with DTE+EDTA as in the third series and were then incubated with bromobimane (thiolyte DB).

Extraction of acanthor shells was done either by boiling eggs in 1.5 M KOH or 0.5 M DTE or 2% SDS+0.5 M DTE+6 M urea or by incubating with 0.1 units chitinase/100 µl citric acid buffer (pH 5.8) at 37° C for 6 or 24 h.

For the electron microscopic localization of carbohydrates the modified PAS reaction according to Thiéry (1967) was used.

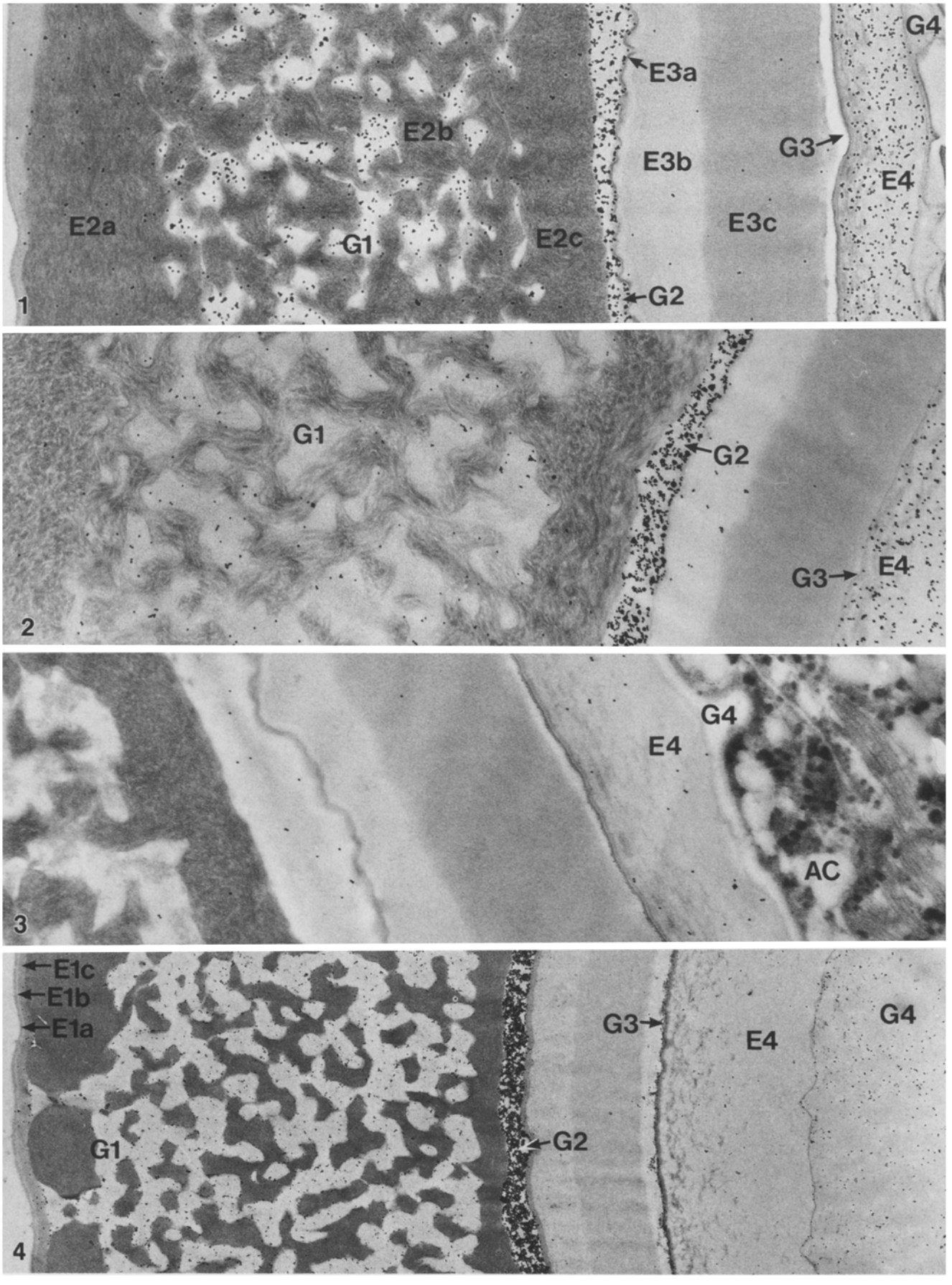
For scanning electron microscopy the worms were fixed as described above, treated with osmium tetroxide, dehydrated in a graded acetone series and critical-point-dried. The dried samples were mounted on specimen stubs, sputtered with gold and observed with a Super 3A scanning electron microscope (ISI).

Results

Mature acanthors of *Macracanthorhynchus hirudinaceus* are enclosed by four electron-dense envelopes (E1–4) separated by four electron-lucent interstices (G1–4). Eggs from the United States did not differ in any respect from those obtained from China. As the eggshells are secreted in different sequences and are reinforced to different degrees, their appearance varies throughout development. To avoid these differences in appearance, as much as possible mature eggs were used.

In *M. hirudinaceus* the outermost shell (E1) has a three-layered appearance (Figs. 1, 4, 6). A rather thin outermost sublayer is followed by an electron-lucent part of similar thickness and then another electron-dense sublayer. The thickness of each of these sublayers differs with the developmental stage and the inner parts appear later than the outermost layer. Only the latter resists extraction with SDS-DTE-urea (Figs. 12, 13). There is no indication that either chitin or keratin or Thiéry-positive substances are present in this triple layer, which is usually destroyed during preparation of the eggs for scanning electron microscopy (Fig. 8). The outermost part of E1, which is present when the zygote detaches from the ovarian ball, is derived from the fertilization membrane and becomes reinforced during further development.

The second shell (E2) is reinforced considerably during development and has a characteristic appearance in both species (Figs. 1, 2, 4). At one end of the egg, however, it is very narrow and forms a fold. This shell consists of a network of irregularly arranged fine filaments in an electron dense matrix. The inner part of E2 forms, a compact, uninterrupted shell. Exteriorly this inner part is supported by radius-like branched and twisted protuberances that are irregularly arranged (Figs. 1–4). At the outer periphery of E2, these protuberances are extended and form longitudinally oriented, prominent ridges that can best be seen in scanning electron micrographs (Fig. 8). Anti-keratin labeled with colloidal gold was


G4
E3a
E2b
E3b
G3
E4
E2a
G1
E2c
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1
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E4
2
G4
E4
AC
3
E1c
E1b
E1a
G3
E4
G4
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4

bound by E2 (Figs. 5, 6), indicating that it contains keratin. The solid inner belt and the dense outer ridges of E2 were more intensely labeled than the spongy bundles of filaments between them. In eggs treated with bromobimane, a specific reagent for thiol groups, E2 showed a marked fluorescence, which is in accordance with anti-keratin-gold labeling; the fluorescence was most significant around the inner solid belt and the outer ridges of E2 (Fig. 7).

The third shell (E3) consists of three sublayers. An outer electron dense sublayer is followed by an electron lucent part and then an interior sublayer which is characterized by a conspicuous cross striation that appears in cross-sections as a hexagonal pattern. Anti-keratin-gold bound only to the cross-striated internal sublayer. Using the bromobimane reaction, we found that the third shell E3 showed a significant fluorescence that was much more intense than that displayed by E2, especially in comparison with the bundles of filaments in E2 (Fig. 7). This indicates that material rich in thiol or disulfide groups is present in the innermost sublayer of E3.

The innermost envelope, E4, consists of fine filaments in a rather regular arrangement. It could be labeled with WGA gold-conjugate (Figs. 1, 2). Competition with *N*-acetylglucosamine resulted only in a minor reduction of label, but competition with triacetyl chitotriose reduced the amount of label almost completely (Fig. 3). When sections were incubated first with chitinase and then with WGA-gold, E4 could no longer be labeled (Fig. 4), indicating that this layer contains chitin.

In the complicated system of interstices (G1) left between the twisted struts of keratin filaments in E2, WGA-gold conjugate was bound (Figs. 1, 2, 4). Competition with 0.3 mol/l *N*-acetylglucosamine resulted in an inhibition of WGA-gold binding. Therefore, these areas seem to contain only glycoproteins but no chitin. G1 was always Thiéry-negative (Fig. 9). The second interstice (G2) was labeled very intensely after incubation with WGA-gold (Figs. 1, 2), even more intensely than the fourth shell (E4; Fig. 1). However, although the label could not be reduced by competition with *N*-acetylglucosamine, it was almost completely reduced by competition with 10 mmol/l triacetyl chitotriose. After incubation of sections with chitinase and subsequent incubation with WGA-gold G2 showed labeling (Fig. 4) that was even more intense than that shown in Figs. 1 and 2; this indicates that chitin cannot be present in this interstice. Competition experiments indicate that glycoproteins occur in G2. Interstices G2 to G4 could not be investigated using the Thiéry method, as the inner parts of the eggshells are usually torn during application of the method. The third and fourth interstice (G3, G4) showed only sparse labeling with WGA-gold (Fig. 4) which was reduced in competition experiments; therefore, only glycoproteins can be present (Fig. 3).

Extraction of proteins including keratin was done by boiling eggs first in either 0.5 M DTE or 2% SDS and then in 2% SDS + 5 M DTE + 6 M urea for 15 or 60 minutes. The outermost sublayer of the first envelope (E1) could not be extracted at all. With increasing time of extraction the second shell (E2) could be disrupted by an osmotic shock. Light microscopy showed that the narrow folded part of E2 is the area in which the acanthor shell is normally disrupted to expel the acanthor (Fig. 11). Electron microscopy revealed that after extraction with SDS + DTE + urea, the disrupted third shell eventually disappeared completely (Figs. 12, 13). Following prolonged extraction, only the outermost sublayer of E1 and the innermost shell (E4) remained (Fig. 13); E1 was folded, whereas E4 surrounded the acanthor. The latter could be labeled intensely with WGA-gold (Fig. 13) and had a filamentous appearance that had not been visible before (Figs. 1–4) as the filaments had probably been masked by matrix proteins. The innermost shell (E4) could not be dissolved by boiling with 1.5 M KOH for 2 h; it dissolved only after eggs that had previously been extracted with SDS + DTE + urea had been embedded and the resultant sections had been incubated with chitinase for 10 and 12 hours. This is additional evidence for the presence of chitin.

Discussion

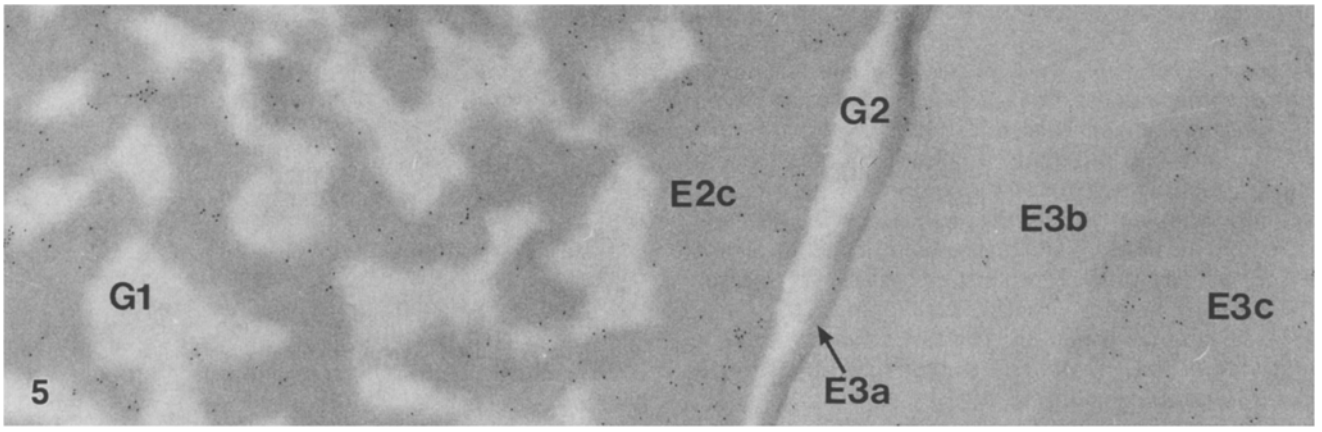
In the present investigation it could be shown that the acanthor larva of the archiacanthocephalan *Macracanthorhynchus hirudinaceus* is surrounded by four eggshells separated by four interstices. In addition to these morphological findings which confirm the results of Marchand (1984), the chemical contents of most of the sheaths and interstices were determined. The outermost sheath (E1) is characterized by its development from the fertilization membrane; however, nothing is known about its constituents. It probably prevents the egg from being attacked by digestive enzymes in the intestinal lumen of the final host. The nomenclature of the eggshells could now be standardized using as a basis the envelopes and interstices that can be characterized with regard to their chemical constituents: keratin, chitin and carbohydrates. Also, considering the results of our investigations on the morphology and chemical composition of palaea-

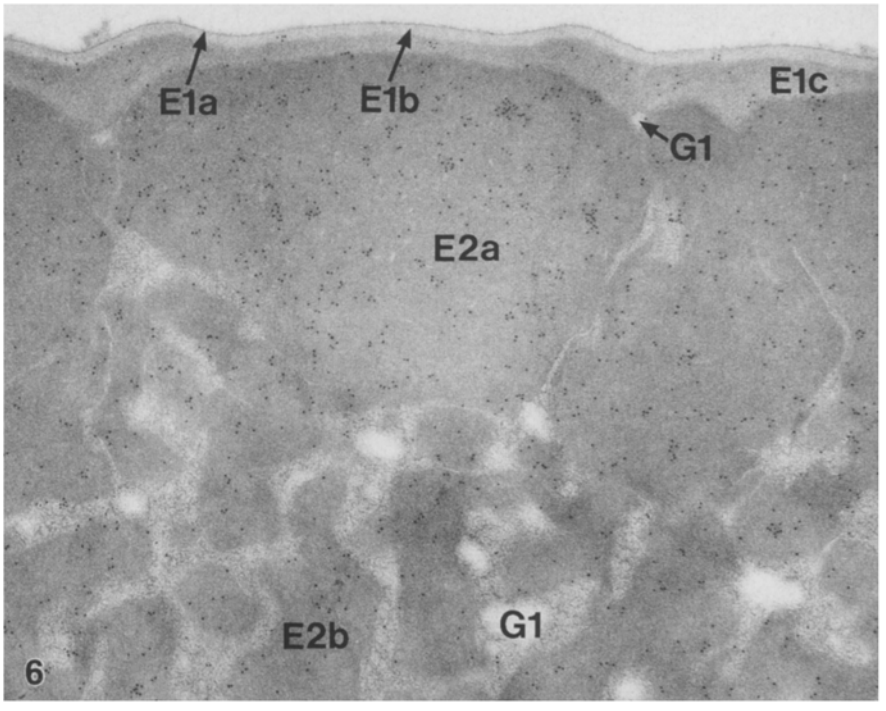
Fig. 1. A section of *Macracanthorhynchus hirudinaceus* after incubation with wheat germ agglutinin (WGA)-gold. Apart from some nonspecific labeling, only the G1 interstices in E2, the interstice G2, and the fourth shell (E4) are labeled. ×17800

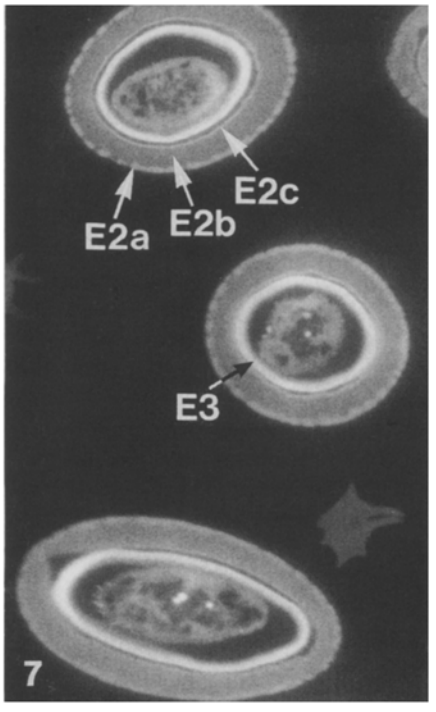
Fig. 2. A less intensely stained section shows more clearly the filamentous nature of the second shell (E2 in Fig. 1). × 26600

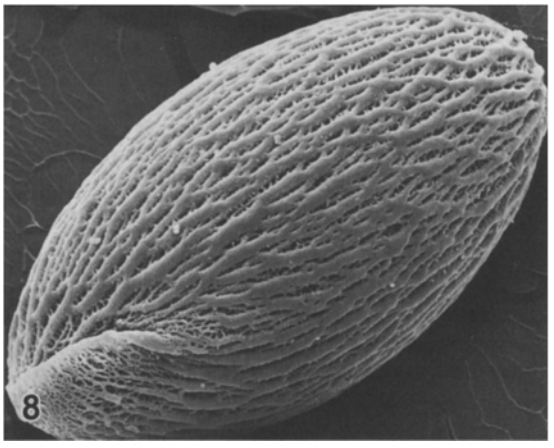
Fig. 3. After competition with 20 mmol/l triacetyl chitotriose only the G1 interstice and the fourth shell (E4) show sparse labeling. ×27500

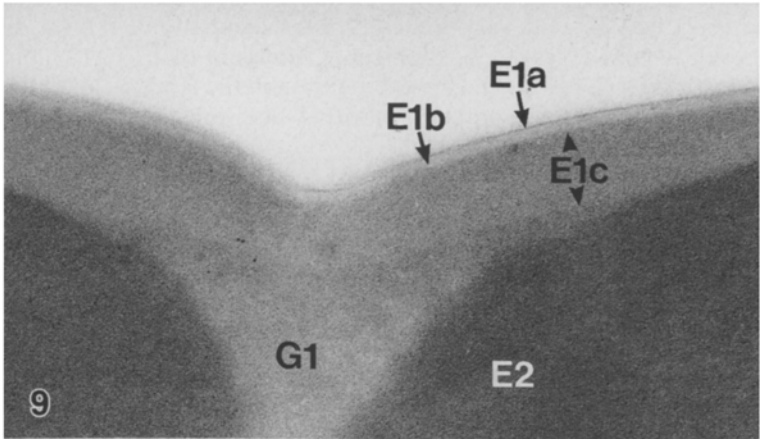
Fig. 4. A section of *M. hirudinaceus* after incubation with chitinase (24 h) and, subsequently, with wheat germ agglutinin (WGA)-gold. Gold label can be seen in the G1 and G2 interstices in E2, and in the embryo, but is almost lacking in the fourth shell (E4). Therefore, the results of incubation of sections with chitinase and of competition with *N*-acetylglucosamine and triacetyl chitotriose show that G1 seems to contain glycoproteins, whereas E4 contains chitin. ×13200









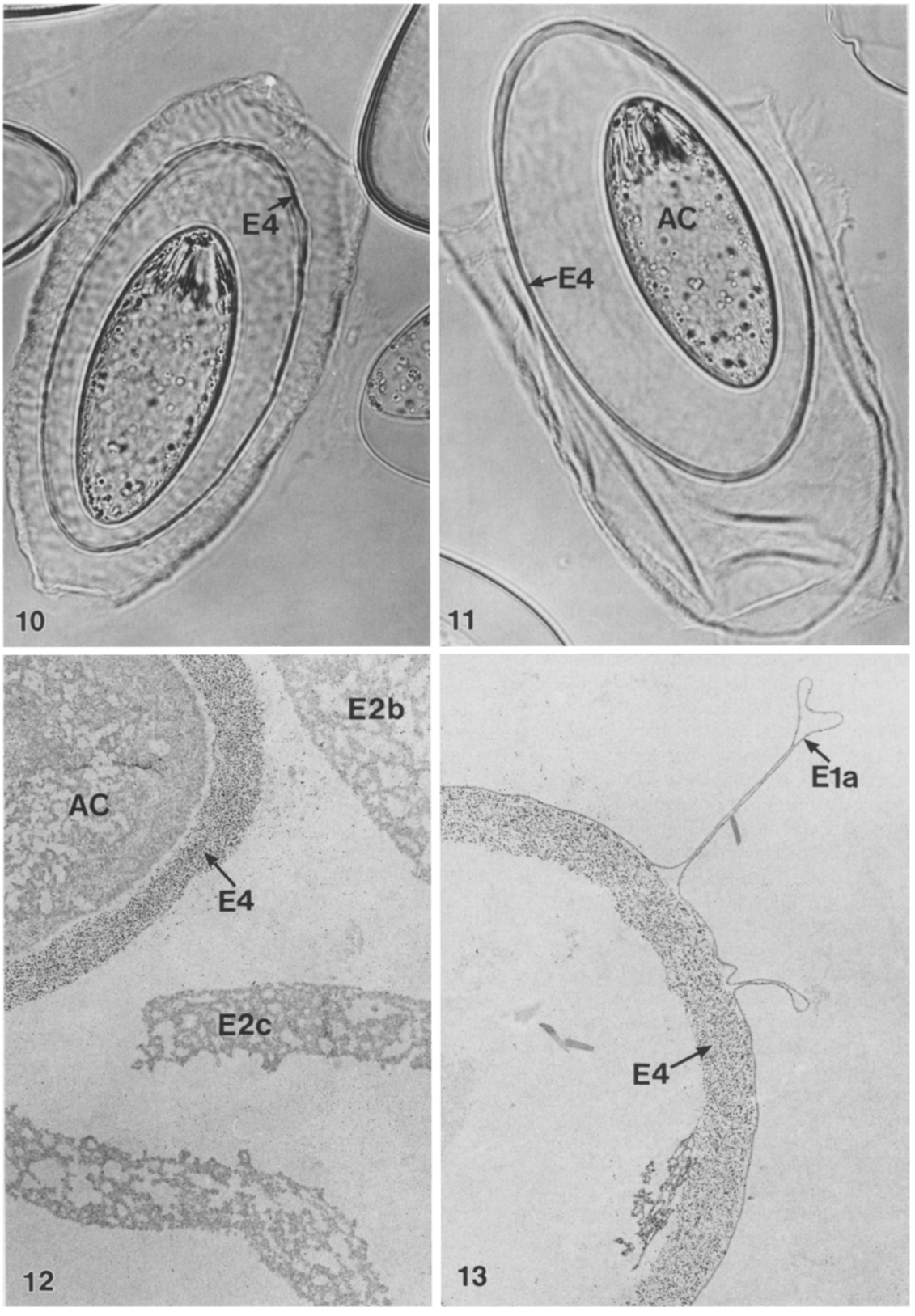


Figs. 5, 6. A section that has been incubated with anti-keratin and, subsequently, with a second antibody labeled with colloidal gold. The gold granules have a diameter of only 5 nm. Apart from some non-specific labeling, the filamentous part of the third envelope (E2) and the inner striated part of the third shell (E3) are labelled. × 35200

Fig. 7. A fluorescence micrograph obtained after the application of bromobimane reveals that thiol groups are present predominantly in E3 and less conspicuously in the thick second shell (E2). × 220

Fig. 8. A scanning electron micrograph of a *M. hirudinaceus* egg that is devoid of the thin outer shell (E1 in Fig. 6), showing the outer network of longitudinally arranged conspicuous ridges in E2. × 400

Fig. 9. An electron micrograph of a section treated according to Thiéry (1967). Neither the outer eggshell (E1) nor the first interstice (G1) reacted positively. × 10500



Figs. 10, 11. After extraction of proteins with SDS + DTE, the first two shells swell considerably and eventually rupture due to osmotic pressure. Therefore, the acanthor (AC) enveloped by the third keratin-containing shell and the fourth chitin-containing shell (E4) is expelled from the outer shells. × 500

Fig. 12. Partially extracted egg shells. The third shell has disappeared, whereas the first shell has thinned considerably and the second (E2) is swollen and has partly ruptured. × 4300

Fig. 13. After prolonged extraction only the thinned first shell (E1a) and the fourth shell (E4) remain. The latter shows intense labeling with wheat germ agglutinin (WGA)-gold because the chitin microfibrils remain after the matrix proteins have been extracted. × 6300

canthocephalans and eoacanthocephalans (Taraschewski and Peters 1991a, b), it appears that the following generalization is possible. The second and third eggshells (E2 and E3) are keratin-containing sheaths. Their functions seem to differ among the systematic and ecological groups of the Acanthocephala. The innermost eggshell, the "embryophore" is the chitin-containing sheath. However, exceptions to this rule exist (see Taraschewski and Peters 1991a, b). Most interstices contain carbohydrates, but the mode of transmission of the different species of acanthocephalans seem to have a strong impact on the distribution of carbohydrates in these interstices (Taraschewski and Peters 1991a, b). Von Brand (1940) has reported that chitin is present in the inner envelope of the eggs of *M. hirudinaceus*, and that this envelope persists even following the treatment of eggs with 60% KOH. This author recognized only three envelopes using light microscopy. He found a total chitin content of 0.18% in a mixed sample of eggs and free-floating ovaries, and suggested that the chitin content of the acanthor shells alone should be considerably higher. The presence of chitin can be demonstrated by x-ray diffraction, by the chitosan reaction, or by enzymatic degradation with chitinase and quantitative determination of the *N*-acetylglucosamine content (DeMets and Jeuniaux 1963, as cited in Jeuniaux 1963). However, it is impossible to use these methods for the localization of chitin. For light and electron microscopic localization of chitin, the lectin wheat germ agglutinin (WGA) coupled with colloidal gold as an electron dense marker has been introduced by several authors. Horisberger and Rosset 1977, Horisberger and Vonlanthen 1977 and Tronchin et al. 1981 used WGA-gold for the localization of chitin in budding yeasts by scanning and transmission electron microscopy. In this way Arroyo-Begovich and Carabez-Trejo 1982 localized chitin in the cyst wall of *Entamoeba invadens*. Peters and Latka (1986) described an improved method and demonstrated its usefulness in the localization of chitin in peritrophic membranes of insects and in the radula of a snail.

WGA possesses a binding site for groups of up to three *N*-acetyl-D-glucosamine residues; therefore it shows a high affinity to polymers of this sugar such as chitin and its precursors (Allen et al. 1973). The specificity of binding can be controlled by competition experiments using *N*-acetylglucosamine and triacetyl chitotriose. Moreover, as a control we used the digestion of chitin with chitinase and subsequent incubation with WGA-gold. Thus, we could demonstrate that the fifth, or innermost, envelope (E5) contains chitin.

Derivatives of bromobimane were introduced to histochemistry in 1979 by Kosower et al. for the localization of proteins with thiol groups. Bromobimane derivatives are non-fluorescent, but their alkylic bromide groups can react with thiol groups under physiological conditions to yield highly fluorescent products. Therefore, they can be used as highly specific and sensitive markers for the thiol groups of proteins. In the past 10 years they have been tested with several types of proteins from different species of animals.

Although it has been emphasized that these compounds do not fade during illumination, as opposed to other well-known markers, such as FITC or rhodamine isothiocyanate, it could be shown by Danielsohn and Nolte (1987) that the reaction products of derivatives of bromobimane also fade, but perhaps less quickly than other fluorescent markers. Moreover, the bromobimanes can react very quickly, with 2 to 10 min being sufficient for the incubation of sections or fixed material such as erythrocytes or eggshells. As bromobimane fluorecence and anti-keratin labeling occurred in the same shells (E2 and 3), these should contain keratin. The localization of keratin by the bromobimane method has not yet been described but seems promising because keratin is characterized by an abundance of thiol groups. However, the results should be confirmed by a different method, in this case by immuno-cytochemistry.

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